Cadherin-11 expression patterns in heart valves associate with key functions during embryonic cushion formation, valve maturation, and calcification

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Abstract

Proper fibroblast cell migration and differentiation are critical for valve formation and homeostasis, but uncontrolled myofibroblastic activation may precede osteogenic differentiation and calcification. Cadherin-11 (cad-11) is a cell-cell adhesion protein classically expressed at mesenchymal-osteoblast interfaces and it participates in mesenchymal differentiation to osteochondral-lineages. This suggests cad-11 may have an important role in heart valve development and pathogenesis, but its expression patterns in valves are largely unknown. In this study, we profiled the spatial and temporal expression patterns of cad-11 in embryonic chick and mouse heart development. We determined that cad-11 is expressed in both endocardial and mesenchymal cells of the atrioventricular (AV) and outflow tract (OFT) cushions (Pre HH30/E14), but becomes restricted to the valve endocardial/endothelial cells during late fetal remodeling and throughout postnatal life. We then investigated changes in Cad-11 expression in a murine aortic valve disease model (the ApoE$^{-/-}$). Unlike wildtype mice, cad-11 becomes dramatically re-expressed in the interstitium. Similarly, in calcified human aortic valve leaflets, cad-11 loses endothelial confinement and becomes significantly re-expressed in the valve interstitium. Double labeling identified that 91% of myofibroblastic and 96% of osteoblastic cells in calcified aortic valves were also cad-11 positive. Collectively, our results suggest that cad-11 is important for proper embryonic cushion formation and remodeling, but may also participate in aortic valve pathogenesis if re-expressed in adulthood.

Keywords

cadherin; heart valve development; calcific aortic valve disease

Introduction

Heart valve disease is a serious and growing clinical problem, both in the United States and globally. Congenital abnormalities of heart valves develop in 1-2% of live births [MEMBERS et al., 2009]. Proper growth and maturation of the valve leaflets are essential for long-term postnatal function. The heart valve leaflets originate as amorphous endocardial cushions rich in glycosaminoglycans and proteoglycans [de Lange et al., 2004]. Remodeling

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and maturation of these cushions into thin fibrous valve leaflets require matrix condensation, extension and eventual trilayer stratification [Ramsdell and Markwald, 1997; Person et al., 2005; Butcher and Markwald, 2007]. However, the mechanisms regulating valve progenitor differentiation into fibroblasts are still poorly understood [de Lange et al., 2004; Kruithof et al., 2007]. Many clinically relevant congenital heart defects arise during later fetal remodeling events, which correlate poorly with specific transcription factor mutations [Mjaatvedt et al., 1998; Camenisch et al., 2000; Kern et al., 2010]. Alternatively, we and other groups have identified extracellular matrix proteins and adhesion ligands, such as periostin and versican, as important regulators of heart valve matrix remodeling [Henderson and Copp, 1998; Butcher et al., 2006; Kruithof et al., 2007; Kern et al., 2010]. These structural and/or matricrine components bind growth factors and provide critical adhesive signals that guide cellular differentiation [Zhang et al., 1998; Horiuchi et al., 1999]. Many of these processes involve interactions between resident cells, but the underlying mechanisms are poorly understood.

Cadherins are calcium dependent membrane glycoproteins that mediate cell-cell interaction. They regulate tissue morphogenesis through endothelial to mesenchymal transition (EMT), mesenchymal-epithelial transition, cell sorting, and cell rearrangement. Their extracellular domain mediates adhesive binding to neighboring cells (homotypic and/or heterotypic), while their cytoplasmic tail interacts with p120, catenins, and other cytoplasmic proteins [Nagafuchi et al., 1987; Friedlander et al., 1989; Levine et al., 1994; Lee and Gumbiner, 1995; Yagi and Takeichi, 2000; Niessen et al., 2011]. Cad-11, also called osteoblastic cadherin, was initially identified in mouse osteoblasts and is a type II classical cadherin [Okazaki et al., 1994]. It is expressed in mesenchymal tissue, maintains bone density, and is essential for formation of the synovial lining [Kimura et al., 1995; Kawaguchi et al., 2001; Lee et al., 2007; Di Benedetto et al., 2010; Kyung Chang et al., 2010; Chang et al., 2011]. Cad-11 mediates synovial fibroblast inflammation by increasing IL-6 production through TNF-α [Chang et al., 2011]. Cad-11 also directly regulates the differentiation of mesenchymal cells into the cells of the osteo-lineage and chondro-lineage [Kii et al., 2004]. Additionally, cad-11 is expressed in several invasive cancer cell lines including breast, prostate, colon, and bone, and it plays a role in tumor invasion and progression [Shibata et al., 1996; Kashima et al., 1999; Pishvaian et al., 1999; Feltes et al., 2002]. We previously discovered that cad-11 is a hemodynamically sensitive protein expressed in adult aortic valve endothelial cells [Butcher et al., 2006]. Yutzey et al. group has also found that the cad-11 gene is expressed in chick endocardium and mitral valves; however these findings were incomplete with respect to cad-11 gene expression patterns throughout the developing heart. [Shelton and Yutzey, 2008]. The localization patterns, such as endothelial-mesenchymal expression differences or the temporal-spatial expression changes in each of the developing valves have not been fully characterized. In addition, cad-11's relationship to the changing valvular shape and resident cell phenotypes is unknown. In this study, we determined the cardiac specific expression pattern of cad-11, at both the gene and protein level across the continuum of development and adulthood. We establish that, in the heart, *cad-11* is expressed exclusively in mesenchymal cells of the early embryonic outflow tract (OFT) and atrioventricular (AV) cushions, but becomes restricted to the cardiac valve endothelium postnatally. We then determine that cad-11 expression reverts to an embryonic pattern in diseased aortic valves, and is co-localized with myofibroblastic and osteogenic phenotypes. Together, these suggest that cad-11 participates in important valve developmental and pathogenic mechanisms.

Materials and Methods

Mouse Strains

Wildtype C57BL/6 and *ApoE*$^{-/-}$ *mice* (The Jackson Laboratory) were bred according to standard protocols. *ApoE*$^{-/-}$ mice were fed a high-fat/high-carbohydrate diet for 30 weeks before sacrificing and collecting their hearts. All animal work was conducted according to relevant national and international guidelines. Full details of this study were reviewed and approved by the Cornell IACUC (protocol #2008-0011).

Immunohistochemistry

Embryos were fixed in 4% paraformaldehyde overnight at 4°C, then dehydrated through an ethanol series and paraffin embedded and sectioned at 8 μm thickness. Following dewaxing and rehydration, sections were blocked with 10% goat serum before primary antibodies used were against cad-11 (rabbit anti-Cadherin 11 from Invitrogen; 1:100 dilution), MF20 (Developmental Studies Hybridoma Bank; 1:200 dilution), and PECAM-1 (Invitrogen; 1:200 dilution). Fluorescence-conjugated Alexa Fluor 488 goat anti-mouse and Alexa Fluor 568 goat anti-rabbit secondary antibodies (Invitrogen, 1:300) were used according to the primary antibody species. Sections were nuclei counterstained with DRAQ5 (Abcam, 1:1000 dilution). Healthy human aortic valve sections were used as positive controls for cad-11 (cad-11 is known to be expressed in endothelial cells of porcine aortic valves [Butcher et al., 2006]). Negative controls were incubated with 10% goat serum instead of primary antibodies. Signals were detected and images were collected with Zeiss 710 confocal microscopy (Cornell University Life Sciences Core Laboratories Center).

In situ Hybridization

Chick embryos and hearts were fixed in 4% paraformaldehyde overnight at 4°C, then dehydrated and stored in methanol. Embryos and hearts were rehydrated, digested with proteinase K, washed & refixed, and hybridized using digoxygenin-labeled in vitro transcribed anti-sense cad-11 reboprobes (corresponding to 501-798 of Gallus gallus cad-11 mRNA, NM_001004371.1) and sense reboprobes.

Quantification of Tissue-Specific Gene Expression

Fertilized eggs were incubated at 37°C and 50% humidity. OFT cushion or aortic valve leaflets from E4, E10 and E14 were dissected away from the surrounding myocardium. RNA was isolated from these tissues using the RNeasy Mini Kit (Qiagen) and transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Inc.) according to the manufacturers' instructions. Cad-11 expression levels were quantified with real-time PCR, using a Mini-Opticon Real-Time PCR Detection System (Bio-Rad, Inc.) and SYBR Green PCR master mix (Bio-Rad, Inc.), normalized to the 18S rRNA housekeeping gene. The following primers were used: cad-11 (F-TGATGGAGATGGCATGGATA, R-TGCCTCTACCTTCAGGCTGT, 79bp), 18S (F-CGGAGAGGGAGCCTGAGAA, R-CGCCAGCTCGATCCCAAGA, 275bp).

Quantitative Analysis of Histological Changes

Immunoreactivity of cad-11 in immunohistochemistry stained tissue sections was measured using ImageJ software version 1.46r (NIH). The area of each cell was selected and the average fluorescence intensity, minus the background, was used for data analysis as previously described [Butcher and Nerem, 2004; Richards et al., 2013]. Statistical significance was determined using Student's t-test ($p < 0.05$).

Results

We first profiled the differential expression of *cad-11* mRNA in avian hearts. We found that *cad-11* mRNA is expressed in the limb bud (Fig. 1A black arrow head), the forming somite (Fig. 1A'), and the pharyngeal arches of early embryos, which is consistent with previous reports [Kimura et al., 1995] and confirmed the specificity of the *cad-11* probe. C*ad-11* is expressed throughout the cushion tissue of the OFT tract and the AV canal at 3 days of incubation (Fig. 1A white arrows). *Cad-11* expression increases with cushion expansion (Day 5), and extends into the atrioventricular sulcus (Fig. 1B white arrows). By day 6, *cad-11* mRNA expression is highly concentrated in the OFT, but decreased in the atrioventricular valve primordia (Fig. 1C). By day 9, *cad-11* expression becomes restricted to the differentiating aortic and pulmonic valve leaflets and shows reduced expression in the atrioventricular valves (Fig. 1D white arrows). Sense probe controls showed no expression as expected (Fig. 1B far right). Real time PCR quantification of mRNA isolated from stage specific aortic valve progenitor cells/leaflets confirms that *cad-11* is elevated during the cushion remodeling period (E4 at 3.2±0.1 fold and E10 at 4.0±0.2 fold, p<0.05), but markedly decreases as the leaflets mature (E14 at 1.0±0.1 fold, p<0.05, Fig. 1E).

Similarly in mouse, cad-11 expression was confined to the endothelium and mesenchyme of the OFT and AV cushions (E11.5, Fig. 2B-E), but not expressed within the myocardium (Fig. 2C box region). [Butcher et al., 2006]. Negative controls showed a clean background (Fig. 2F). During semilunar valve remodeling (E14.5) however, cad-11 becomes increased in the endothelium but decreased in the mesenchyme of both the semilunar and atrioventricular valves (Fig. 3D-K). Cad-11 is progressively more restricted as valves condense and mature (E18.5, Fig. 4D-K), and virtually exclusively expressed in the valve endothelium postnatally (Fig. 5D, F and G). This expression patterns was largely consistent with previous reports in porcine aortic valve endothelial cells[Butcher et al., 2006], confirming antibody specificity (Fig. 2G).

Cad-11 expression changed dramatically under diseased heart valve conditions. In the ApoE$^{-/-}$ hyperlipidemic mouse model that presents with aortic valve disease [Plump et al., 1992; Nakashima et al., 1994; Reddick et al., 1994; Hjortnaes et al., 2010], cad-11 expression was markedly increased (2.4±0.2 fold higher in aortic valve and 2.3±0.1 fold higher in mitral valve, p<0.05) compared to wildtype controls (Fig. 5C-E). While still expressed in the endothelium, cad-11 became re-expressed in the interstitial cells of ApoE$^{-/-}$ aortic valves (Fig. 5E).

Cad-11 expression was similarly restricted to the endothelium of healthy human aortic valves (Fig. 6A, D, and F). The valve leaflets are composed of three layers: the ventricularis, on the ventricular side of the lealflets, the fibrosa, on the aortic side of the leaflet, and the spongiosa between the two. In calcified human aortic valves, mineralized lesions appear preferentially in the fibrosa (Fig. 6B). Double staining of cad-11 and PECAM-1 confirmed co-localization of the two proteins in endothelial cells on both the fibrosa and ventricularis layers of the healthy valves (Fig 6D and F). Cad-11 expression was statistically lower (27.3±3.4 fold less on the fibrosa, p<0.05, Fig. 6D, D'; 22.7±2.6 fold less on the ventricularis, p<0.05, Fig. 6F, F', and C) on a per cell basis in the endothelium layer of calcified aortic valves compared to healthy controls. However, significantly fewer valve endothelial cells were present (3.6±0.3 fold less PECAM-1 surface expression on the fibrosa, p<0.05, Fig. 6D, D' and C, 2.6±0.2 fold less on the ventricularis, p<0.05, Fig. 6F, F' and C) in endothelial cells on both the fibrosa and ventricularis layers of the calcified valve leaflets. These results suggest that the endothelial expression of cad-11 in healthy aortic valves may be involved in maintaining the integrity of the endothelial monolayer.

Cad-11 expression was statistically higher (4.2±0.6 fold, p<0.05, Fig. 6E, E', and C) in the interstitial cells of calcified aortic valves, where the cells are mostly part of the spongiosa. As expected, both myofibroblastic (αSMA positive) and osteoblastic (Runx2 positive) cells were present within the diseased valves, but not in healthy valves (Fig. 7 and Suppl Fig. 1). We identified the phenotypes of the interstitial cells in calcified aortic valves by co-labeling cad-11 with αSMA, vimentin, or Runx2 (Fig. 7A-C). 91.3%±1.4% of myofibroblasts and 96.0%±2.5% of osteoblastic cells were also cad-11 positive (Fig. 7D). These results suggest that cad-11 is associated with myofibroblastic activation and osteoblastic differentiation of the valve interstitial cells (VICs) in diseased valves.

Discussion

Cad-11 is a well-established mesenchymal cadherin involved in skeletal morphogenesis, osteogenic differentiation, and arthritic fibrosis, suggesting a role at the interface between fibrous and osseous tissues. Heart valve formation and disease also involves proper fibroblastic differentiation, and in pathological conditions leads to matrix mineralization, which suggests that cad-11 may be involved. Our results here clarify that cad-11 is expressed in avian and mouse heart valves with unique spatial and temporal characteristics. Cad-11 is found in the endothelium and mesenchyme of embryonic atrioventricular cushions and outflow tract cushions, but it becomes confined to the endothelial layers of semilunar, and to lesser degree, atrioventricular valves as they mature. Mesenchymal cells from different origins proliferate and migrate into the cardiac jelly and become endocardial cushions, valve primordia, and the cardiac septa of the adult heart [Markwald et al., 1975; Markwald et al., 1977]. Considering the highly migratory ability of the mesenchymal cushion cells and of cancer cells, and the up-regulation of cad-11 coinciding with tumor progression in adult tissue [Pishvaian et al., 1999; Tomita et al., 2000], our results suggest cad-11 may play a role in promoting cell migration into the cardiac jelly during early development [Valencia et al., 2004].

Recent studies have highlighted similarities in the transcriptional regulation of embryonic valve maturation and bone-tendon morphogenesis [Chakraborty et al., 2008]. Hutcheson and colleagues [Hutcheson et al., 2013] showed that cad-11 was upregulated in human calcified aortic valves, but cell specific expression was not investigated. Our findings in this study indicate similar results in diseased aortic valves: cad-11 is upregulated in both diseased mouse and human calcified aortic valves on the protein level. We further specified the cell types that over express cad-11 in calcified aortic valves. In ApoE$^{-/-}$ mice, cad-11 is increased in the endothelial and interstitial cells, suggesting that cad-11 may be involved in early inflammation activation of VECs. The first stage of CAVD is an inflammation activation of the valve endothelial cells (VECs), characterized by the recruitment of circulating inflammatory monocytes and T cells [Muller et al., 2000; Guerraty et al., 2010] and by EMT [Mahler et al., 2013]. EMT occurs in postnatal valves and creates a type of progenitor-like cell that maintains the interstitial cell population [Paranya et al., 2001]. We have previously reported that both TNF-α and IL-6 induce EMT in embryonic and adult valve endothelium via an AKT/NFκB-dependent pathway [Mahler et al., 2013]. Schneider and colleagues shown cad-11 knockdown can decrease EMT in A549 epithelial cell lines [Schneider et al., 2012]. Further researches on whether cad-11 is involved in the EMT process in the adult healthy and/or calcified valves will yield interesting results.

The second stage of CAVD is characterized by VICs undergoing myofibroblastic activation, depicted by an increase in αSMA, increased migration, increased proliferation, and increased traction force based matrix compaction [Kaden et al., 2005; Chen et al., 2009; Yip et al., 2009]. Late stage CAVD is portrayed by lesions of large calcified regions with osteogenic differentiating VICs that express a panel of osteoblast-related genes, including

osteocalcin, osteonectin, and the transcription factor cbfa1/runx2 [Mohler et al., 1999; Osman et al., 2006]. Cad-11 upregulation is seen in VICs in human calcified aortic valves, with above 90% myofibrolastic-active and 96% osteogenic-active VICs expressing cad-11, suggesting cad-11 may trigger VIC myofibrolastic and osteogenic activation. The VIC family of cells can be divided into several groups according to their functions in normal valve physiology and in pathological processes—embryonic progenitor cells, quiescent VICs, progenitor VICs, and osteoblastic VICs [Liu et al., 2007]. A subset of valve interstitial cells is from hematopoietic stem cell origin [Visconti et al., 2006]. Cad-11 transfected E-cadherin$^{-/-}$ embryonic stem cells directly differentiate into chondrogenic and osteogenic phenotypes [Kii et al., 2004]. It is unclear whether cad-11 exclusively triggers resident valvular interstitial cells or if it triggers other sources, such as hematopoietic stem cells, to differentiate into osteogenic phenotype in diseased valves. Given the high proportion of mesenchymal cell expression of cad-11 in diseased valves in our study, we believe that each cell group has this capacity.

In our previous research, we have demonstrated that cad-11 is a mechanosensitive protein expressed in the aortic valve endothelium that is upregulated by steady shear stress [Butcher et al., 2006]. Cad-11 is also expressed in osteoblast cells [Okazaki et al., 1994] but it is unknown why VECs maintain elevated cad-11 expression during normal valve function. Cad-11 has been shown to function at lower Ca^{++} concentrations than normal cadherins [Hutcheson et al., 2013] and cad-11 junctions withstand 2-fold higher forces when compared with connections formed with N-cadherin [Pittet et al., 2008]. We did not find side specific differences in cad-11 expression in valve endothelial cells in mice. One explanation could be that mouse valves are thin - there may not be much of a "side" with respect to fibrosa vs. ventricularis (In contrast, in larger mammals/humans, there is a higher likelihood for a side difference). The co-expression of cad-11 and PECAM-1 in healthy aortic valve leaflets suggests that cad-11 may function to maintain the endothelial layer's monolayer integrity. Interestingly, we found co-expression of cad-11 and PECAM-1 inside diseased aortic valves consistent with angiogenic vessels. Hematopoietic stem cells have been reported to promote angiogenesis through angiopoietin-1during embryonic development [Takakura et al., 2000]. Whether cad-11 expressing cells are involved in stem-cell-associated angiogenesis in the calcification process, or if cad-11 expressing cells are involved in the formation of vessels derived from endothelial cells that underwent EMT is still unknown.

In conclusion, our results illustrate the expression pattern of cad-11 on both the gene and protein levels. Our data supports the hypothesis that cad-11 may be involved in mesenchymal cell migration during embryonic heart valve development. Cad-11 may also be involved in myofibroblastic and osteogenic activation during heart valve disease. More research is warranted to better elucidate how cad-11 interacts with other proteins in the promotion of valve cell migration and calcification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

AVC	atrioventricular cushion
ApoE	Apolipoprotein E
CAVD	calcified aortic valve disease
Cad-11	cadherin-11
EMT	endothelial to mesenchymal transformation
OFT	outflow tract
PECAM-1	platelet endothelial cell adhesion molecule
TGFβ1	transforming growth factor β1
VEC	valve endothelial cell
VIC	valve interstitial cell

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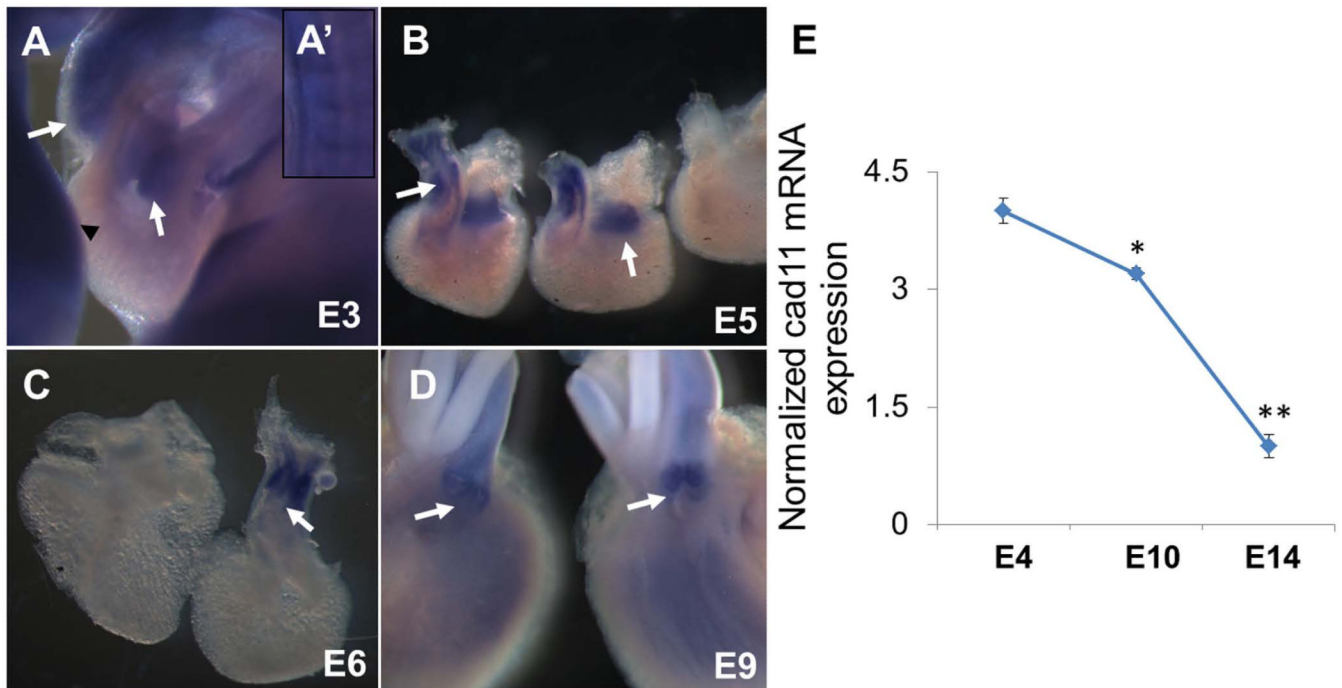
Fig. 1.
RNA level expression of *cad-11* in different stages of chick hearts. **A-D,** *In situ* hybridization of *cad-11* at E3 (A), E5 (B), E6 (C), and E9 (D). **A',** *Cad-11* expression in somites at E3, consistent with previous research. White arrows indicate strong probe binding at the valve progenitor cells and valve cells. **E,** Real-time PCR quantification of *cad-11* expression during aortic valvulogenesis. The values are *cad-11* expression normalized to 18S rRNA and are the mean ± SEM (n=3, *p<0.05, between E4 and E10; **p<0.05, between E10 and E14).
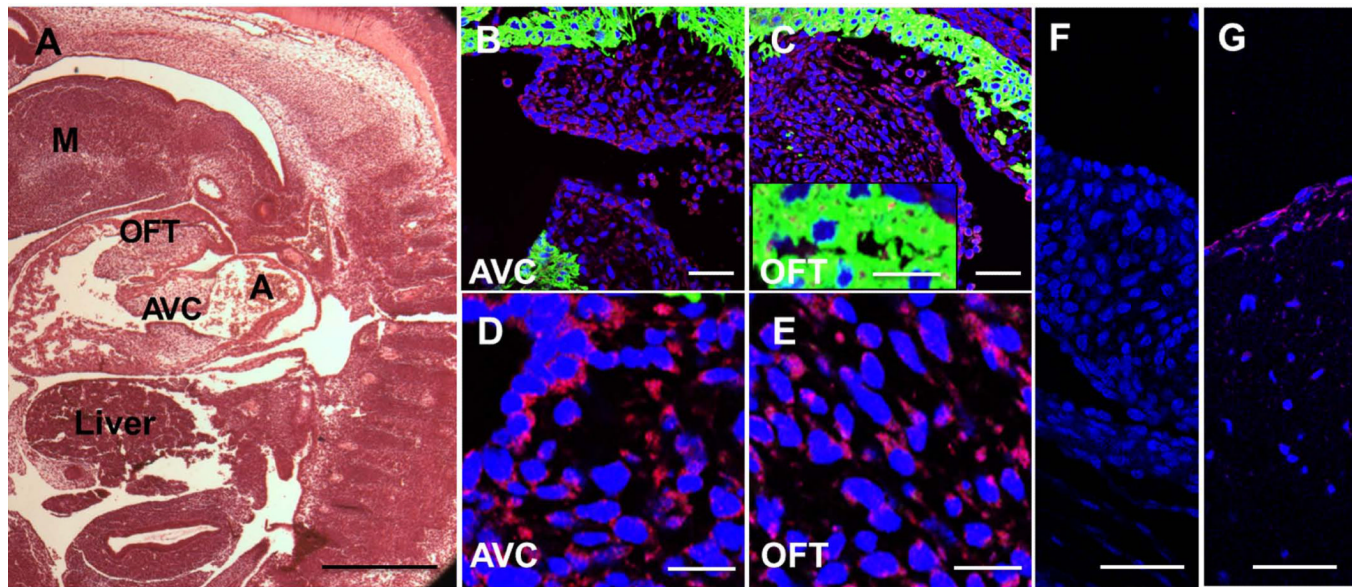
Fig. 2.
Mesenchymal expression of cad-11 in E11 mice. **A,** Hematoxylin and eosin staining of the heart valve progenitor region-the OFT and AV cushion of the heart in sagittal sections. **B, C,** Immunofluorescence staining of cad-11 protein (red) and MF20 (green) in the mesenchymal cells of the OFT (B) and the AV cushion (C). **D, E,** Higher magnification of immunofluorescence staining of cad-11 protein (red) and MF20 (green) in the mesenchymal cells of the AV cushion (D) and the OFT (E). **F,** Negative control without primary antibody showed a clean background. **G,** Positive staining of cad-11 (red) is visible in endothelial cells of healthy human aortic valve leaflets. Abbreviations: A, atrium; OFT, outflow tract; AVC, atrioventricular cushion; M, Mandibular component of first branchial arch. Scale bar = (A) 0.5mm, (B,C, F,G) 50 μm, (D, E) 20 μm.
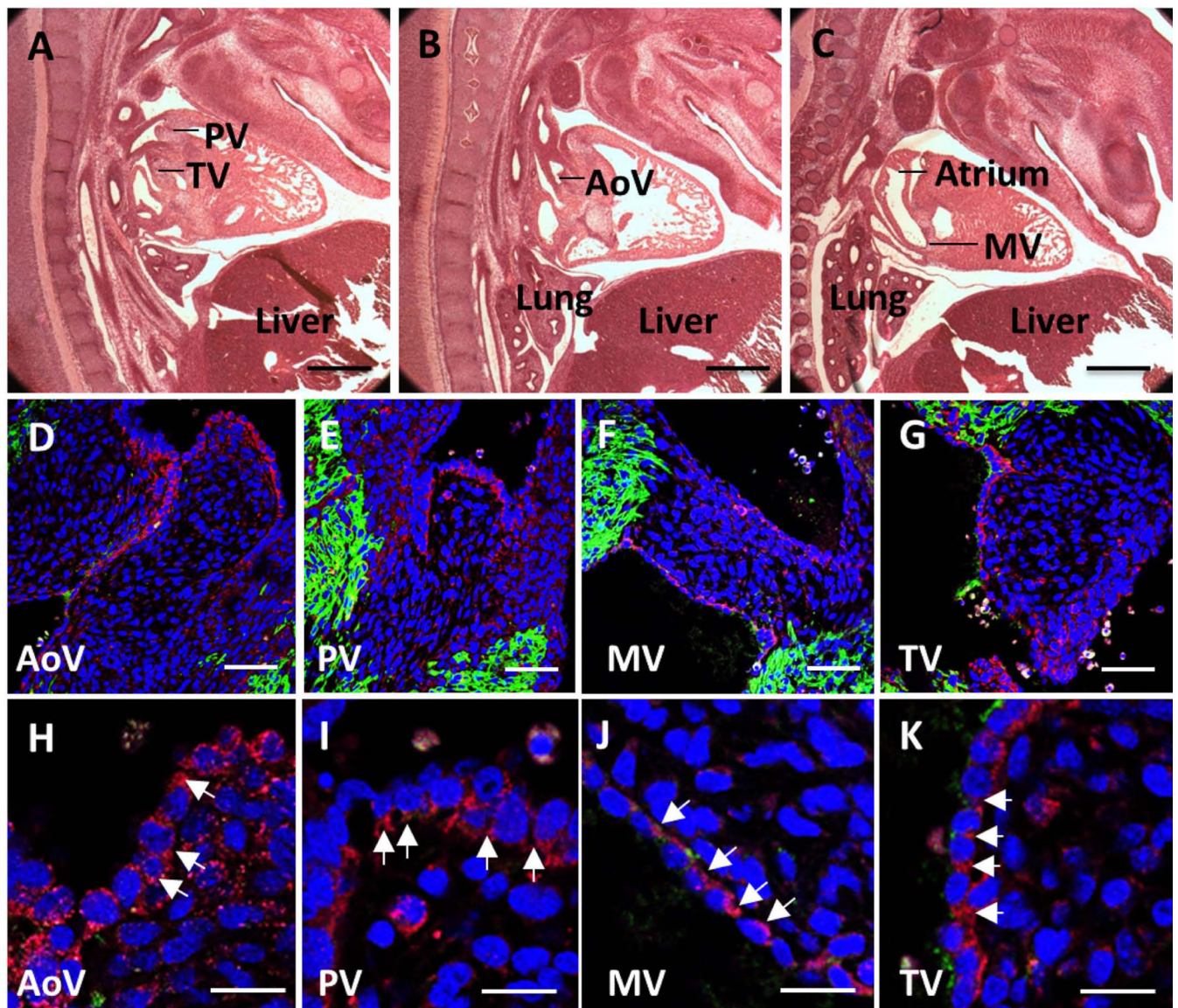
Fig. 3.
Endothelial expression of cad-11 in E14 mice. **A-C,** Hematoxylin and eosin staining of the aortic valve, pulmonic valve, mitral valve, and tricuspid valve in sagittal sections. **D, H,** Immunofluorescence staining of cad-11 protein (red) and MF20 (green) at low (D) and high (H) magnification of the aortic valve. **E, I,** Immunofluorescence staining of cad-11 protein (red) and MF20 (green) at low (E) and high (J) magnification of the pulmonic valve. **F, J,** Immunofluorescence staining of cad-11 protein (red) and MF20 (green) at low (F) and high (J) magnification of the mitral valve. **G, K,** Immunofluorescence staining of cad-11 protein (red) and MF20 (green) at low (G) and high (K) magnification of the tricuspid valve. White arrows indicate endothelial cells expressing cad-11. Abbreviations: AoV, aortic valve; PV, pulmonic valve; MV, mitral valve; TV, tricuspid valve. Scale bar = (A-C) 0.5mm, (D-G) 50 µm, (H-K) 20 µm.
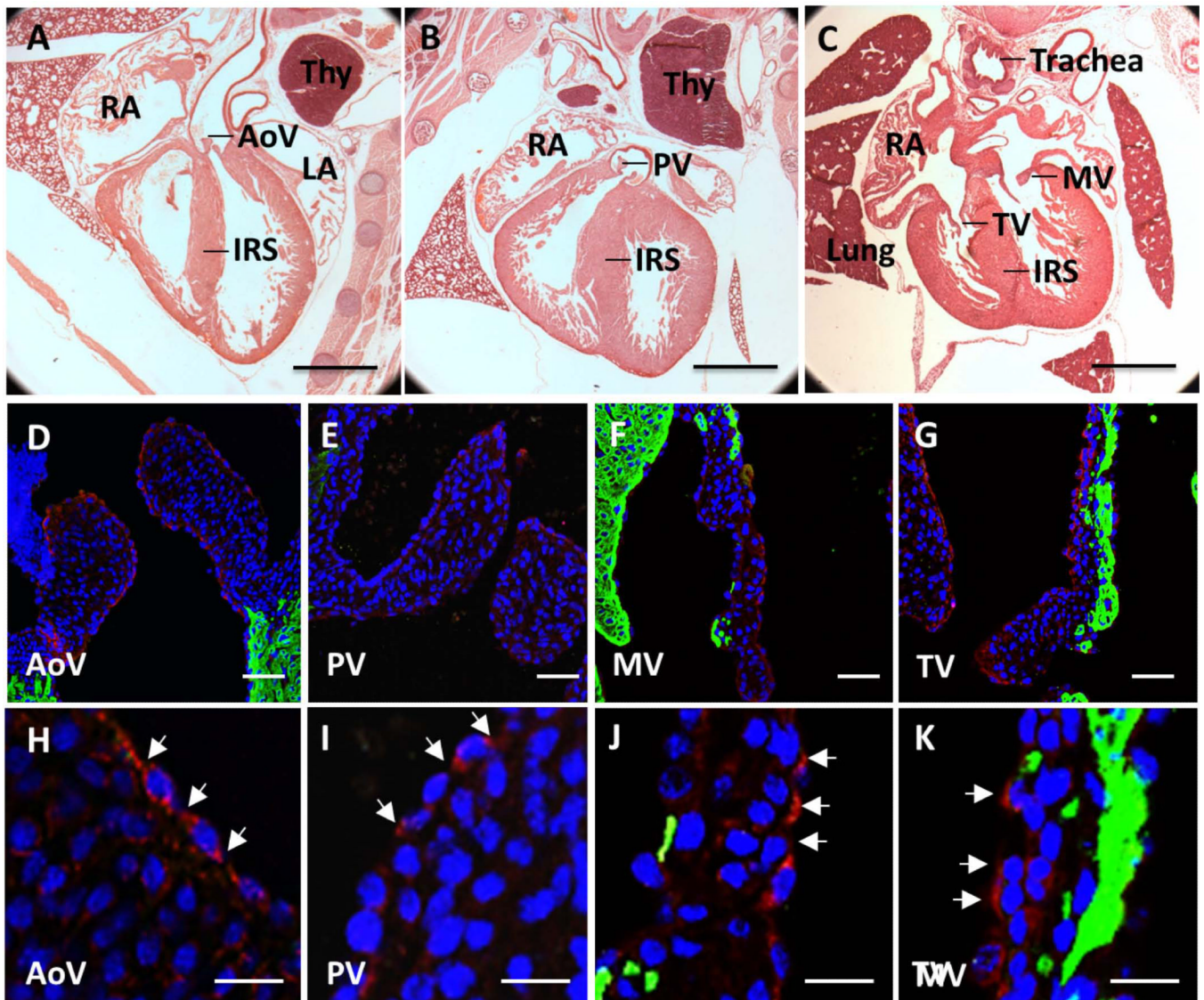
Fig. 4.
Endothelial expression of cad-11 in E18 mice. **A-C,** Hematoxylin and eosin staining of the aortic valve, pulmonic valve, mitral valve, and tricuspid valve in frontal sections. **D, H,** Immunofluorescence staining of cad-11 protein (red) and MF20 (green) at low (D) and high (H) magnification of the aortic valve. **E, I,** Immunofluorescence staining of cad-11 protein (red) and MF20 (green) at low (E) and high (J) magnification of the pulmonic valve. **F, J,** Immunofluorescence staining of cad-11 protein (red) and MF20 (green) at low (F) and high (J) magnification of the mitral valve. **G, K,** Immunofluorescence staining of cad-11 protein (red) and MF20 (green) at low (G) and high (K) magnification of the tricuspid valve. White arrows indicate endothelial cells expressing cad-11. Abbreviations: AoV, aortic valve; PV, pulmonic valve; MV, mitral valve; TV, tricuspid valve; LA, left atrium; RA, right atrium; Thy, thymus; IRS, Interventricular septum. Scale bar = (A-C) 1mm, (D-G) 50 µm, (H-K) 20 µm.
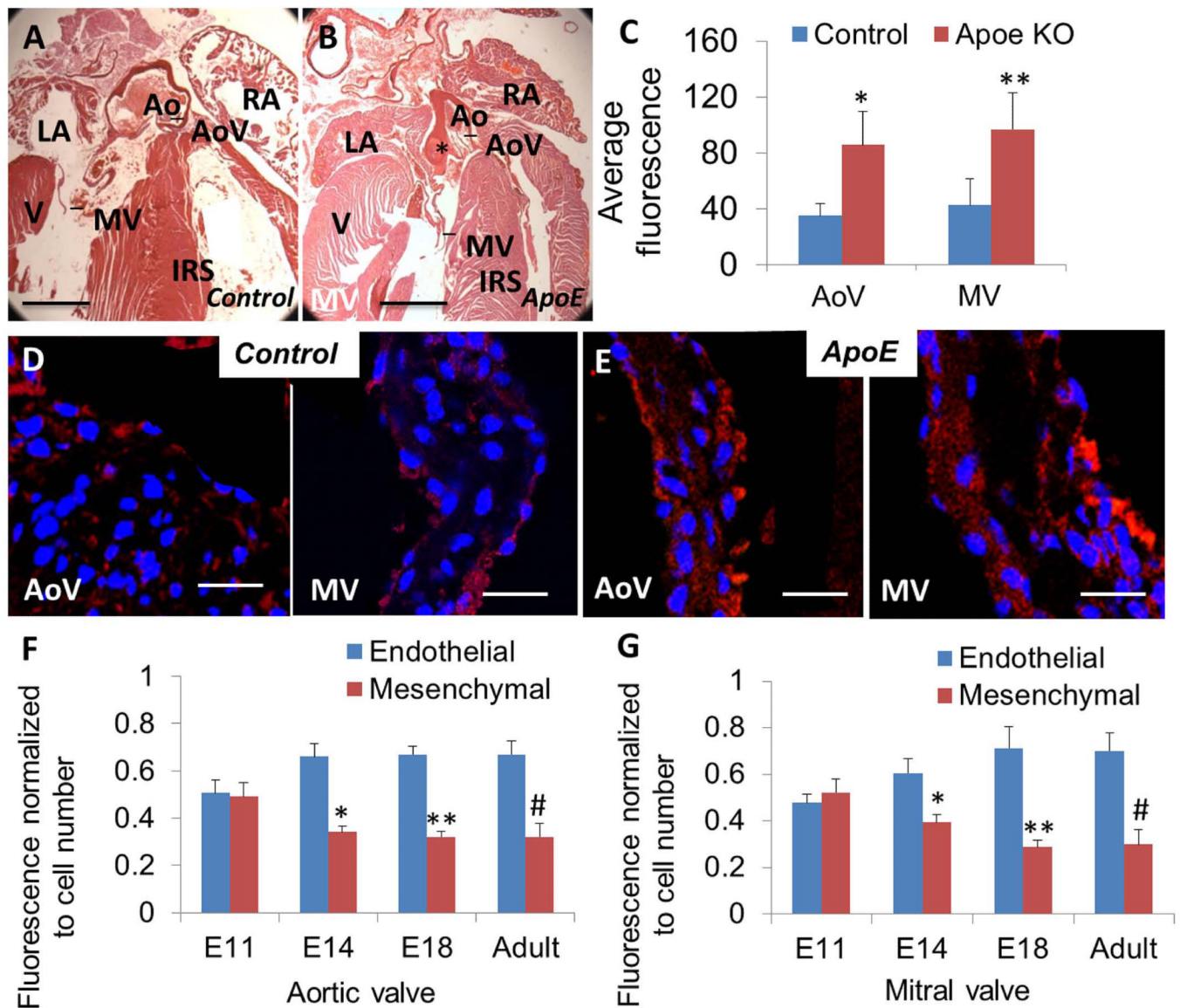
Fig. 5.
Histological and immunohistochemical analysis of control and ApoE$^{-/-}$ heart valve leaflets. **A, B,** Hematoxylin and eosin staining of control (A) and ApoE$^{-/-}$ (B) adult mice transverse sections. Notice the ApoE$^{-/-}$ aortic artery exhibits large plaques (indicated by *). **C-E,** Immunofluorescence staining of cad-11 protein (red) of ApoE$^{-/-}$ aortic and mitral valves (E) showed increased expression (C) compared to control aortic and mitral valves (D). Values are average fluorescence intensity and are means ± SEM (*p<0.05, section number = 3; **p<0.05, section number =3). **F,** Trends in expression of cad-11 protein from E11 to adult in the aortic valve progenitor cells and leaflets. **G,** Trends in expression of cad-11 protein from E11 to adult in the mitral valve progenitor cells and valve leaflets. Values are average fluorescence intensity and are means ± SEM (*p<0.05, between E11 and E14 mesenchymal fluorescence; **p<0.05, between E11 and E18 mesenchymal fluorescence; #p<0.05, between E11 and adult mesenchymal fluorescence; E11, n=9 (AoV), 9 (MV); E14, n=9 (AoV), 10 (MV); E18, n=10 (AoV), 11 (MV); Adult, n=10 (AoV), 9 (MV)). Abbreviations: AoV, aortic valve; MV, mitral valve. Scale bar = (A, B) 1mm, (D, E) 20 µm.
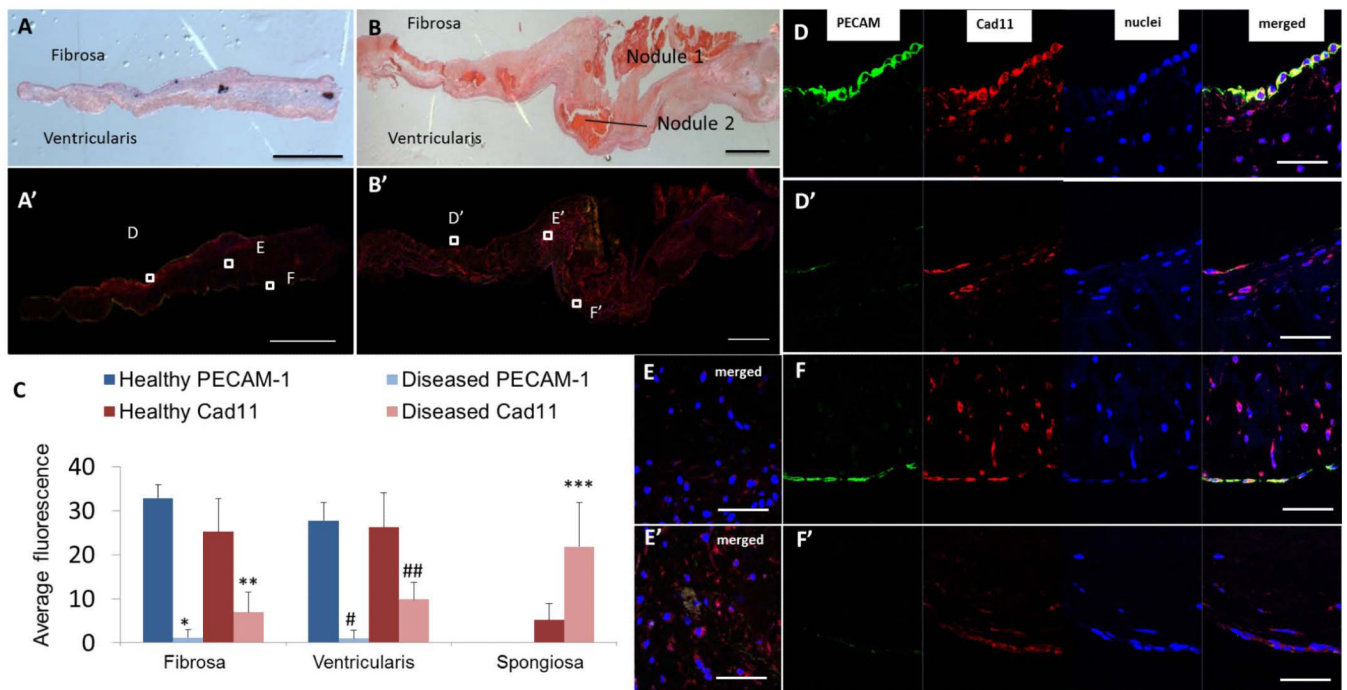
Fig. 6.
Histological and immunohistochemical analysis of normal and calcified human aortic valve leaflets. **A, B,** Hematoxylin and eosin staining of normal (A) and calcified (B) human aortic valve leaflets. Notice the calcified aortic valves exhibit disturbed endothelium layers and several nodules. **A', B',** Double immunofluorescence staining of cad-11 protein (red) and PECAM-1 (green) of normal (A') and calcified (B') human aortic valve leaflets. **C–F',** Co-localization of cad-11 and PECAM-1 in normal human aortic valve endothelial cells of fibrosa (D) and ventricularis (F). Decreased PECAM-1 and cad-11 expression was found in calcified aortic valve leaflets of fibrosa (D') and ventricularis (F'). Increased cad-11 expression in calcified aortic valve leaflets (E'), compared to normal leaflets (E). Values are average fluorescence intensity and are means ± SEM. (*$p<0.05$, between healthy and diseased PECAM-1 fluorescence on the fibrosa, n=10; **$p<0.05$, between healthy and diseased cad-11 fluorescence on the fibrosa, n=10; #$p<0.05$, between healthy and diseased PECAM-1 fluorescence on the ventricularis, n=10; ##$p<0.05$, between healthy and diseased cad-11 fluorescence on the ventricularis, n=10; ***$p<0.05$, between healthy and diseased cad-11 fluorescence in the intersititium, n=6). Scale bar = (A–B') 1 mm, (D–F') 50 µm.
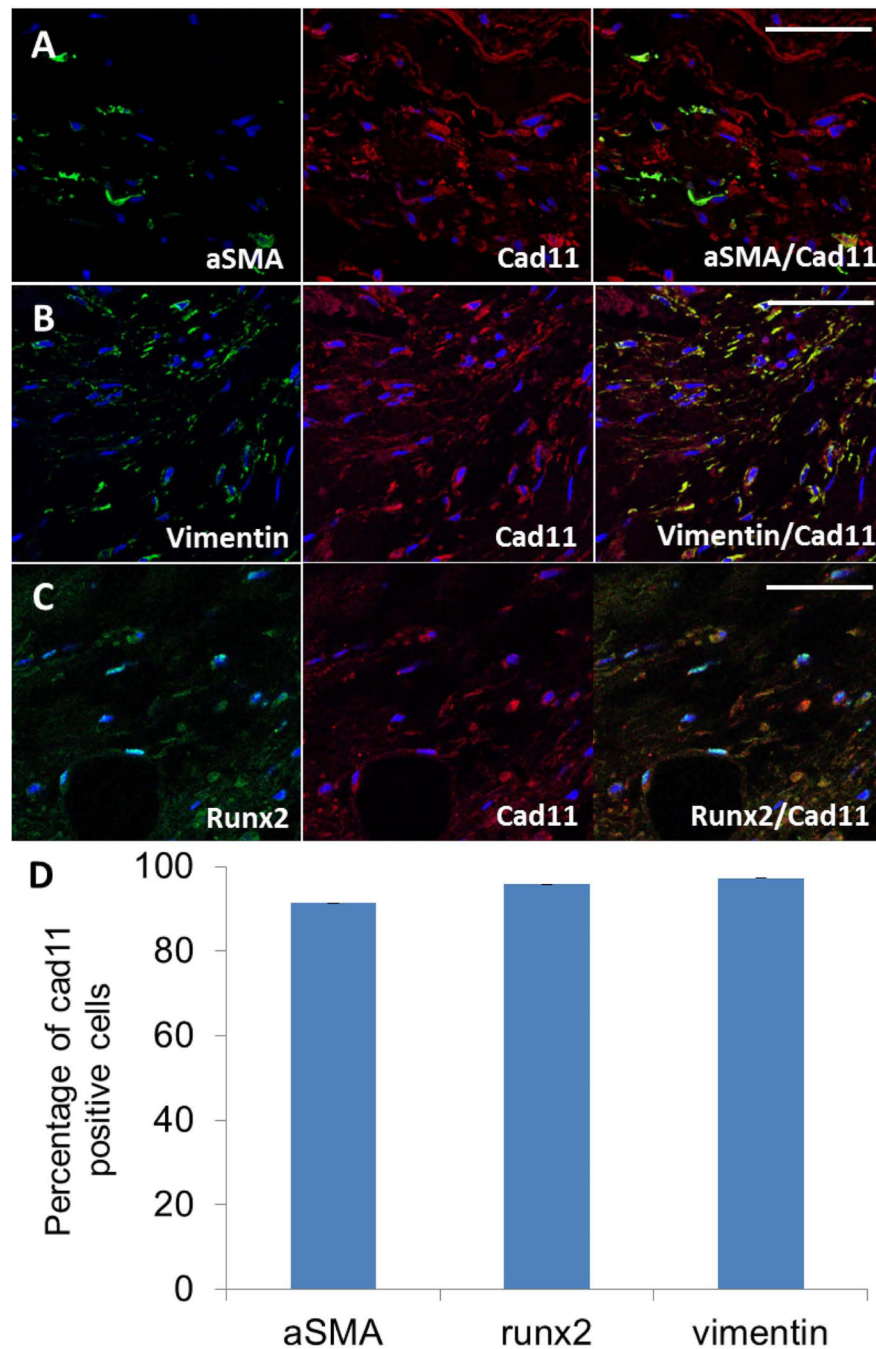
Fig. 7.
Co-staining of cad-11 with αSMA, vimentin, or runx2 in the valve interstitial cells (VICs) of human calcified valve samples. **A,** Double immunofluorescence staining of cad-11 protein (red) with αSMA protein (green) in the VICs of human calcified valve samples. **B,** Double immunofluorescence staining of cad-11 protein (red) with vimentin protein (green) in the VICs of human calcified valve samples. **C,** Double immunofluorescence staining of cad-11 protein (red) with runx2 protein (green) in the VICs of human calcified valve samples. **D,** Quantification of αSMA, vimentin, and runx2 expressing cells in cad-11 positive VICs. Values are average percentage of cad-11 positive cells and are means ± SEM. (αSMA,

section number =3; vimentin, section number =3; runx2, section number =3). Scale bar = 50 µm.