

Heterogeneity in Pkp2-Containing Junctions in the Adult Epicardium

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Abstract

The epicardium, a thin layer of mesothelial cells that encapsulate the heart, is a major source of cardiac fibroblasts during development as a result of epithelial to mesenchymal transition (EMT). EMT requires the loss of cell-to-cell adhesion and conditions and/or diseases that destabilize cellular junctions. Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC) is an inherited disorder associated with mutations in desmosomal proteins and characterized by myocardial accumulation of fibrous or fibrofatty tissue. It has been hypothesized that expression of mutant desmosomal proteins in epicardial cells might contribute to the pathogenesis of ARVC. However, this has not been tested nor has regional variation in epicardial mechanical junctions been examined. We used ultrastructural analysis and immunohistochemistry to examine the expression of desmosomal proteins in mouse epicardium. The number of structures containing Plakophilin 2 (Pkp2), a desmosomal protein that is frequently mutated in ARVC, was quantified in whole mount preparations of epicardium and normalized to the number of epicardial cell nuclei. Ultrastructural analysis revealed mechanical junctions of indeterminate morphology were in both atrial and ventricular epicardium, but were more abundant in atrial epicardium. In frozen sections, Pkp2 was expressed in the epicardium and there was more Pkp2 immunoreactivity in atrial epicardium. In whole mount preparations, both the number and density of Pkp2 containing epicardial structures were greater in the atria than the ventricles. This suggests that the epicardium exhibits heterogeneity in the abundance of Pkp2-containing mechanical junctions. Future work will determine if these junctions are bona fide desmosomes or a different type of Pkp2-containing junction. By understanding which desmosomal proteins are expressed in the adult epicardium, we also hope to gain insight into the potential role of the epicardium in the development of fibrosis in ARVC patients with mutations in other desmosomal genes.

Introduction

Arrhythmogenic Right Ventricular Cardiomyopathy (ARVC) is a genetic cardiac disease associated with mutations in desmosomal proteins in the intercalated discs, which are found between two adjacent cardiac myocytes. The disease is characterized by myocardial accumulation of fibrous and fibrofatty tissue and can result in severe ventricular tachyarrhythmias and sudden cardiac death (Saffitz et al. 2011). As a result, the heart loses its contractibility and ability to function as a pump. These arrhythmias often develop early in the progression of the disease even before significant right ventricular degeneration and fibrotic tissue accumulation occur (Saffitz et al. 2011). In more than 50% of ARVC patients, mutations in one or more genes are involved. It appears that one of the most commonly mutated proteins in ARVC is Plakophilin 2 (Pkp2), which is mainly found in the desmosome junctions in the cardiac intercalated discs. A mutation in such proteins causes a mechanical change in the desmosome due to the breakdown of junctions and the loss of cell-to-cell adhesions. These can lead to the loss of interaction between junctional proteins and cellular deformation in the cardiac tissue (Saffitz, 2011; Basso et al. 2006). This could contribute to an increase in fibrous and fibrofatty tissue in the heart and the inability of the cardiac muscle to properly contract in patients with ARVC.

Fibrosis is defined as the excess accumulation of the extracellular matrix. In the heart, cardiac fibroblasts are the main producers of the extracellular matrix. In excess, the extracellular matrix can prevent the cardiac muscles from contracting properly (Figure 1). There are two ways in which existing cardiac cells can give rise to new cardiac fibroblasts. First, existing fibroblasts can proliferate to produce more fibroblasts. Second, endothelial cells and epicardial cells can

undergo a process called epithelial-to-mesenchymal transition (EMT) to further differentiate into fibroblasts, and smooth muscle cells can also dedifferentiate into fibroblasts (Figure 2).

The epicardium, a single layer of mesothelial cells that encapsulate the heart, makes important contributions to the development of the fetal myocardium and the formation of the coronary vasculature and the fibrous skeleton that support the heart. During cardiac development, the epicardium originally encapsulates the mesenchymal myocardium. Then later during the developmental stage, epicardial cells can undergo epithelial-to-mesenchymal transition (EMT) and migrate into the myocardium (Stamm et al. 2009). During EMT, the epicardial cells first become epicardial derived progenitor cells (EDPCs). Then, as they migrate from the outer surface of the heart into the myocardium, they further differentiate into various cells types, including adipocytes, endothelial cells, smooth muscle cells, and fibroblasts or myofibroblasts (Figure 3) (Zhou et al. 2011). Furthermore, EMT is characterized by the loss of adhesion and epithelial characteristics of epicardial cells and the acquisition of a mesenchymal phenotype (Figure 4).

Normally, epithelial cells are organized as flat sheets with tight and adheren junctions anchoring the cells along the apical-basal axis, perpendicular to the epithelial plane (von Gise et al. 2012). On the other hand, mesenchymal cells do not have the tight junctions and the flat morphology of epithelial cells. These characteristics allow them more free movement as individual cells in the extracellular matrix. In addition, mesothelial cells have the potential to further differentiate into multiple cell types and acquire various cellular morphologies (von Gise et al. 2012). Therefore, in EMT, epithelial cells lose their flat and stable conformation and transition to acquire a more mobile and dynamic cell shape. Often, errors in EMT can contribute to the development of congenital heart diseases (von Gise et al. 2012).

While it is known that in the developing heart EMT occurs, it is uncertain whether EMT also occurs in the post-developmental heart. Thus, it is unclear whether the epicardium can give rise to new cardiac non-myocytes or is involved in myocardial regeneration. There is a growing body of evidence that shows that disease-mediated activation of EMT may contribute to the development of certain diseases that are characterized by cardiac fibrosis (von Gise et al. 2012). While studies have shown that the epicardium does not undergo EMT in the adult mammalian heart (Zhou et al. 2011), it has been suggested that in some cases, epicardium can be activated during myocardial injury, such as myocardial infarction (MI), which causes epithelial cells to develop into an expanded layer of EDPCs. The EDPCs stay in the epicardial mesenchyme and promote coronary vessel growth. The EDPCs then secrete paracrine factors that participate in the injury response. However, they do not further differentiate into other cell types (Zhou et al. 2011). On the other hand, some other studies have shown that some epicardial cells can further differentiate into cardiac myocytes. Due to myocardial injury, some molecules and inflammatory mediators, such as Tgf β , can trigger the epicardium to undergo EMT (Stamm et al. 2009). Then the generation of EDPCs can contribute to myofibroblast and fibroblast proliferation, which can further contribute to an increased amount of extracellular matrix in the myocardium, resulting in the development of fibrosis in hearts with congenital disorders (Figure 5).

In ARVC, protein mutations occur in the intercalated discs of cardiac myocytes. More specifically, mutations occur in the desmosomes, a type of cellular mechanical junction that is present in various cell types. They are normally found between epithelial cells and the intercalated discs of the cardiac myocytes. Unlike adheren junctions, which anchor actin filaments, desmosomes anchor intermediate filaments (either keratin or desmin) to the cell surface, forming strong mechanical attachments (spot welds) between neighboring cells

(Thomason et al. 2010). Cell-to-cell adhesion is provided by the desmosomal cadherins (desmocollins and desmogleins). The desmosomal complex (Figure 6) includes two armadillo repeat proteins, plakophilin (Pkp) and plakoglobin (Jup), which are essential proteins for the proper function of desmosomes. Together, plakoglobin, plakophilin, and desmoplakin link the structure of the intermediate filament network. Plakophilin 2 (Pkp2) is the most ubiquitously expressed of the plakophilin proteins, and is the only plakophilin expressed in ventricular cardiac myocytes (Saffitz et al. 2011). Pkp2 contains two long splice variants (Pkp2a, 92 kDa and Pkp2b, 97 kDa). These two isoforms co-localize in the nucleus and desmosomal plaque (Mertens et al. 1996). Without functional Pkp2, desmosomes are unable to form proper cell-to-cell contacts (Grossmann et al. 2005). It has been reported that Pkp2 mutations are present in 70% of cases of ARVC with an identifiable genetics link (Saffitz et al. 2011).

Since the epicardium consists of a thin layer of epithelial cells, it is possible that desmosomes are also found in the epicardium. Since Pkp2 mutations prevent desmosomes from forming proper junctions, it is possible that these mutations may also impact the structure and integrity of mechanical junctions in the epicardium. The destruction of desmosomal integrity in the epicardium may then alter the propensity of epicardial cells to undergo EMT and contribute to remodeling of the heart. However, the composition of epicardial cell junctions has not yet been determined in the adult heart epicardium. The expression of desmosomal protein genes in the epicardium may influence the effect of ARVC mutations on the cardiac epicardium. Our hypothesis is that if epicardial cells express the desmosomal protein isoforms that are mutated in ARVC, then the epicardium may contribute to the pathogenesis of ARVC. In addition, chamber specific differences in epicardial desmosome abundance may contribute to the localization of fibrosis in ARVC.

We used scanning electron microscopy and immunohistochemistry to locate and visualize Pkp2 in the wild-type mouse epicardium. In addition, we developed a novel epicardium whole mount technique that enabled us to better characterize the Pkp2 localized in the epicardium and quantify the varying abundance of Pkp2 in different chambers of the heart. These findings contribute to the evidence that Pkp2 is indeed present in the epicardium, and its heterogeneity in distribution in different locations of the heart may contribute to the pathogenesis of ARVC.

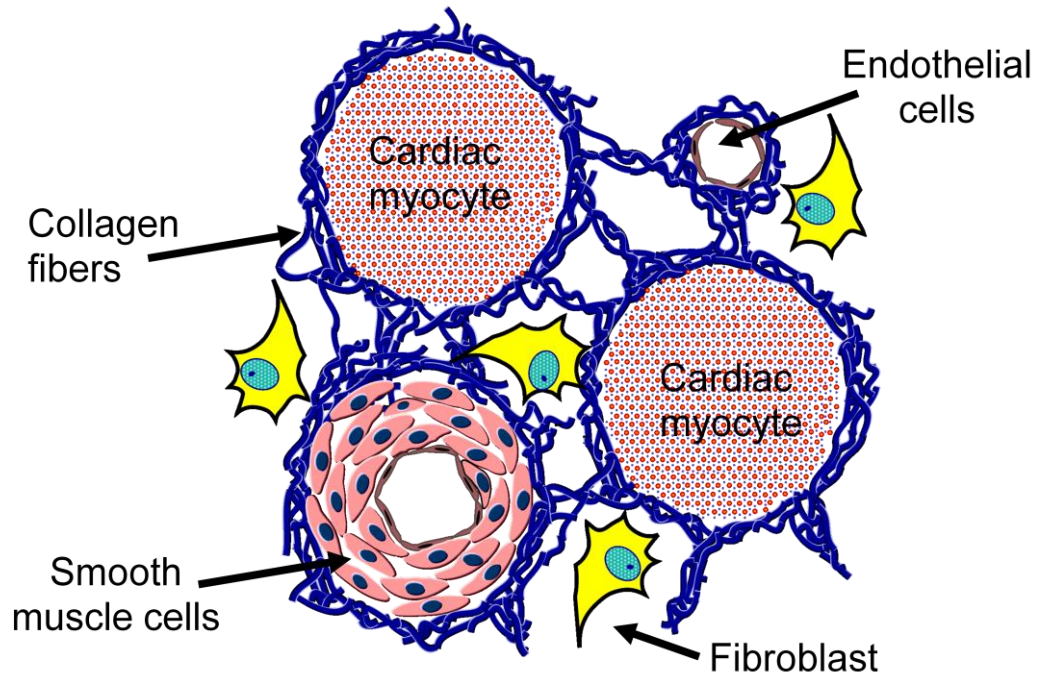


Figure 1. Cardiac tissue structure. In the heart, the cardiac myocytes, smooth muscle cells, and endothelial cells are surrounded by the extracellular matrix. The extracellular matrix consists mainly of collagen fibers and is produced by cardiac fibroblasts. In ARVC, an excess aggregation of the extracellular matrix contributes to fibrosis and fibrofatty tissue accumulation, which can lead to cardiac death. (Adapted from lab slides by Dr. Karen Vikstrom)

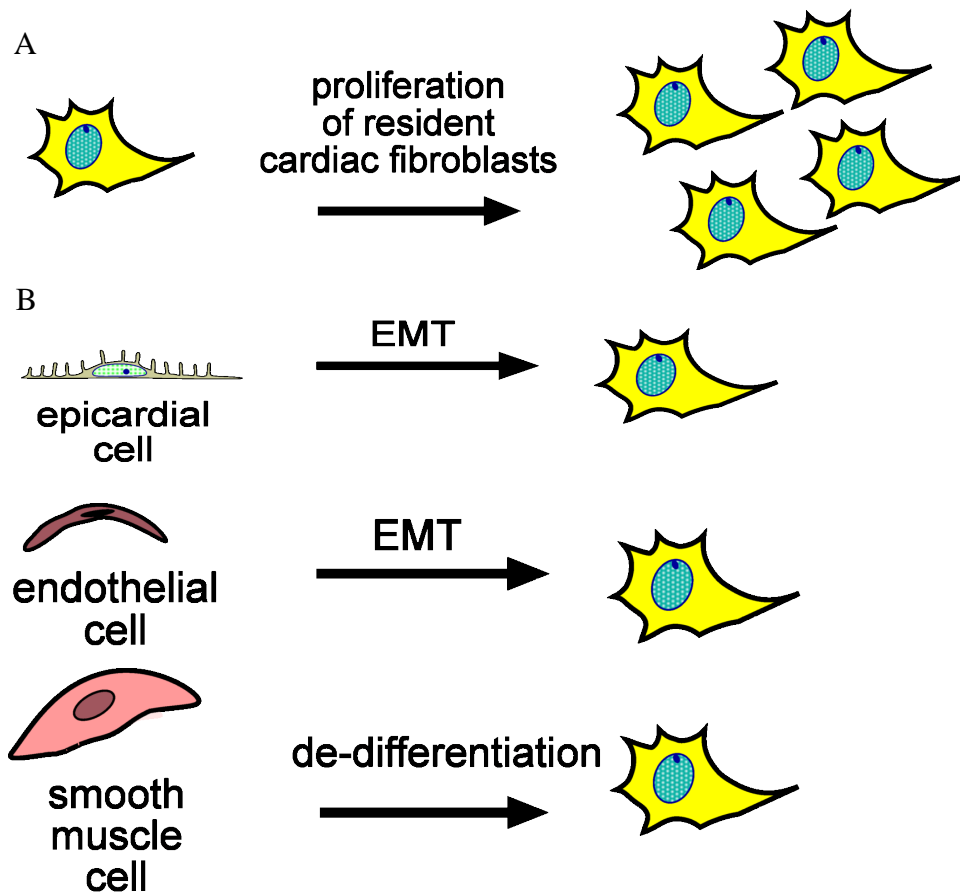


Figure 2. Fibroblast origins. A) Existing fibroblasts proliferate to produce additional fibroblasts. B) Epicardial cells and endothelial cells undergo EMT to produce new fibroblast. Smooth muscle cells can undergo de-differentiation to produce fibroblasts.

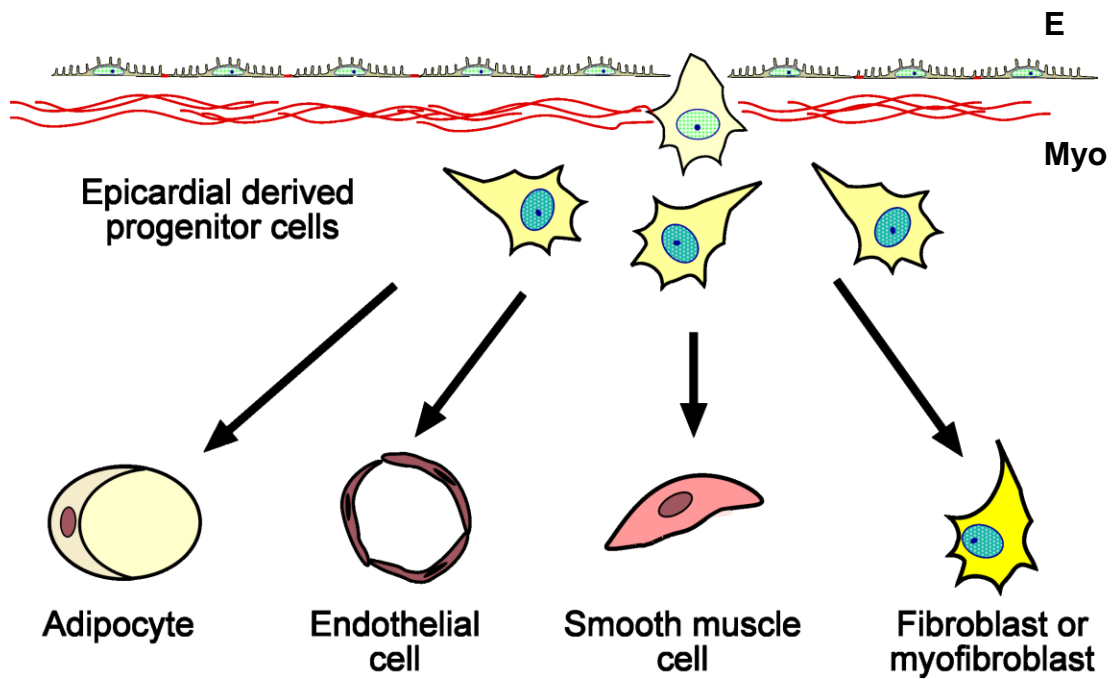


Figure 3. *During development, the epicardium undergoes EMT.* Epicardial cells migrate from the epicardium (E) towards the myocardium (Myo) and become epicardial derived progenitor cells (EDPCs). Epicardial derived progenitor cells then differentiate into adipocytes, endothelial cells, smooth muscle cells, and fibroblasts or myofibroblasts. (Adapted from lab slides by Dr. Karen Vikstrom)

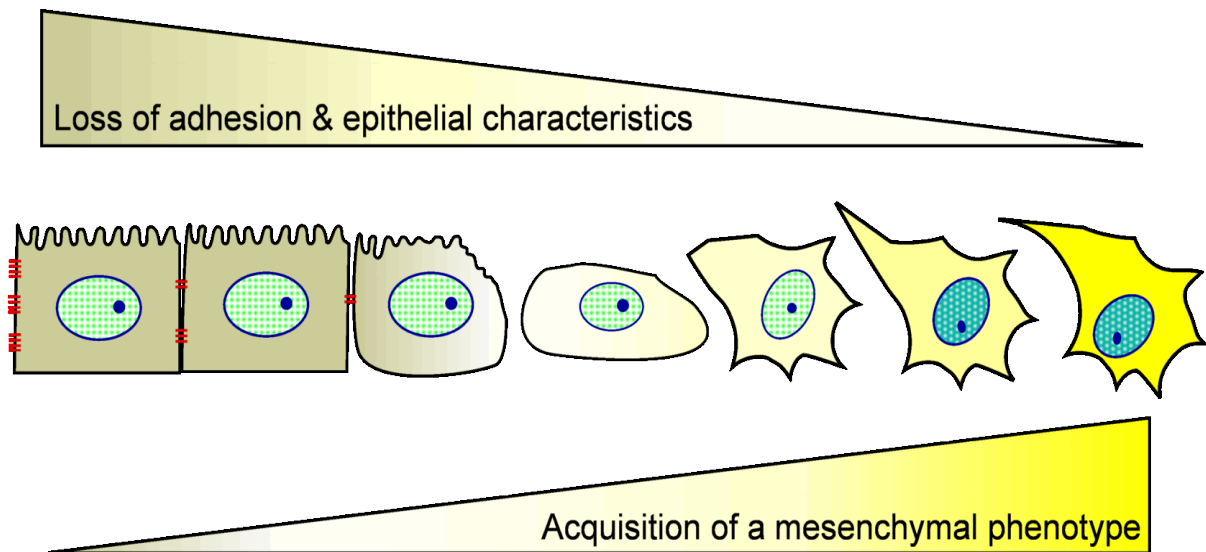


Figure 4. During EMT, epicardial cells change their cell morphology. EMT is characterized by the loss of adhesion and epithelial characteristics and the gradual acquisition of a more mobile and dynamic mesenchymal phenotype. (Adapted from lab slides by Dr. Karen Vikstrom)

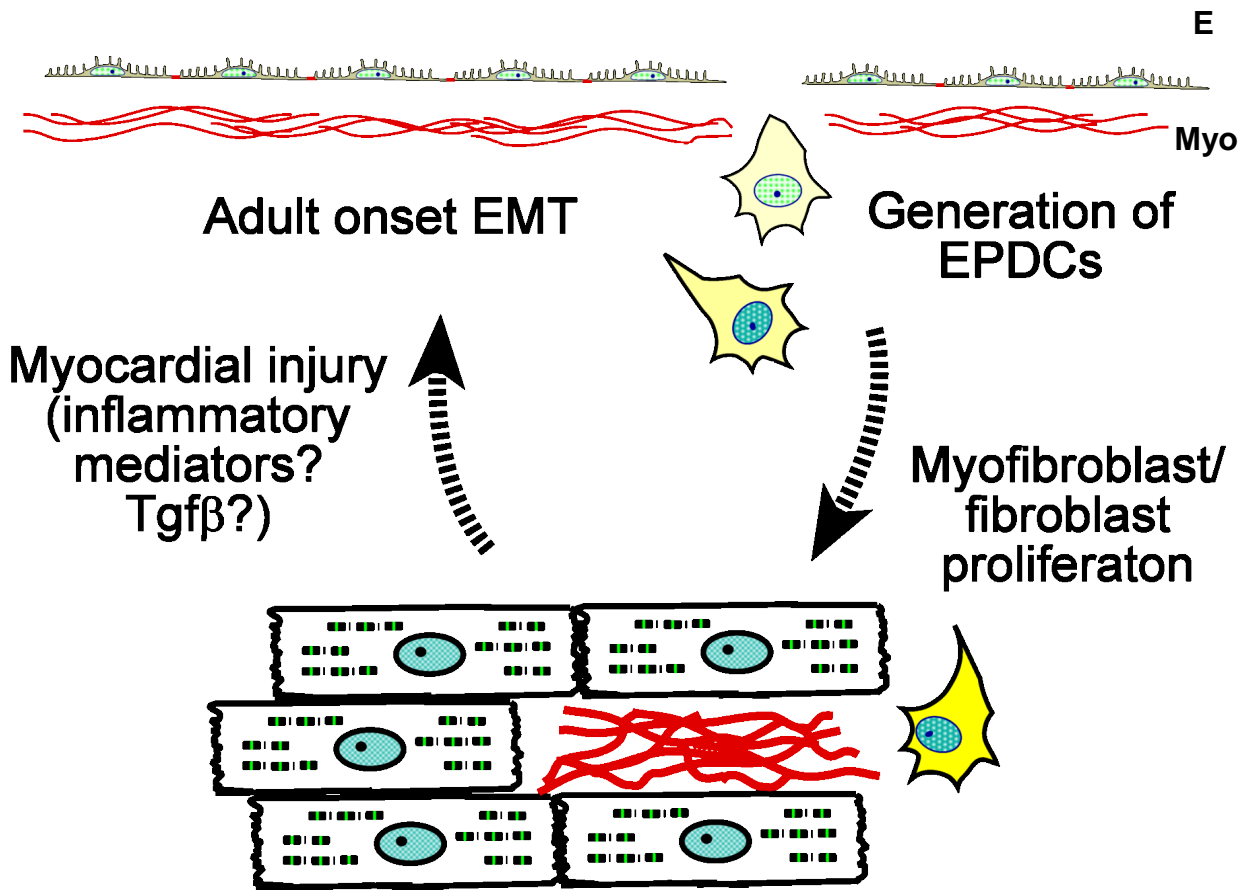


Figure 5. Adult epicardium may undergo EMT in response to injury. Myocardial injury may release inflammatory molecules that will trigger adult epicardium cells to undergo EMT. In turn, this may also cause fibroblast proliferation and an increased amount of extracellular matrix surrounding cardiac myocytes, contributing to the pathogenesis of ARVC. Epicardium, E; Myocardium, Myo. (Adapted from lab slides by Dr. Karen Vikstrom)

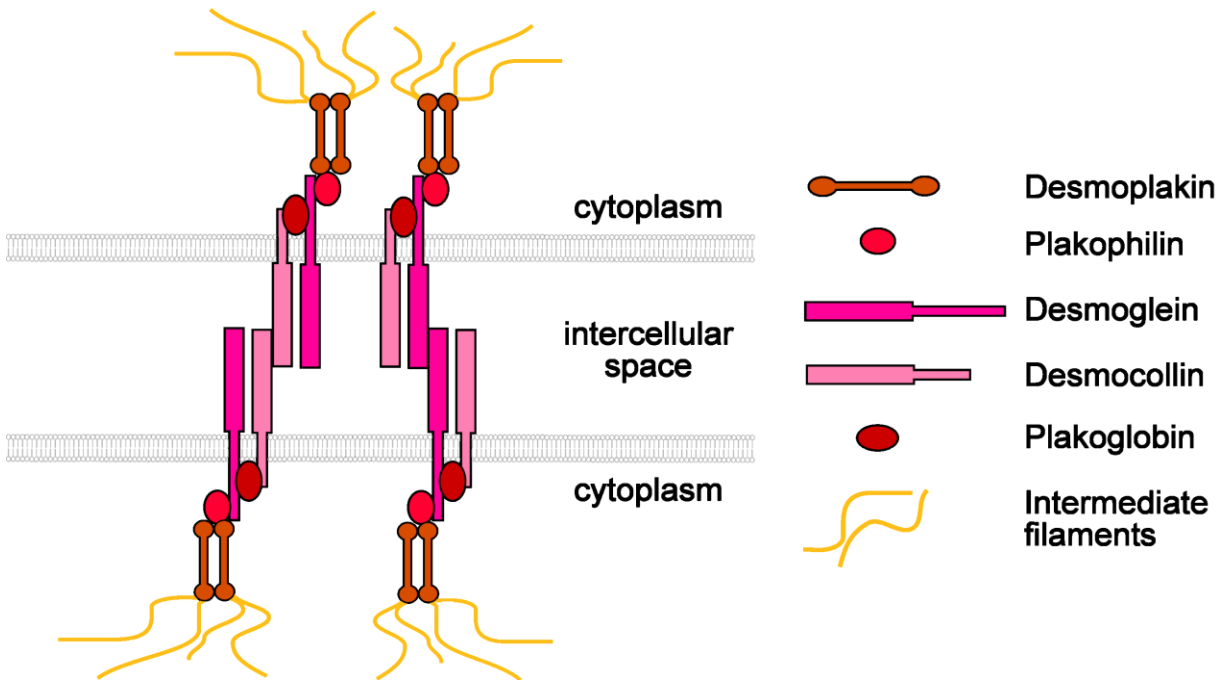


Figure 6. The Desmosome. The desmosome forms strong mechanical adhesions between two adjacent cardiac or epithelial cells. The cadherins (desmoglein and desmocollin) provide the cell-to-cell adhesion in the intercellular space. Plakoglobin, plakophilin, and desmoplakins link the intercellular structure to the intermediate filament network within the cells.

Materials and Methods

Animals. Male ICR mice (Harlan Laboratories, Haslett, Michigan) were used for light and electron microscopic analysis. Mice on a C57B16 background were used for epicardial cell isolation. All animal use was approved by the University Committee on the Care and Use of Animals (UCUCA) of the University of Michigan.

Antibodies. Primary antibodies included Mouse Anti-Pkp2 (Meridian Life Science, Inc. Memphis, Tennessee), Rabbit Anti-Collagen IV (Millipore, Billerica, Massachusetts), Guinea Pig Anti-Pkp2 (Acris Antibodies, Inc. San Diego, California), Rat Anti-Mesothelin (MBL International, Woburn, Massachusetts). DyLight-labeled secondary antibodies include Donkey Anti-Mouse Dylight 488, Donkey Anti-Rabbit Dylight 549, and AffiniPure Donkey Anti-Guinea Pig IgG Dylight 488 (Jackson ImmunoResearch, West Grove, Pennsylvania).

Epicardial Whole Mounts. Animals were euthanized and hearts were removed. Blood was flushed from the heart with phosphate buffered saline (PBS), and the atria and ventricle were separated and pinned flat, fixed in 4% formaldehyde for 1 hour, and then stored in PBS overnight. Specimens were dehydrated with methanol. Each piece of tissue was picked up with fine forceps, drained of excess methanol, dipped in acetone for 5 seconds, placed with epicardium side up on tissues paper, and allowed to dry for approximately 5 to 10 seconds. Next, the tissues were adhered to a glass slide (epicardium side down) with a thin layer of superglue and pressed down gently with another glass slide. The tissue was then picked up at one corner with fine forceps and the myocardium was peeled off, leaving the epicardium attached to the superglue. The slide was next placed in PBS and then processed for immunohistochemistry.

Immunohistochemistry. 8 μm axial frozen heart sections were collected using a Microm 550 cryostat (Thermo Fischer Scientific, Inc. Hampton, New Hampshire). Frozen sections were air

dried, fixed in 75% acetone/25% ethanol for 5 minutes and rehydrated with PBS. Frozen sections were blocked with 10% Normal Donkey Serum/0.1% Triton X-100/PBS while whole mounts were blocked with 10% Normal Donkey Serum/0.05% Tween-20/PBS at room temperature (RT) for 2 hours. Both preparations were then blocked with M.O.M. Mouse IgG Blocking Reagent (1 drop in 1.25 ml of 0.01% Triton X-100/PBS, MKB-2213, Vector Laboratories, Inc. Burlingame, California) at RT for 2 hours with shaking. Next, slides were incubated with primary antibodies at 4° C overnight. Frozen sections were incubated with Monoclonal Mouse Anti-Pkp2 (1:20, dilution, K44262M, Meridian Life Science, Inc. Memphis Tennessee) and Polyclonal Rabbit Anti-Collagen IV (1:3,000 dilution, Millipore, Billerica, Massachusetts). Whole mounts were incubated with Polyclonal Guinea Pig Anti-Pkp2 (1:50 dilution, AP09554SU-N, Acris Antibodies, Inc. San Diego, California). Next, the preparations were washed with 0.05% Tween-20/PBS and incubated with fluorescence-labeled secondary antibodies for 1 hour at RT. The secondary antibody against Monoclonal Mouse Anti-Pkp2, Polyclonal Rabbit Anti-Collagen IV, and Polyclonal Guinea Pig Anti-Pkp2 were Donkey Anti-Mouse IgG Dylight 488 (1:200 dilution, Jackson ImmunoResearch, West Grove, Pennsylvania), Donkey Anti-Rabbit Dylight 549 (1:1,000 dilution, Jackson ImmunoResearch, West Grove, Pennsylvania), and AffiniPure Donkey Anti-Guinea Pig IgG Dylight 488 (1:100 dilution, 94393, Jackson ImmunoResearch, West Grove, Pennsylvania), respectively. Slides were washed with 0.05% Tween-20/PBS. Next, all slides were counterstained with DAPI (1:1,000 dilution) for 10 minutes at RT. Slides were washed again with 0.05% Tween-20/PBS and dipped once in dH₂O. Then cover slips were mounted using Fluomount-G (0100-01, SouthernBiotech, Birmingham, Alabama). Images were obtained using a Carl Zeiss Axioplan 2e microscope (Germany) equipped with structured illumination (Apotome).

Electron Microscopy. Mouse heart specimens were fixed in Karnovsky's fixation and then processed for electron microscopy on a Philips CM-100 electron microscope by the Microscopy & Image Analysis Laboratory (University of Michigan).

Epicardial Cell Isolation and FACS. Isolated mouse hearts were trimmed of fat and briefly rinsed in PBS. Hearts were bisected, incubated at RT for 40 min in Hanks buffered salt solution (HBSS) (Mediatech Inc. Manassas, Virginia), containing a combination of Accumax and Accutase (Innovative Cell Technologies Inc. San Diego, California). Hearts were pinned in a dish epicardial side up and the epicardium scraped using a rubber policeman. Hearts were rinsed with HBSS with 10% FBS, scrapings were collected and cells were pelleted. Pellets were resuspended in PEB buffer (PBS, 0.5% BSA and 2mM EDTA) and Red Blood Cell Lysis Solution (Sigma-Aldrich, St. Louis, Missouri), incubated for 2 min and pelleted. This process was repeated until all red blood cells were eliminated from the pellet which looks white when all red blood cells have been lysed. Cells were resuspended in PEB buffer and counted. Cell preparations were then processed for FACS using a PE labeled rat anti-mesothelin antibody and the sorted cells were collected in RNAlater (Ambion, Carlsbad, California).

Results

Mouse Epicardium Ultrastructure. In order to characterize the morphology of the mouse epicardium, transmission electron microscopy images were taken of cross-sections of the mouse atria and ventricles to examine the ultrastructure of the epicardium (E) (Figure 7). In both the atria and ventricles, mechanical junctions (arrows) were observed between epicardial cells. It was apparent that there was overlapping of cells in the epicardium with protrusion of microvilli, most obviously seen in the atria (Figure 7A). Due to such overlapping, it was difficult to identify the specific morphology of the cellular junctions. Furthermore, the atrial epicardium was significantly thicker than the ventricular epicardium. The sub-epicardial mesenchyme (M) was also thicker in the atria than in the ventricles (Figure 7B, C). These features may contribute to the different distribution of junctional proteins in the atrial and ventricular epicardium.

Pkp2 Localization in Atria and Ventricles. The difference in morphology between the atrial and ventricular epicardium suggested that there may be a difference in Pkp2 density in the atria and ventricles, if there is any Pkp2 localization at all. Using immunohistochemistry techniques, we localized Pkp2 in cross-sections of the mouse heart (Figure 8). Pkp2 was found to be present in the epicardium of both the atria and ventricles (Figure 8A, B). The cardiac intercalated discs were stained for Pkp2 as a positive control (Figure 8C). It is known that Pkp2 is localized with great abundance in the intercalated discs (Sato et al. 2011; Sato et al. 2009). Immunoreactivity of Pkp2 was significantly greater in the intercalated discs than in the epicardium (Figure 8C). In atria, Pkp2 stains were seen at a greater density and had greater amount of fluorescence. However, in the ventricles, Pkp2 stains were located sparsely in the epicardium and had less amount of fluorescence. In both the atria and ventricles, Pkp2 localization was characterized by punctuate stains in the epicardium. However, it was observed that the atria have a greater

abundance of Pkp2 immunoreactivity than the ventricles. Pkp2 localization in the atria appeared to have both higher density and higher fluorescent intensity. An antibody to Collagen IV was used to visualize the sub-epicardial space. The staining of Collagen IV was more robust in the atria than the ventricles, suggesting a thicker sub-epicardial space in the epicardium (Figure 8A, B). This observation coincided with the previous observation of the epicardium and sub-epicardial space using transmission electron microscopy. Due to the morphology of the epicardium, it was difficult to accurately visualize the punctuate staining of Pkp2 in cross-section of the heart. Therefore, it was not possible to determine whether the Pkp2 stains were junctional, cytoplasmic or nuclear.

Quantification of Pkp2 in Atrial and Ventricular Epicardium. In order to obtain a clearer visualization of Pkp2 in the epicardium, a protocol for an epicardial whole mount preparation was developed. In the literature, there are numerous numbers of studies that have shown ways to produce *en face* isolation and preparation of various tissues (Poole and Florey, 1958; Cotton and Wartman, 1961; Warren, 1965; Obase and Wright, 1968; Pugatch and Saunders, 1968; Tsutsumi and Gore, 1969; Sade and Folkman, 1972; Hiarsch et al. 1980; Fornas and Fortea, 1987). Many of these studies developed methods that produced *en face* preparation of endothelial cells of vasculature. An *en face* preparation involves peeling off a flat single layer of intact cells from tissue. Currently, there is no other method to produce whole mounts of heart epicardium, a very thin layer of delicate cells. Thus, we decided to develop a simple technique to strip off the epicardium and visualize Pkp2 localization. This technique involved several simple steps (Figure 9). First, the heart was dissected and cannulated. The atria and ventricles were separated and fixed. Next, the atria and ventricles were treated with methanol then acetone and laid down flat on a glass slide covered with superglue with the epicardial side down. After the superglue has dried,

the myocardium was peeled off, leaving the epicardium on the surface of the glass side. Immunohistochemistry was then performed according to protocol. When the epicardium was stained with DAPI, it was observed that the epicardial cells were flatly attached to the glass slide. It appeared that the superglue did not interfere with the morphology and the structure of the epicardial cells. Since the epicardium is a thin layer of epithelial cells, it appeared to have a flat morphology under the microscope. Using our technique, the epicardium retained its shape. Through immunohistochemistry, the epicardial cells retained its cellular and nuclear structure when stained with DAPI. Similar to what was observed in the frozen sections, there appeared to be greater immunoreactivity in the atria than in the ventricles (Figure 10). At high magnification, there was more non-junctional (nuclear) Pkp2 localization, and at lower magnification, junctional stains were also observed. This allowed for the quantification of the amount of Pkp2 in the atria and ventricles. Quantification of Pkp2 demonstrated that the atria contained significantly more Pkp2 per nuclei than the ventricles (1.72 Pkp2 structures/atrial cell vs. 0.42 Pkp2 structures/ventricular cell, $p < 0.05$) (Figure 11). This quantification suggested that perhaps there could be a relationship between the number of Pkp2 per epicardium cell and the morphology of the epicardium in the atria and ventricles. Furthermore, it suggested that such differences between the atria and ventricles could contribute to the development of ARVC if mutations were to occur in the epicardial junctions.

Identifying a Marker for the Adult Mouse Epicardium. The epicardium is a single layer of mesothelial cells that surround the heart. In order to isolated epicardial cells, we needed to find a fluorescent marker that would specifically target the epicardium. Frozen cross-sections of the heart were stained with anti-mesothelin antibody. Antibody immunoreactivity demonstrated localization in the epicardium, confirming mesothelin as an epicardial marker (Figure 12).

Epicardial cells were isolated using an epicardium scraping protocol and stained with PE-labeled rat anti-mesothelin antibody (Figure 13). FACS allowed for the isolation of a population of mesothelin-positive cells, indicating successful isolation of the mouse epicardium.

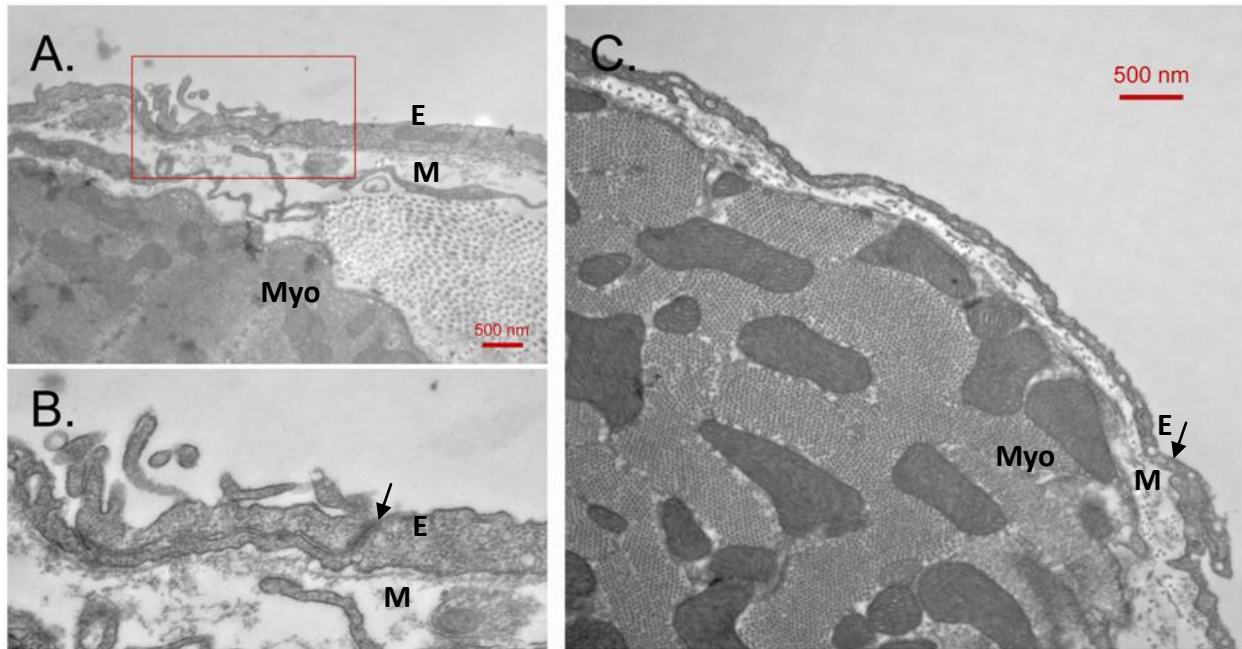


Figure 7. Regional differences in mouse epicardial ultrastructure. Transmission electron microscopy was used to examine the structure of atrial (A and B) and ventricular (C) epicardium (E). Mechanical junctions (arrows) of indeterminate morphology were seen in areas of overlap between cells (B) while the epicardium of the atrial free wall was significantly thicker with a more extensive subepicardial mesenchyme (M) than in the ventricle. The boxed area in (A) is shown at higher magnification in (B). Epicardium, E; Sub-epicardial mesenchyme, M; Myocardium, Myo.

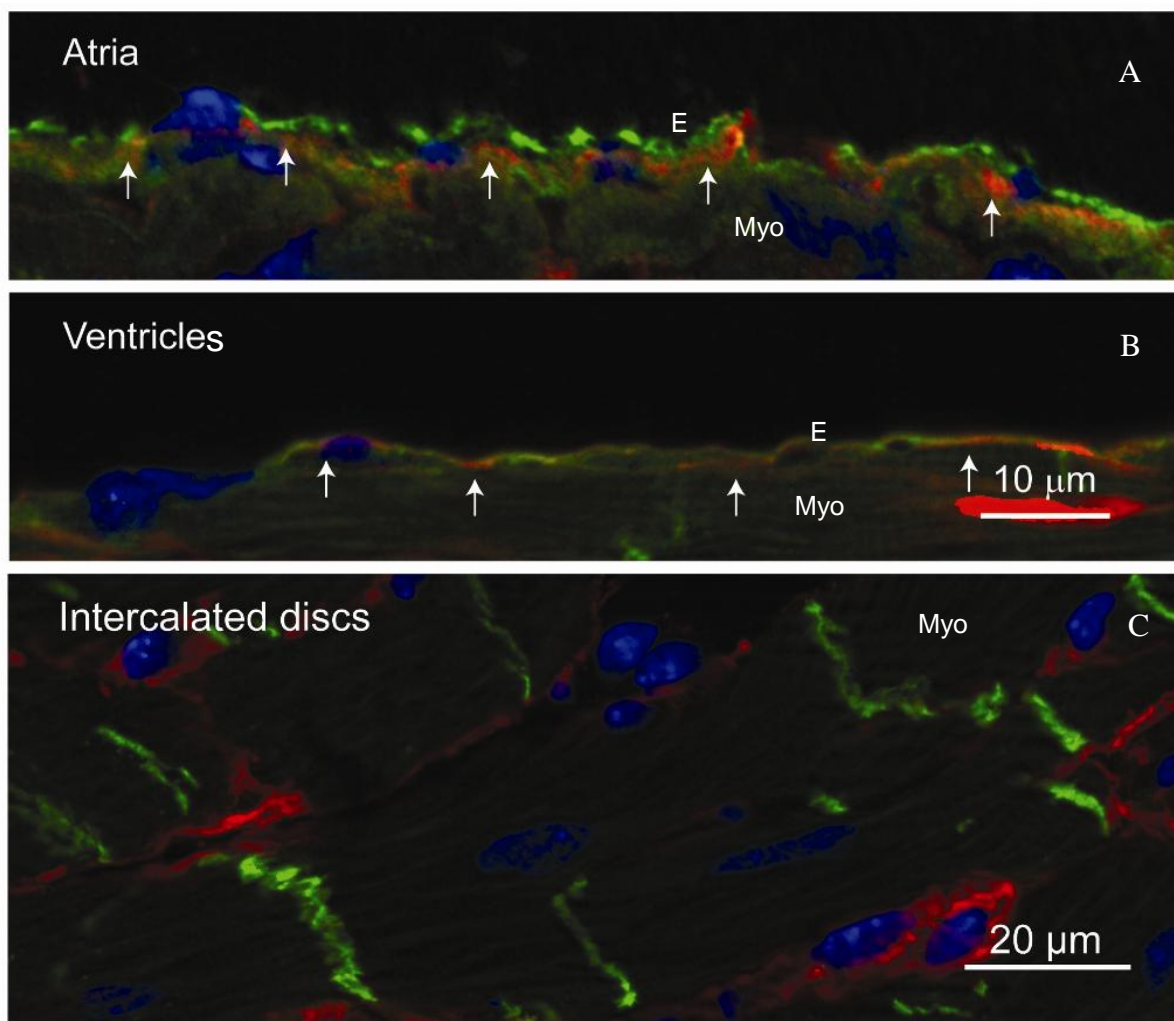


Figure 8. Localization of Pkp2 in the adult mouse epicardium. Pkp2 immunoreactivity was present in both atrial (A) and ventricular (B) epicardium and in the cardiac intercalated discs (C). Pkp2 immunoreactivity in the epicardium was significantly less than that in the intercalated discs of cardiac myocytes. However, Pkp2 and Collagen IV (a marker for the basement membrane) immunoreactivity were greater in the atrial epicardium than in the ventricular epicardium. Pkp2 localization in the atrial epicardium was both more robust and closer in proximity to each other than in the ventricular epicardium. The more robust Collagen IV immunoreactivity beneath the epicardial cell layer conveyed a wider and more undulating sub-epicardial space in the atria than in the ventricles. Due to the plane of section, junctional, cytoplasmic, and nuclear staining could not be distinguished in these images. Pkp2, green; Collagen IV, red; DAPI, blue. Epicardium, E; Myocardium, Myo.

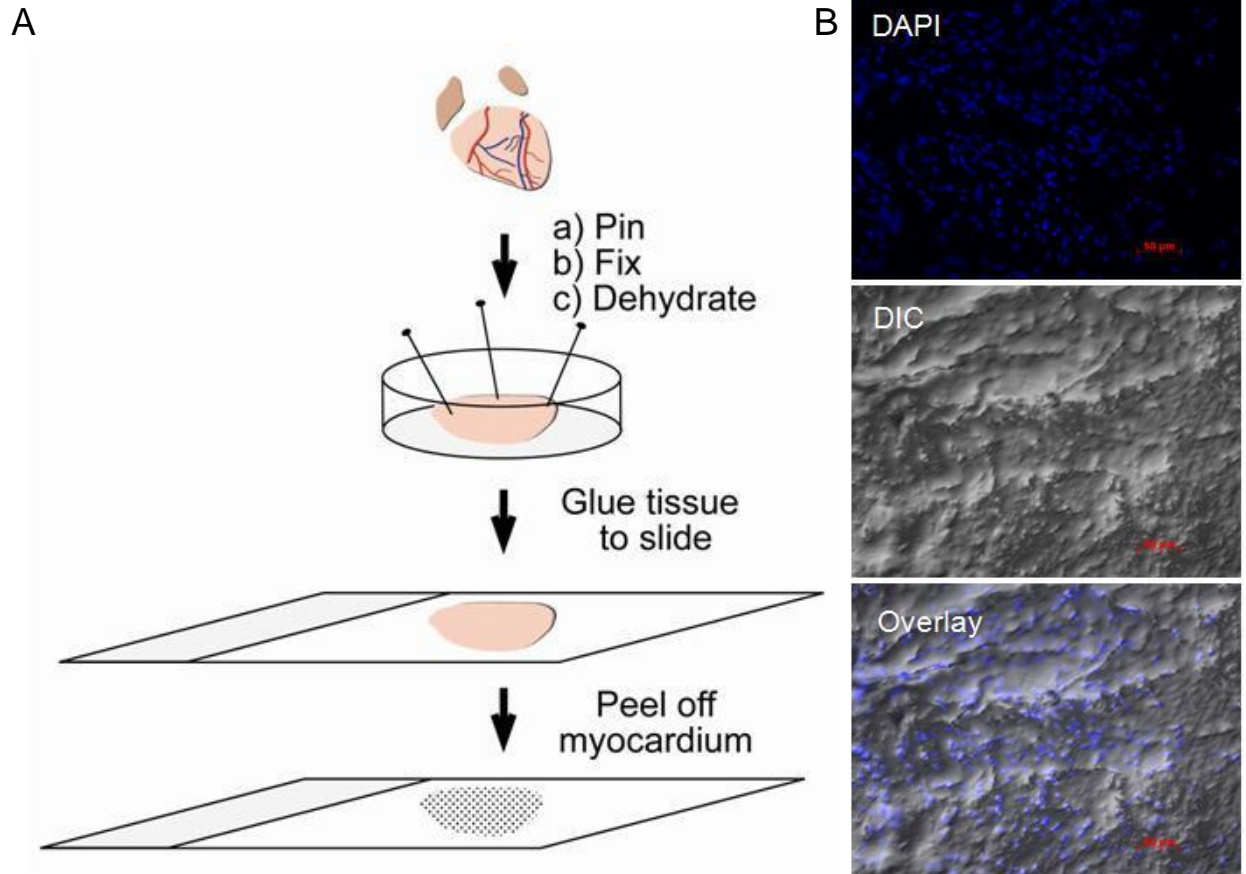


Figure 9. Epicardial whole mounts. Whole mount preparations of adult mouse epicardium was prepared from atria, right and left ventricles. The heart was first dissected and fixed in formaldehyde. Then the tissue was dipped once in acetone and glued onto a glass slides covered with superglue with the epicardial side down. After the tissue has dried for 5 to 10 seconds, the myocardium was peeled off, leaving the epicardium on the surface of the glass slide. Immunohistochemistry was then performed according to standard protocol. DAPI staining of atrial epicardium is shown in the figure. Epicardial cells retained their shapes and were not physically disturbed by their physical interaction with the superglue.

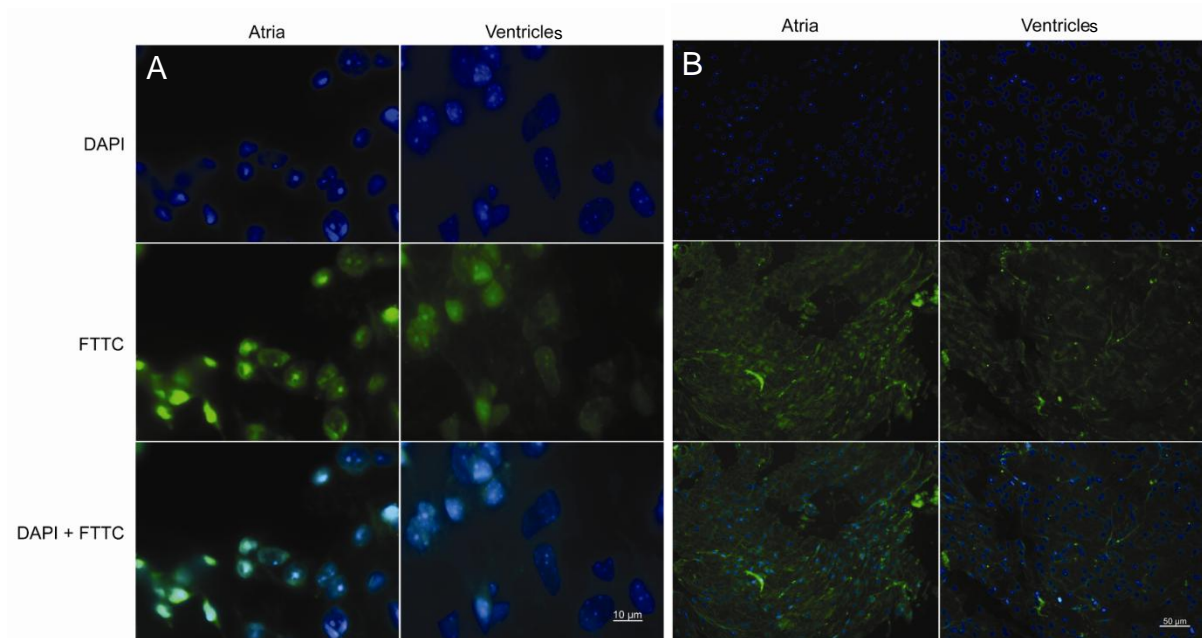


Figure 10. *Pkp2 immunolocalization in whole mount epicardial preparations.* Greater immunoreactivity was observed in the atria than in the ventricles. Non-junctional (nuclear and cytoplasmic) Pkp2 localization can be clearly distinguished in whole mount epicardial preparations at high magnification (A). Labeling of some junctional Pkp2-containing structures was also revealed at low magnifications (B). Pkp2, green; DAPI, blue. High magnification, A; Low magnification, B.

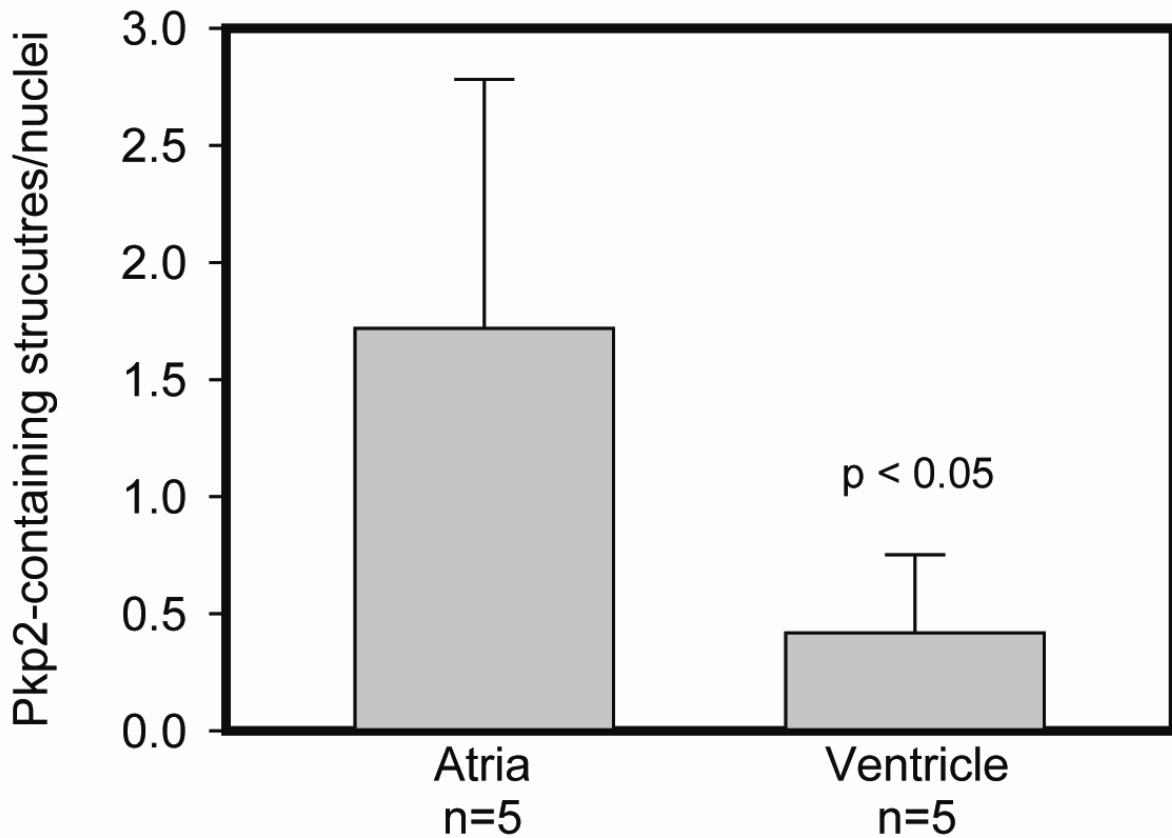


Figure 11. *Quantification of Pkp2 immunolocalization in whole mount epicardial preparations revealed more immunoreactivity in atria than ventricles.* Quantification was obtained by the direct counting of Pkp2-containing structures per nuclei in the whole mount preparations. In the atria, it was found that there were 1.72 Pkp2 structures per atrial cell. In the ventricles, it was found that there were only 0.42 Pkp2 structures per ventricular cell.

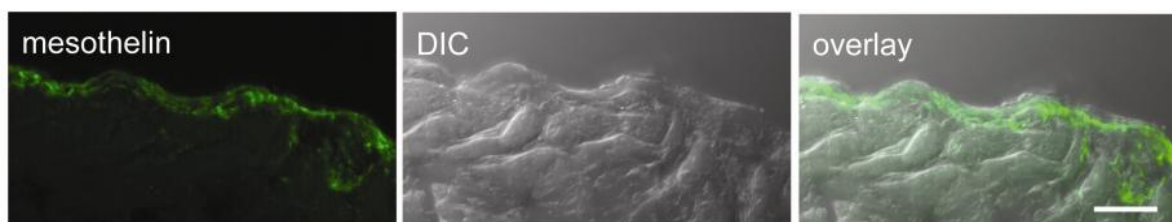


Figure 12. Mesothelin as a marker for adult mouse epicardium. Immunolocalization of mesothelin to the epicardial cell surface in the healthy adult mouse was accomplished by staining cross-sections of the mouse heart by an anti-mesothelin antibody.

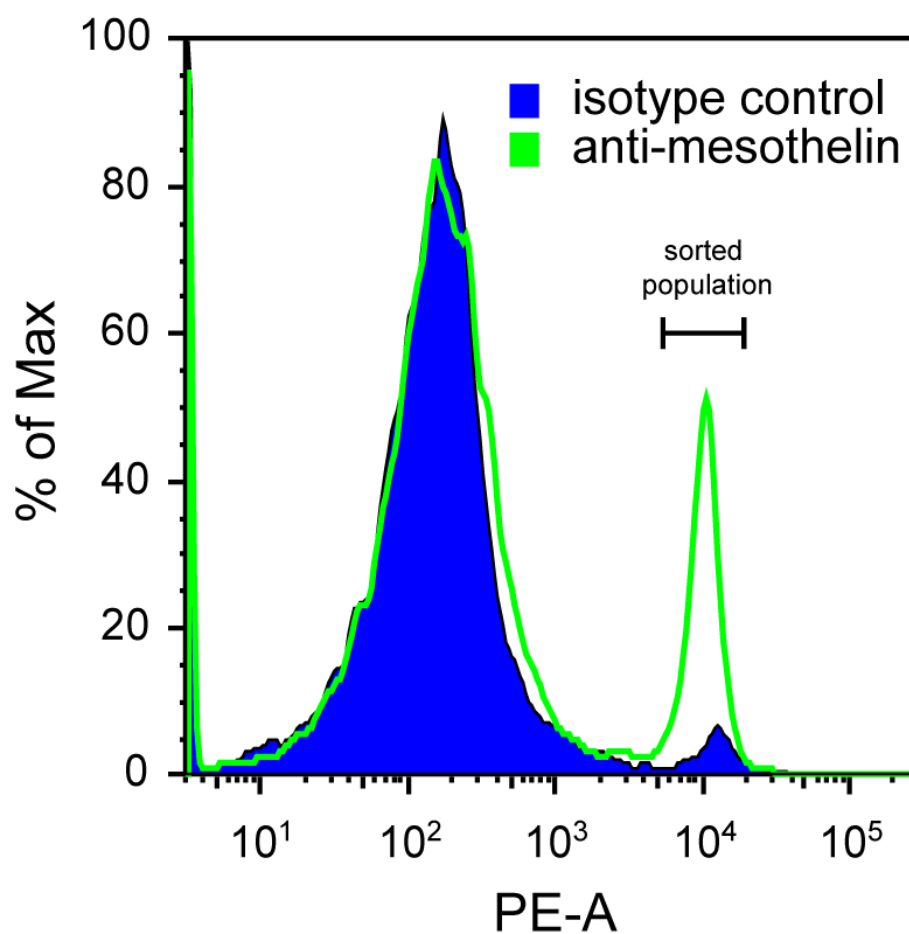


Figure 13. Isolation of adult epicardial cells by FACS. Epicardial cells were freshly isolated from adult mice, stained with a PE-labeled rat anti-mesothelin or PE-labeled isotype control and the mesothelin positive population was isolated using FACS.

Discussion

It is known that Pkp2 is a protein of the desmosome that is normally found in the cardiac intercalated discs (Saffitz, 2011). A mutation in Pkp2 and/or with a few other desmosomal proteins may contribute to the pathogenesis of ARVC (Saffitz, 2011). ARVC is characterized by myocardial accumulation of fibrous and fibrofatty tissues, a likely result from over accumulation of cardiac fibroblasts. Since these fibroblasts can be derived from epicardial cells that undergo EMT during cardiac development, it is possible that the epicardial cells may play a role in ARVC development. It is not known whether adult epicardial cells undergo EMT and epicardial cell junctions have never been characterized. If epicardial cell junctions are in fact desmosome-like junctions and contain Pkp2, then perhaps the epicardium plays an important role in ARVC.

We have begun to examine variability in epicardial structure as a function of anatomical location. Immunohistochemistry of cross sections of wild-type mouse heart revealed that Pkp2 was indeed localized in the epicardium of both the atria and the ventricles. In fact, there was more immunoreactivity, both in density and fluorescence, in the atria than in the ventricles. Collagen IV immunoreactivity beneath the epicardial cell layer was also more robust in the atria than in the ventricles, which indicated a wider and more undulating interface between the epicardium and the underlying myocardium (Figure 8). In addition, ultrastructural analysis of the epicardium showed that atrial epicardium and sub-epicardial mesenchyme were significantly thicker than those in the ventricle (Figure 7). In both chambers, epicardial cells were extremely thin with numerous microvilli, overlapping cell margins (Figure 7). Based on these observations, there seemed to be a morphological difference between the atrial and the ventricular epicardium, which may contribute to the observed difference in abundance of Pkp2 that was present in each chamber.

Furthermore, the whole mount preparation was important because it allowed for a basic characterization of epicardial junctions. First of all, the preparation was the first preparation that accomplished the stripping of the epicardium. It enabled better visualization of epicardial cells because the epicardium can now be viewed from the bird's-eye perspective. The preparation did not disturb the physical and nuclear structure of the epicardium, despite the epicardium being a thin delicate layer of epithelial cells. Second, the whole mount preparation revealed that the Pkp2 stains in the epicardium were mostly nuclear (at high magnification) and junctional (at low magnification). This was something that could not be characterized with merely the cross-section stains. In general, this technique can be used to isolate intact epithelial cells and retain their junctional structure. It does not require the cells to undergo treatment with harsh chemicals that would disturb their cellular integrity.

In our experiments, we determined that Pkp2 was found in the cardiac epicardium in wild-type mice, in addition to being present in the cardiac intercalated discs. Because a mutation of Pkp2 in the cardiac intercalated discs can cause ARVC, it is possible that a mutation of Pkp2 in the epicardium may also contribute to the development of ARVC. If this were the case, then such mutations may disrupt the mechanical junctions between epicardial cells. Once the junctions are disrupted, then it is possible that the epicardium may be triggered to undergo EMT to acquire a more dynamic cellular shape due to the loss of cell-to-cell adhesion between epicardial cells. This suggests that perhaps the epicardium is capable of undergoing EMT even after cardiac development due to cardiac injury. The fact that the atria and the ventricles contain differing amounts of Pkp2 may also determine the location at which ARVC is first triggered and the pathological progression of the disease. It is possible that some expression of Pkp isoforms may in fact compensate for ARVC mutations. In general, the characterization of epicardial

junctions and the identification of their protein components may lead to more understanding of the pathogenesis of ARVC and contribute to finding an effective treatment for ARVC.

Future Directions

Future work could include the use of PCR to determine the expression of Pkp2 in isolated adult mouse epicardial cells. We could use immunohistochemistry and the whole mount preparation to determine whether Pkp2 is expressed in mutant ARVC mice. If it is expressed, then we would want to determine whether its expression level is different from that in the wild-type mice. We could also determine which splice variants of Pkp2 are present in the epicardial cells and if these cells express other Pkp isoforms, such as Pkp1 and Pkp3, that may compensate for ARVC mutations. If different splice variants of Pkp2 are expressed at different levels in wild-type and mutant mice, then we could learn more about the genetic mechanism behind ARVC mutations. By studying Pkp1 and Pkp3, we could determine whether and how the Pkp isoforms are functionally and structurally related and which one(s) contribute(s) more to the pathogenesis of ARVC.

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