Published in final edited form as:

Cardiovasc Pathol. 2015 ; 24(3): 160–167. doi:10.1016/j.carpath.2014.10.006.

Claudin-5 levels are reduced from multiple cell types in human failing hearts and are associated with mislocalization of ephrin-B1

Sarah A. Swager, B.S.^{#1}, Dawn A. Delfín, Ph.D.^{#1,&}, Neha Rastogi, M.S.^{1,2,4}, Honglan Wang, Ph.D.^{2,4}, Benjamin D. Canan, M.P.H.^{2,4}, Vadim V. Fedorov, Ph.D.^{2,4}, Peter J. Mohler, Ph.D.^{2,4}, Ahmet Kilic, M.D.³, Robert S.D. Higgins, M.D.³, Mark T. Ziolo, Ph.D.^{2,4}, Paul M.L. Janssen, Ph.D.^{2,4}, and Jill A. Rafael-Fortney, Ph.D.^{1,2,4,*}

¹Dept. of Molecular & Cellular Biochemistry, The Ohio State University, Columbus, OH 43210.

²Dept. of Physiology & Cell Biology, Wexner Medical Center, The Ohio State University, Columbus, OH 43210.

³Dept. of Surgery, Wexner Medical Center, The Ohio State University, Columbus, OH 43210.

⁴Davis Heart & Lung Research Institute, Wexner Medical Center, The Ohio State University, Columbus, OH 43210.

[#] These authors contributed equally to this work.

Abstract

Claudin-5 is transcriptionally downregulated resulting in dramatically reduced protein levels in human heart failure. Studies in mice have demonstrated that reduced claudin-5 levels occur prior to cardiac damage and far before reduced whole heart function. Therefore, claudin-5 may be a useful early therapeutic target for human heart failure. However, the cell types in which claudin-5 is localized in human heart and from which claudin-5 is reduced in heart failure is not known. The recent identification of claudin-5's interaction with ephrin-B1 in mouse hearts has also not been investigated in non-failing or failing human hearts. In this study we collected human left ventricular midmyocardium histological samples from 7 non-failing hearts and 16 end-stage failing hearts. Immunoblots demonstrate severe reductions of claudin-5 protein in 14 of 16 failing hearts compared to non-failing controls. Claudin-5 was observed to localize to cardiomyocytes, endothelial cells, and a subset of fibroblasts in non-failing human heart sections. In isolated cardiomyocytes, the transmembrane claudin-5 protein localized in longitudinal striations in lateral

© 2014 Elsevier Inc. All rights reserved.

* Address for correspondence: Jill A. Rafael-Fortney, Ph.D. Dept. of Molecular and Cellular Biochemistry College of Medicine, The Ohio State University 410 Hamilton Hall, 1645 Neil Ave. Columbus, OH 43210 (614) 292-7043 (phone); (614) 292-4118 (fax) rafael-fortney.1@osu.edu.

&Current address: Assistant Professor, Clinical & Translational Sciences Center KL2 Scholar, Dept. of Pharmaceutical Sciences, College of Pharmacy, University of New Mexico

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

DISCLOSURES

The authors have no competing interests with industry.

membranes. In failing heart, both cardiomyocyte and endothelial claudin-5 localization was severely reduced, but claudin-5 remained in fibroblasts. Absence of claudin-5 staining also correlated with the reduction of the endothelial cell marker CD31. Ephrin-B1 localization, but not protein levels, was altered in failing hearts supporting that claudin-5 is required for ephrin-B1 localization. These data support that loss of claudin-5 in cardiomyocytes and endothelial cells is prevalent in human heart failure. Investigating claudin-5/ephrin-B1 protein complexes and gene regulation may lead to novel therapies.

Keywords

claudin-5; ephrin-B1; human myocardium; heart failure; cell junctions; human heart explants

1. INTRODUCTION

The five-year survival rate for heart failure is about fifty percent overall and decreases with advancing stages of disease [1]. Understanding the molecular mechanism of human cardiomyopathy may lead to earlier novel treatment targets and approaches. We previously identified a cardiac reduction of the cell junction protein claudin-5 in a mouse model of muscular dystrophy that displays physiological, histological, and biochemical indicators of heart failure [2-5]. Claudin-5 transcript and protein levels are reduced at a very early stage of pathology, and sustained expression of claudin-5 in this model is able to prevent the onset of cardiomyopathy [5, 6]. These data suggest that claudin-5 may represent a novel treatment target early in the pathway to heart failure.

We then demonstrated potential clinical relevance by identifying reduced claudin-5 protein levels in a large collection of failing human cardiac explant samples compared to non-failing controls [7]. Reductions of claudin-5 levels were independent of, and much more common than alterations in other cell junction and membrane associated proteins known to be involved in human heart failure, including connexin-43 and dystrophin [7-9]. No other cell junction proteins analyzed were commonly reduced in these human failing samples [7]. An unbiased screen was recently performed using two independent computational analyses of gene expression and promoter microarray data comparing human dilated cardiomyopathy (DCM) and non-failing hearts [10]. The claudin-5 promoter was one of only four genes in DCM found to be hypermethylated in conjunction with reduced claudin-5 gene expression. Together, these studies suggest that changes in claudin-5 regulation may be one of the most common alterations in human heart failure.

Claudin-5 is a 23 kilodalton four-transmembrane protein known to form tight junctions between endothelial cells at the blood-brain-barrier [11]. However, the function of claudin-5 in the heart is unknown. A recent study identified ephrin-B1, a member of the transmembrane ephrin family that act as ligands for Eph receptor protein tyrosine kinases, to be in a complex with claudin-5 [12]. In an ephrin-B1 knockdown mouse model, claudin-5 is concomitantly reduced and is no longer localized normally in heart [12]. Mice with reduced ephrin-B1 display increased cardiomyocyte lateral membrane stiffness, increased expression of extracellular matrix components, left ventricular dilation, and an increased sensitivity to

pressure overload. Eph-Ephrin signaling is known to play essential roles in nerve and vascular development [13], but their molecular functions in adult heart remain undefined.

Although our previous study was able to identify claudin-5 reductions in failing heart protein homogenates [7], no corresponding histological sections were available. We have now collected samples from failing human hearts at transplant and non-failing donor controls to allow parallel analyses of protein levels and localization. To begin to understand the functions of claudin-5 in heart, we sought to define the various cardiac cell types with claudin-5 localization, whether claudin-5 is reduced in all cell types, and whether its reduction influences the localization of putative interacting proteins.

2. METHODS

2.1. Human tissue samples

Failing human cardiac tissue was obtained after informed consent and used per Institutional Review Board approval of The Ohio State University (IRB 2008H0113 and IRB 2012H0197). Additional human cardiac tissue was obtained in collaboration with the Lifeline of Ohio Organ Procurement program. For these tissues, the Institutional Review Board waived the need for consent and these tissues were used according to the Ohio State University guidelines regarding the use of data and/or specimens. Whole human hearts were collected within minutes after removal from tissue donors (non-failing, NF) and from explants at heart transplant surgeries (failing, F). Hearts were immediately flushed with cold cardioplegic solution, and transported in oxygenated solution to the lab. Within 1 hour, samples were frozen in Optimal cutting temperature cryoprotectant medium (OCT) in liquid nitrogen-cooled isopentane or directly in liquid nitrogen. Left ventricular samples were isolated by removing a ~1 cm² piece from the free wall at the midpoint between the left ventricular base and apex. The removed piece was then subsequently divided into 3 parts and frozen labeled as LV-mid endocardium, LV-mid myocardium, and LV-mid epicardium. A panel of 7 NF and 14 F LV-mid myocardium tissue samples were used for analyses. Two failing samples were excluded from histological analyses due to freezing damage (303122 and 278931). In addition, LV-mid epicardium and endocardium sections were analyzed for claudin-5 and ephrin localization from 3 NF and 3 F representative hearts. Clinical data (Table 1) was not collected until after analyses of all samples in this study were completed.

To assess viability of the myocardium, small isolated muscles were used to test contractile function *in vitro* from the majority of hearts collected [14]. Contracting muscles from failing hearts showed the phenotypic hallmarks of failing tissue: a negative force-frequency relationship [15, 16], and a desensitized isoproterenol response. Muscles isolated from most non-failing hearts showed a typical normal positive force-frequency response, and preserved beta-adrenergic responsiveness [17].

2.2. Histochemistry and immunofluorescence staining of tissue sections

8 µm LV-mid myocardium sections were stained with hematoxylin and eosin using standard methods and used in immunofluorescence experiments for analysis of protein localization. Immunostaining was conducted by blocking sections for 15 min in potassium phosphate

buffered saline (KPBS) plus 1% gelatin, incubating in primary antibody for 2 hours and secondary antibody for 1 hour. Antibodies were diluted in KPBS + 0.2% gelatin (KPBSG) plus 1% normal goat serum, except for ephrin-B1. For claudin-5, ephrin-B1, and ER-TR7, the sections were first post-fixed with 100% ice cold acetone followed by 0.1% Triton X-100. For ephrin-B1, DAKO Superblock and fetal bovine serum (FBS) were used. Slides were coverslipped in 2 µg/ml DAPI in vectashield (Vector).

Primary antibodies used include: polyclonal rabbit antibodies against β -catenin (Sigma 2206, 1:1000, St. Louis, MO), CD31 (Abcam AB28364, 1:50), claudin-5 (Acris AP15490PU-N, 1:300), collagen I (Abcam Ab292, 1:100), and connexin-43 (LifeTechnologies 71-07000, 1:200), monoclonal mouse anti-claudin-5 (Life Technologies 35-2500, 1:50), polyclonal goat anti-ephrin-B1 (R&D AF473, 1:25), monoclonal rat anti-ER-TR7 (Abcam 51824, 1:100), monoclonal rabbit anti-PDGFR β (Cell Signaling 3169, 1:50) and monoclonal mouse anti- α -actinin (Sigma A7811, 1:200). Secondary antibodies used include: Alexa 555 goat anti-rabbit, Alexa 488 goat anti-mouse, Alexa 555 rabbit anti-goat (Life Technologies; A11029, A21429, A21431; 1:200), Cy3 goat anti-mouse, Cy3 goat anti-rat, and Dylight 488 donkey anti-rabbit (Jackson ImmunoResearch; 115-165-146, 112-165-167, 711-485-152; 1:200). The polyclonal and the monoclonal anti-claudin-5 antibodies show the same localization pattern in tissue sections and demonstrate the same pattern of protein levels on western blots. The polyclonal anti-ephrin-B1 antibody was the same antibody validated to be specific to ephrin-B1 using a knockout mouse model in a previously published study [12]. The same anti-ephrin-B1 antibody is used for immunofluorescence and western blots. Secondary antibody-only controls showed no specific staining, only the presence of lipofuscin autofluorescence, which is observed with no antibody (Supplemental Figure 1).

Claudin-5 and ephrin-B1 staining was performed in at least duplicate on the whole panel of non-failing and failing samples; connexin-43 and β -catenin staining was carried out on the panel of 7 non-failing and 12 failing independent samples; co-stains were performed at least in duplicate on either the whole panel of independent samples (ER-TR7) or in duplicate on 4 non-failing and 4 failing representative independent samples (CD31; PDGFR β). Scoring of all collagen I stained human heart slides was carried out by 2 independent investigators.

Wide-field images were taken on a Nikon Eclipse 800 microscope using a SPOT RT slider digital camera and SPOT software. Confocal images were taken using an Olympus FV1000 Spectral Confocal microscope under a 60X oil-immersion objective with or without optical zoom.

2.3. Cardiomyocyte isolation and immunofluorescence staining

Ventricular cardiomyocytes were isolated from C57BL/10 mice under an IACUC approved protocol as described previously [18]. Immunofluorescence was performed using the antibodies and protocol similar to that described above after a saponin solubilization step [19].

Images were taken using an Olympus FV1000 Spectral Confocal microscope using a 60X oil-immersion objective with a 2X optical zoom. Z-stacks were taken for all images using a 0.39 μm step-size.

2.4. Western blots

Proteins from human LV-mid tissue homogenates (100 μg per lane) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoprobed with monoclonal mouse anti-claudin-5 (Life Technologies 35-2500, 1:125), polyclonal goat anti-ephrin-B1 (R&D AF473, 1:5000), and monoclonal α -sarcomeric actin (Sigma A2172, 1:5000) as previously described [7]. Actin was detected as a normalization control for cardiomyocyte content on the same blot as claudin-5 cut at the 37 kDa marker. Westerns of all samples were run in at least 2 independent experiments. Protein quantification of claudin-5 in each sample was obtained by densitometric analysis using Image Studio Odyssey software and was normalized to α -sarcomeric actin levels from the same blot. The failing samples were normalized to the same internal control NF samples run on each blot (685884 and 474083). Protein quantification of ephrin-B1 in representative samples with low claudin-5 were quantified using similar methodology, except for using the software NIH Image J.

3. RESULTS

Human heart explant samples ($n = 23$) were obtained from patients undergoing heart failure transplantation or from organ donors and used to assess the levels and localization of claudin-5 along with its potential protein interactor, ephrin-B1. Clinical data showed that of the failing explant hearts obtained at transplantation, 6 were classified as ischemic cardiomyopathy, 6 as dilated cardiomyopathy, and 4 as nonischemic (**Table 1**). Age, race, gender, and cause of death for donors was also collected for all samples (**Table 1**).

Histological sections of left ventricular (LV) mid-myocardium were first stained with hematoxylin and eosin and for Collagen I to assess the quality, gross morphology, and fibrotic content of the samples (**Figure 1**). Fibrosis was present in addition to intact myocardial tissue in all failing samples (**Table1**).

Western blots confirmed the reduction of claudin-5 protein levels in failing human hearts in the samples collected for the current study (**Figure 2A**). Five of the seven non-failing samples exhibited high levels of claudin-5 protein. Claudin-5 protein levels were observed to be low in 14 of the 16 protein homogenates from failing samples. Nine of the 16 failing samples showed less than 25% normal claudin-5 levels and five of 16 showed claudin-5 levels between 25-80% of normal (**Table1**). One of the two donor samples with lower claudin-5 protein levels had an ejection fraction of 25%, an abnormal ECG and an enlarged heart (352041) and the other showed an enlarged heart on X-ray (947202).

We performed claudin-5 immunofluorescence staining to assess whether reductions were focal or uniform throughout the LV mid-myocardium sections analyzed (**Figure 2B**). Non-failing samples with high claudin-5 protein levels also had uniformly bright claudin-5 staining. Claudin-5 staining was punctate between cardiomyocytes, which likely represents non-myocyte cells. Claudin-5 staining in myocytes was more diffuse and appeared localized

in longitudinal striations (**Figure 2C**). Additional immunostaining for claudin-5 in LV mid-endocardial and -epicardial samples also demonstrated a uniform pattern of staining (not shown). All failing samples exhibited reduced claudin-5 staining, including the two samples without dramatically reduced levels by western. These data support that claudin-5 reductions may have some variability throughout the ventricle.

Of note, auto-fluorescent bodies were observed in human heart sections, but could be distinguished from antibody staining by their peri-nuclear localization, cluster of grapes-like appearance, and presence in unstained sections (**Supplemental Figure 1**). Lipofuscin, a wide-spectrum fluorescent non-digestable material within perinuclear lysosomes and marker of aging in postmitotic cells including cardiomyocytes [20], is the likely cause of this autofluorescence.

To determine whether ephrin-B1 and claudin-5 may be associated in human heart failure, we investigated the levels and localization of ephrin-B1 in the same collection of protein homogenates and histological sections used for claudin-5 analyses. Ephrin-B1 levels were not reduced by western analysis in failing samples (**Figure 2A**). However, immunofluorescence showed that ephrin-B1 localization was reduced in parallel with claudin-5 from failing human heart (**Figure 2B**) and co-localizes with claudin-5 in non-failing human heart sections (**Figure 2D**). Localization of the cell junction proteins β -catenin and connexin-43 retained localization to intercalated discs in failing samples and demonstrated the presence of intact cardiomyocytes. These data further support that reduced localization of claudin-5 and ephrin-B1 in human heart are strongly correlated with human heart failure.

The bright claudin-5 staining seen throughout normal LV-mid myocardium in human heart sections is the same pattern previously observed in mouse heart [5, 6, 12] and supports that claudin-5 localizes to multiple cell types (**Figure 2B**). Claudin-5 staining appeared to be present in longitudinal striations, likely on the lateral membranes of myocytes since it is a 4-transmembrane protein (**Figure 2C**). Claudin-5 also appeared to localize to vasculature throughout cardiac muscle.

To begin to delineate the cell-type localization of claudin-5 in heart, we performed immunofluorescence staining on isolated normal myocytes, and co-immunostaining of claudin-5 with myocyte and non-myocyte cell type markers in human histological sections. Confocal images of non-failing heart sections co-stained with claudin-5 and the myocyte marker α -actinin demonstrate that the light longitudinal striations of claudin-5 staining localize to cardiomyocytes (**Figure 3A**). Myocyte claudin-5 staining appears in different optical sections from the cytoplasmic α -actinin, supporting that the transmembrane claudin-5 protein localizes to the plasma membrane. Staining of isolated mouse cardiomyocytes with two claudin-5 antibodies confirmed that claudin-5 forms longitudinal striations along the lateral membranes of cardiomyocytes (**Figure 3B**). Confocal microscopy shows that claudin-5 striations were present at the top and bottom optical sections of myocytes, but not within the middle of myocytes, further confirming that claudin-5 is present on the membrane and not within a cytoplasmic structure (**Supplemental Figure 2**). Ephrin-B1 also appeared to have a similar localization pattern of longitudinal striations as

claudin-5 (**Figure 3C**). These data corroborate previous immunogold labeling showing ephrin-B1 localization to cardiomyocyte lateral membranes in mice [12].

Cardiac fibroblasts, endothelial cells, and pericytes were co-immunostained with claudin-5 in human heart sections to further delineate claudin-5 localization in other cardiac cell types. Co-staining of claudin-5 with the fibroblast marker, ER-TR7 [21], in non-failing sections showed that some fibroblasts are positive for both ER-TR7 and claudin-5 (**Figure 4**). Even when claudin-5 localization is mostly lost in failing samples, there are still claudin-5 / ER-TR7 positive fibroblasts present (**Figure 4A**). ER-TR7 positive cells that are claudin-5 negative are also present in both non-failing and failing samples (**Figure 4A, B**), suggesting that some, but not all fibroblasts express claudin-5. The reduction of claudin-5 associated with heart failure does not appear to have a major contribution from the loss of its expression in fibroblasts.

Pericytes are another cell type present in heart, but their functions are not well defined. These cells are distinguished as being perivascular in location and have an unclear role in providing support to endothelial cells [22]. The pericyte marker PDGFR β was used to identify any localization of claudin-5 in cardiac pericytes. Coimmunostaining of non-failing and failing human heart sections for PDGFR β and claudin-5 showed that claudin-5 does not localize within pericytes, although pericytes surround vasculature containing endothelial cells that are claudin-5 positive (**Figure 5A, B, C**). Both non-failing and failing sections contained PDGFR β -positive pericytes. Therefore, pericytes do not appear to be related to the reduction of claudin-5 in human heart failure.

To confirm that claudin-5 localizes to endothelial cells in the heart and to determine whether this cell type may be reduced in parallel with claudin-5 levels, we co-stained the non-failing and failing human heart sections with antibodies against claudin-5 and CD31, an endothelial cell marker. CD31 and claudin-5 non-myocyte staining patterns almost completely overlapped in non-failing sections (**Figure 6A, B**). These data suggest that the punctate claudin-5 staining throughout non-failing myocardium (**Figure 2B**) mostly represents endothelial cells. Furthermore, when claudin-5 localization and levels are reduced in failing human heart samples, CD31 positive cells also appeared qualitatively to be reduced in number (**Figure 6A**). This data is in alignment with recent evidence implicating the loss of CD31 microparticles from endothelial cells as an important biomarker for cardiovascular disease [23].

4. DISCUSSION

The experiments in this study demonstrate the normal localization of claudin-5 in cardiomyocytes and endothelial cells in mammalian heart and show that claudin-5 protein is dramatically reduced in human heart failure. Reduction of claudin-5 levels in 2 of the donor (NF) samples, which were later identified to have cardiac pathology, supports that claudin-5 levels may even be predictive of undiagnosed heart pathology.

We also show that ephrin-B1 co-localizes with claudin-5 in non-failing human heart. Ephrin-B1 is abnormally localized in cardiomyocytes and endothelial cells in human failing

hearts with reduced claudin-5 levels, further supporting a previously shown interaction between claudin-5 and ephrin-B1 in mouse hearts [12]. Since ephrin-B1 protein levels are similar in failing and non-failing human heart samples, claudin-5 appears to be necessary for stabilizing ephrin-B1 at cell membranes, but not for regulating its transcript or protein levels.

In contrast to cardiomyocytes, which lose claudin-5 expression, the situation in endothelial cells is less clear. Although claudin-5 appears to be present in all CD31 positive cells in non-failing human hearts, CD31 positive cells appear reduced in parallel with claudin-5 reduction from failing hearts (**Figure 6**). Previous studies show that CD31 positive cells were significantly reduced in both ischemic and dilated human cardiomyopathy [24, 25]. Recent studies support that CD31 positive microparticles are shed from vascular endothelial cells into the serum in several forms of human cardiovascular disease, and that CD31 microparticle concentration in serum predicts adverse events in coronary artery disease [23, 25]. Further investigation will be required to distinguish between the loss of endothelial cells and a change in endothelial cell phenotype that results in loss of both the CD31 cell marker and claudin-5 gene expression during heart failure.

In addition to all cardiomyocytes and endothelial cells, claudin-5 also localizes to a subset of ER-TR7 positive cardiac fibroblasts. The low levels of claudin-5 retained in human failing hearts appear to be retained specifically in these fibroblasts. Previous studies suggest that PDGFR β also stains a subset of cardiac fibroblasts [22], and small PDGFR β positive cells negative for claudin-5 were also observed in this study (**Figure 5**). The presence or absence of claudin-5 localization may help to define subclasses of cardiac fibroblasts and their roles in the cardiac microenvironment.

We recently demonstrated that claudin-5 reductions occur prior to the onset of cardiac damage and that sustained expression of claudin-5 in a mouse model prevented the onset of cardiomyopathy [6]. These data, together with the prevalence of claudin-5 reductions in human heart failure, support that claudin-5 may represent a useful therapeutic target for preventing cardiomyopathy early in its progression. Since claudin-5 was one of only four genes identified to be hypermethylated and have reduced expression in failing versus non-failing human hearts [10], drugs that alter the chromatin structure at the claudin-5 promoter may show a beneficial effect. Future epigenetic studies of claudin-5 gene regulation could form a foundation for developing novel therapeutics for heart failure. In addition, modulating Eph/ephrin-B1 signaling may provide another potential therapeutic avenue to modulate claudin-5 expression, since ephrin-B1 knockdown mice show reduced claudin-5 gene expression. Ephrin and claudin protein family members are also known to interact in other tissues and disease states. Drugs targeting other ephrins involved in Eph/Ephrin signaling are already in clinical trials for other diseases including cancer [13]. Continuing to define the functions of the claudin-5/ephrin-B1 protein complex and the regulation of the claudin-5 gene in heart may aid in the development of future therapeutic strategies designed to maintain normal claudin-5 localization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was funded by the Muscular Dystrophy Association, NIH/NHLBI RC1HL099538 and R01HL113084, and the OSU Center for Muscle Health and Neuromuscular Disorders. We thank The Ohio State University Campus Microscopy and Imaging Facility. Donor hearts were obtained through collaboration of the Dorothy Davis Heart and Lung Research Institute with the Lifeline of Ohio Organ Procurement program.

REFERENCES

1. Yancy CW, Jessup M, Bozkurt B, Butler J, Casey DE Jr, Drazner MH, et al. 2013 ACCF/AHA guideline for the management of heart failure: a report of the American College of Cardiology Foundation/American Heart Association Task Force on practice guidelines. *Circulation*. 2013; 128:e240–327. [PubMed: 23741058]
2. Delfin DA, Zang KE, Schill KE, Patel NT, Janssen PM, Raman SV, et al. Cardiomyopathy in the dystrophin/utrophin-deficient mouse model of severe muscular dystrophy is characterized by dysregulation of matrix metalloproteinases. *Neuromuscul Disord*. 2012; 22:1006–14. [PubMed: 22749475]
3. Hainsey TA, Senapati S, Kuhn DE, Rafael JA. Cardiomyopathic features associated with muscular dystrophy are independent of dystrophin absence in cardiovascular. *Neuromuscul Disord*. 2003; 13:294–302. [PubMed: 12868498]
4. Janssen PM, Hiranandani N, Mays TA, Rafael-Fortney JA. Utrophin deficiency worsens cardiac contractile dysfunction present in dystrophin-deficient mdx mice. *Am J Physiol Heart Circ Physiol*. 2005; 289:H2373–8. [PubMed: 16024571]
5. Sanford JL, Edwards JD, Mays TA, Gong B, Merriam AP, Rafael-Fortney JA. Claudin-5 localizes to the lateral membranes of cardiomyocytes and is altered in utrophin/dystrophin-deficient cardiomyopathic mice. *J Mol Cell Cardiol*. 2005; 38:323–32. [PubMed: 15698839]
6. Delfin DA, Xu Y, Schill KE, Mays TA, Canan BD, Zang KE, et al. Sustaining cardiac claudin-5 levels prevents functional hallmarks of cardiomyopathy in a muscular dystrophy mouse model. *Mol Ther*. 2012; 20:1378–83. [PubMed: 22547149]
7. Mays TA, Binkley PF, Lesinski A, Doshi AA, Quaile MP, Margulies KB, et al. Claudin-5 levels are reduced in human end-stage cardiomyopathy. *J Mol Cell Cardiol*. 2008; 45:81–7. [PubMed: 18513742]
8. Dupont E, Matsushita T, Kaba RA, Vozzi C, Coppen SR, Khan N, et al. Altered connexin expression in human congestive heart failure. *J Mol Cell Cardiol*. 2001; 33:359–71. [PubMed: 11162139]
9. Vatta M, Stetson SJ, Perez-Verdia A, Entman ML, Noon GP, Torre-Amione G, et al. Molecular remodelling of dystrophin in patients with end-stage cardiomyopathies and reversal in patients on assistance-device therapy. *Lancet*. 2002; 359:936–41. [PubMed: 11918913]
10. Koczor CA, Lee EK, Torres RA, Boyd A, Vega JD, Uppal K, et al. Detection of differentially methylated gene promoters in failing and nonfailing human left ventricle myocardium using computation analysis. *Physiol Genomics*. 2013; 45:597–605. [PubMed: 23695888]
11. Morita K, Sasaki H, Furuse M, Tsukita S. Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells. *J Cell Biol*. 1999; 147:185–94. [PubMed: 10508865]
12. Genet G, Guilbeau-Frugier C, Honton B, Dague E, Schneider MD, Coatrieux C, et al. Ephrin-B1 is a novel specific component of the lateral membrane of the cardiomyocyte and is essential for the stability of cardiac tissue architecture cohesion. *Circ Res*. 2012; 110:688–700. [PubMed: 22302788]
13. Boyd AW, Bartlett PF, Lackmann M. Therapeutic targeting of EPH receptors and their ligands. *Nat Rev Drug Discov*. 2014; 13:39–62. [PubMed: 24378802]

14. Milani-Nejad N, Janssen PM. Small and large animal models in cardiac contraction research: advantages and disadvantages. *Pharmacol Ther.* 2014; 141:235–49. [PubMed: 24140081]
15. Janssen PML, Lehnart SE, Prestle J, Hasenfuss G. Preservation of contractile characteristics of human myocardium in multi-day cell culture. *J Mol Cell Cardiol.* 1999; 31:1419–27. [PubMed: 10423340]
16. Rossman EI, Petre RE, Chaudhary KW, Piacentino V 3rd, Janssen PM, Gaughan JP, et al. Abnormal frequency-dependent responses represent the pathophysiologic signature of contractile failure in human myocardium. *J Mol Cell Cardiol.* 2004; 36:33–42. [PubMed: 14734045]
17. Ziolo MT, Maier LS, Piacentino V 3rd, Bossuyt J, Houser SR, Bers DM. Myocyte nitric oxide synthase 2 contributes to blunted beta-adrenergic response in failing human hearts by decreasing Ca²⁺ transients. *Circulation.* 2004; 109:1886–91. [PubMed: 15037528]
18. Kohr MJ, Wang H, Wheeler DG, Velayutham M, Zweier JL, Ziolo MT. Targeting of phospholamban by peroxynitrite decreases beta-adrenergic stimulation in cardiomyocytes. *Cardiovasc Res.* 2008; 77:353–61. [PubMed: 18006474]
19. Zhang M, Takimoto E, Lee DI, Santos CX, Nakamura T, Hsu S, et al. Pathological cardiac hypertrophy alters intracellular targeting of phosphodiesterase type 5 from nitric oxide synthase-3 to natriuretic peptide signaling. *Circulation.* 2012; 126:942–51. [PubMed: 22829024]
20. Han L, Li M, Liu X. Effects of long-term atorvastatin treatment on cardiac aging. *Exp Ther Med.* 2013; 6:721–6. [PubMed: 24137254]
21. Van Vliet E, Melis M, Foidart JM, Van Ewijk W. Reticular fibroblasts in peripheral lymphoid organs identified by a monoclonal antibody. *J Histochem Cytochem.* 1986; 34:883–90. [PubMed: 3519751]
22. Bergers G, Song S. The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol.* 2005; 7:452–64. [PubMed: 16212810]
23. Sinning JM, Losch J, Walenta K, Bohm M, Nickenig G, Werner N. Circulating CD31+/Annexin V + microparticles correlate with cardiovascular outcomes. *Eur Heart J.* 2011; 32:2034–41. [PubMed: 21186238]
24. Hunt MJ, Aru GM, Hayden MR, Moore CK, Hoit BD, Tyagi SC. Induction of oxidative stress and disintegrin metalloproteinase in human heart end-stage failure. *Am J Physiol Lung Cell Mol Physiol.* 2002; 283:L239–45. [PubMed: 12114184]
25. Schafer R, Abraham D, Paulus P, Blumer R, Grimm M, Wojta J, et al. Impaired VE-cadherin/beta-catenin expression mediates endothelial cell degeneration in dilated cardiomyopathy. *Circulation.* 2003; 108:1585–91. [PubMed: 12963640]

Figure 1.

Gross histology and fibrosis in representative human heart sections. Hematoxylin & eosin (H&E) staining shows overall histology of representative non-failing and failing human heart sections. An antibody against Collagen I (red) demonstrates the fibrosis in representative failing samples compared to non-failing controls. Perivascular fibrosis associated with normal aging present in some of the non-failing sections can be seen in sample 488240. Bar = 100 μ m.

Figure 2.

Claudin-5 and ephrin-B1 levels and localization in human heart failure. **A)** Western blots show reduced claudin-5 protein levels in most cardiac explant failing samples compared with most non-failing samples. Ephrin-B1 levels in failing and non-failing human heart samples are not significantly different, as determined by quantification of western blots. Alpha-sarcomeric actin levels were used as internal normalization controls on the same blot as claudin-5 and show relatively equal cardiomyocyte content between samples. NF1=474083; NF2=685884; NF3=947202; F1=222565; F2=733252; F3=825654;

F4=278931; F5=625899; F6=565411. **B)** Immunofluorescence of claudin-5 (red) exhibits reduced staining in failing heart sections compared with non-failing sections. Ephrin-B1 (red) also shows reduced localization in failing compared with non-failing samples. In non-failing sections, claudin-5 and ephrin-B1 have punctate staining in non-myocytes and lighter striated staining in cardiomyocytes. The cell junction proteins β -catenin and connexin-43 were used as controls to show cardiomyocyte integrity and localize to intercalated discs in both non-failing and failing samples. Bar=100 μ m. **C)** A higher magnification confocal image shows the localization of claudin-5 protein (green) in cardiomyocytes in longitudinal striations (arrow) in a representative non-failing human heart section. Blue = DAPI stained nuclei. Bar = 20 μ m. **D)** Confocal images shows claudin-5 protein (green) and ephrin-B1 protein (red) co-localizing in longitudinal striations (arrows) on myocytes in the same representative non-failing human heart section. The asterisks show co-localization of both claudin-5 and ephrin-B1 in an endothelial cell. Blue = DAPI stained nuclei and white = autofluorescence. Bar = 10 μ m.

Figure 3.

Localization of claudin-5 in mammalian cardiomyocytes. **A)** Confocal images of a representative non-failing human heart section co-stained for claudin-5 and cardiomyocytes. Stacked optical slices show localization of claudin-5 protein (red) and the cardiomyocyte marker α -actinin protein (green). Claudin-5 striations (arrows) on myocytes are perpendicular to the cytoplasmic α -actinin striations and are not present in the same z-plane as α -actinin, supporting localization of claudin-5 in the plasma membrane. The first 2 panels show stacked optical sections equaling a 3.7 μ m thickness. The last panel shows a complete z-stack image of 10 μ m thickness. Bar = 20 μ m. **B)** Confocal images of isolated C57BL/10 normal mouse cardiomyocytes stained with claudin-5 monoclonal (mono) and claudin-5

polyclonal (poly) antibodies are shown. Longitudinal stripes of claudin-5 protein on the lateral membranes of cardiomyocytes are observed with both antibodies. These striations are only observed in optical sections near the top and bottom of the myocytes corresponding to the lateral plasma membranes, but are not present in optical sections within cardiomyocyte cytoplasm. C) Ephrin-B1 polyclonal antibody stained isolated mouse cardiomyocytes also shows localization in striations along cardiomyocyte membranes in a similar pattern to claudin-5. Cardiomyocytes stained with fluorescently-conjugated secondary antibody only are shown as negative controls for each experiment. Red = antibody; blue = DAPI stained nuclei. Single optical Z-section images and full Z-stack images are shown for each antibody. Top and bottom single optical sections for claudin-5 mono and poly, and ephrin-B1 are 12, 8, and 6 μm apart, respectively. Bar = 10 μm .

Figure 4.

Co-staining of claudin-5 and fibroblasts in human heart sections. **A)** ER-TR7 (red) positive and claudin-5 (green) positive fibroblasts (arrowheads) are present in both non-failing and failing heart sections. ER-TR7 positive and claudin-5 negative fibroblasts (arrows) are also present in both non-failing and failing samples. ER-TR7 also stains non-cellular reticular fibers in the extracellular matrix between myocytes. Cellular and non-cellular staining can be distinguished by co-localization with DAPI (blue merged images). Bar = 100 μ m. **B)** Higher magnification Z-stack confocal images show both an ER-TR7 positive, claudin-5

positive cell (arrowhead) and an ER-TR7 positive, claudin-5 negative cell (arrow) in a representative non-failing sample. Bar = 20 μ m.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5.

Co-staining of claudin-5 and pericytes in human heart sections. **A)** PDGFR β (red) positive pericytes surround blood vessels (arrow). No claudin-5 (green) localization is observed in PDGFR β positive cells identified as pericytes. DAPI = blue. Bar = 50 μ m. **B)** Higher magnification Z-stack confocal images of a representative non-failing heart sample confirm the lack of claudin-5 staining in pericytes. Bar=20 μ m. **C)** An even higher magnification confocal image shows no claudin-5 co-staining in a PDGFR β positive pericyte surrounding a blood vessel, but shows claudin-5 staining in the endothelial cell supported by the pericyte. Bar = 10 μ m.

Figure 6.

Co-staining of claudin-5 and endothelial cells in human heart sections. **A)** The pattern of CD31 (red) and non-myocyte claudin-5 (green) are almost completely overlapping in a representative non-failing Z-stack confocal image. CD31 positive cells appear to be reduced in parallel with claudin-5 in failing samples. DAPI = blue. Bar = 20 μ m. **B)** One single optical Z-section image in a non-failing sample shows an endothelial cell stained with both claudin-5 and CD31. Bar = 10 μ m.

Table 1

Clinical Data for Human Heart Samples.

Sample	Gender	Age	Cause of Death	Type of CM	Race	Col I	Claudin-5
222565	M	56		Non-Ischemic	C	+++	+
278931 *	M	64		Dilated	C	-	+
303122	F	51		Dilated	C	+++	++
328163	M	63		Ischemic	C	+	+
371558	M	36		Non-Ischemic	AA	++	+++
450564	M	30		Dilated	C	+++	+
479062	M	50		Ischemic	C	+++	++
565411 *	M	61		Non-Ischemic	C	-	+
625899	F	68		Ischemic	C	++	++
709841	M	50		Ischemic	C	+	+++
733252	M	61		Dilated	A	+++	+
814562	M	55		Ischemic	C	+++	++
825654	M	63		Non-Ischemic	C	+++	+
869123	M	30		Dilated	C	+++	++
906620	M	65		Dilated	C	+++	+
961536	F	61		Ischemic/Radiation	C	+	+
168021	F	62	CVA; ICH		C	+	+++
352041	F	28	CVA		AA	+	+
474083	F	41	CVA; SAH		AA	++	+++
488240	F	51	Anoxia; s/p CA		C	++	+++
618200	F	58	Head trauma; s/p fall		C	++	+++
685884	M	36	Anoxia; DI; CA		C	+	+++
947202	M	34	Head trauma; MVA		C	+	+

Gender, age, race, collagen I staining, and claudin-5 level data for each human heart sample is listed. The cause of death is included for non-failing donor samples and the type of cardiomyopathy necessitating transplantation for each failing sample is included.

For collagen I (Col I), + = normal amounts of cardiac Col I; ++ = 3 or more large areas of Col I staining surrounded by intact cardiomyocytes; +++ = Col I stained area representing a majority of the cardiac tissue. For claudin-5, quantitation of claudin-5 western blots normalized to α -sarcomeric actin; + = < 25% normal claudin-5 levels; ++ = 25-80% normal claudin-5 levels; +++ > 80% normal claudin-5 levels. CM = cardiomyopathy; MVA=Motor vehicle accident; CVA=Cerebral vascular accident; s/p=status post; CA=cardiac arrest; DI=drug intoxication; SAH=subarachnoid hemorrhage; ICH=intracerebral hemorrhage; C= Caucasian; AA=African American; A= African; Col I= Collagen I staining.

* The two samples excluded from histological analysis are indicated with an asterisk.