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Desmoplakin is Important for Proper Cardiac Cell-Cell Interactions

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Abstract

Normal cardiac function is maintained through dynamic interactions of cardiac cells with each other and with the extracellular matrix. These interactions are important for remodeling during cardiac growth and pathophysiological conditions. However, the precise mechanisms of these interactions remain unclear. In this study we examined the importance of desmoplakin (DSP) in cardiac cell-cell interactions. Cell-cell communication in the heart requires the formation and preservation of cell contacts by cell adhesion junctions called desmosome-like structures. A major protein component of this complex is DSP, which plays a role in linking the cytoskeletal network to the plasma membrane. Our laboratory previously generated a polyclonal antibody (1611) against the detergent soluble fraction of cardiac fibroblast plasma membrane. In attempting to define which proteins 1611 recognizes, we performed two-dimensional electrophoresis and identified DSP as one of the major proteins recognized by 1611. Immunoprecipitation studies demonstrated that 1611 was able to directly pulldown DSP. We also demonstrate that 1611 and anti-DSP antibodies co-localize in whole heart sections. Finally, using a three-dimensional *in vitro* cell-cell interaction assay, we demonstrate that 1611 can inhibit cell-cell interactions. These data indicate that DSP is an important protein for cell-cell interactions and affects a variety of cellular functions, including cytokine secretion.

Keywords

desmoplakin; cell-cell interactions; cardiac cells; desmosomes; fibroblasts

Introduction

The cellular components of the heart are organized into a dynamic, three-dimensional (3D) network that are involved in chemical, mechanical, and electrical regulation of cardiac function (Banerjee et al., 2006; Camelliti et al., 2006; Porter & Turner, 2009; Bowers et al., 2010). Mechanical coupling of these cells occurs through cell-cell junctions. Desmosomes are organized intercellular junctions of different degrees of complexity that link the

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Supplementary Figure 1, which shows that 1611 specifically stains fibroblasts in the heart, can be found online. Please visit journals.cambridge.org/jid_MAM.

cytoskeletal network to the plasma membrane at specialized sites of cell-cell interactions (Farquhar & Palade, 1963; Garrod, 1993; Garrod & Collins, 1994; Cowin & Burke, 1996). Desmosomes are predominantly found in tissues that undergo mechanical stress, such as the heart and skin. Disruption of either desmosomes or the intermediate filament (IF) network can have devastating effects on tissue architecture and integrity (Coulombe & Fuchs, 1994; Fuchs, 1994; McLean & Lane, 1995; Bierkamp et al., 1996; Ruiz et al., 1996; Norgett et al., 2000; den Haan et al., 2009). Desmosomes consist of multiple membrane proteins including desmogleins, desmocollins, and the most abundant desmosomal protein, desmoplakin (DSP) (O'Keefe et al., 1989; Cowin & Burke, 1996).

It has been shown in mice that genetic deletion of *dsp* results in embryonic lethality (Gallicano et al., 1998). These studies demonstrated that DSP is critical in anchoring the IF network to desmosomes, as well as playing a key role in desmosome assembly (Gallicano et al., 1998). To better understand the importance of DSP, Gallicano and colleagues performed tetraploid rescue experiments on *dsp*-deficient embryos (Gallicano et al., 2001). These animals survived longer than conventional *dsp* knockout mice but still died shortly after gastrulation from defects in heart and skin epithelium (Gallicano et al., 2001). In humans, it has been shown that mutations in DSP can disrupt IF-DSP interactions (Norgett et al., 2000). In addition, mutations or loss of DSP have been shown to cause arrhythmogenic left ventricular cardiomyopathy (Norgett et al., 2000; Norman et al., 2005; Uzumcu et al., 2006). Moreover, cardiac-specific $dsp^{-/-}$ mouse embryos exhibit growth arrest at E10–E12, have no circulating red blood cells, and are approximately 50% smaller than heterozygous and wild-type embryos (Garcia-Gras et al., 2006). Cardiac-specific $dsp^{-/-}$ embryos also displayed poorly formed hearts with no chamber specification; however, the cardiac phenotype is normal in wild-type and $dsp^{-/-}$ embryos (Garcia-Gras et al., 2006). Furthermore, it was demonstrated that the cardiac-specific *dsp*-deficient mice recapitulated the phenotype of human arrhythmogenic right ventricular cardiomyopathy by exhibiting fibroadipocytic replacement of myocytes, increased myocyte apoptosis, ventricular dysfunction, and ventricular arrhythmias (Garcia-Gras et al., 2006). Finally, several studies have demonstrated that DSP is required for microvascular tube formation (Valiron et al., 1996; Zhou et al., 2004).

To better examine cell-cell interactions involving fibro-blasts in the heart, a polyclonal antibody, termed 1611, was raised against the detergent soluble fraction of the cardiac fibroblast plasma membrane. We have previously used 1611 antibody to inhibit cell-cell interactions (between myocytes and cardiac fibroblasts), to alter cytokine secretion *in vitro*, and to specifically demark cardiac fibroblasts in whole heart sections *in vivo* (Banerjee et al., 2006; Bowers et al., 2010). In the current study, we demonstrate *in vivo* cell-cell interactions between various cardiac cells using transmission electron microscopy (TEM). We also demonstrate that DSP is one of the major proteins recognized by 1611 antibodies, as determined by two-dimensional (2D) gel electrophoresis, proteomic analyses, immunoprecipitation (IP) studies, and confocal microscopy. Furthermore, we show that both 1611 antibody and knockdown of DSP can block cell-cell interactions *in vitro* and alter cytokine secretion. From these studies we conclude that DSP is an important protein involved in cardiac cell-cell interactions and communication, and that 1611 is a novel antibody to further study these dynamic cellular relationships.

Materials and Methods

Cardiac Cell Isolation and Culture

The Institutional Animal Care and Use Committee approved these studies. Myocytes were isolated from day 3 neonatal rat pups as previously described using collagenase digestion and Percoll gradient purification (Borg et al., 1997; Sharp et al., 1997; Bullard et al., 2005).

Briefly, animals were sacrificed according to Institutional Animal Care and Use Committee guidelines and hearts were minced and subjected to multiple digestions in 0.01% collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA). Myocytes were separated from fibroblasts by Percoll (GE Healthcare Biosciences, Piscat-away, NJ, USA)gradient purification as previously published (Borg et al., 1997; Sharp et al., 1997; Bullard et al., 2005). Myocytes and fibroblasts were counted using a hemocytometer and plated on aligned collagen. Collagen was aligned on tissue culture plates as previously described (Simpson et al., 1994; Baudino et al., 2008). Plates were coated with liquid collagen (Gibco, Langley, OK, USA) in a tissue culture hood and then angled at approximately 30° to allow the collagen to flow gently from top to bottom, giving the collagen an aligned appearance. The myocytes were then plated onto these collagen-coated dishes to achieve an *in vivo*–like phenotype and used in cell-cell interaction assays as described below.

Knockdown of Desmoplakin

Ambion Silencer Select siRNA for DSP was purchased from Applied Biosystems (Foster City, CA, USA). Control siRNA (nontargeting siRNA) was used as a negative control. Cardiac fibroblasts were plated onto six well plates and transfected with a final concentration of 10 nM for the corresponding siRNA using Lipofectamine RNAi MAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cells were used 48 h after transfection. The efficiency of DSP knockdown was determined by western blotting and real-time RT-PCR analysis.

Cell-Cell Interaction Assays

Interactions between cardiac fibroblasts and myocytes, between fibroblasts, and between fibroblasts and endothelial cells (ECs) were assayed as previously described (Baudino et al., 2008; Bowers et al., 2010). Briefly, cells were co-cultured; media were collected at 1, 2, 4, 6, and 8 h; and viable cells were counted. Data were analyzed by plotting the number of unattached cells as a function of time. For studies involving disruption of cell-cell interactions, we preincubated cells with 50 mg of our 1611 antibody. Cardiac fibroblasts were preincubated with rabbit IgG or with 1611 antibody for 20 min prior to co-culture with myocytes, fibroblasts, or ECs (Baudino et al., 2008; Bowers et al., 2010). In addition, cardiac fibroblasts that had been treated with DSP siRNA were used in these studies as indicated.

Immunoprecipitation Assays

Total protein was isolated from whole heart, cardiac fibroblasts, or myocytes, and each protein lysate was subjected to IP and western blot analyses as previously described (Uzumcu et al., 2006). Briefly, tissue or cells were lysed using lysis buffer (20 mM Tris HCL pH 8.0, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM EDTA plus protease inhibitor cocktail [Sigma-Aldrich, St. Louis, MO, USA]) and precleared using normal rabbit IgG (Cell Signaling Technology, Beverly, MA, USA). 100 mg of precleared lysate was then incubated with 4 mg of 1611 antibody or 2 mg of anti-DSP antibody at 4° C overnight with rotation. 20 mL of Protein A/G PLUS-Agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) was added to the lysate/antibody mix and incubated with rotation at 4° C for 4 h. Each sample was centrifuged, and the beads were washed three times with cold lysis buffer. Following the last wash, 25 mL of 1X Laemmli sample buffer was added, the sample was vortexed, heated at 95° C for 5 min, and then subjected to western blot analyses.

Two-Dimensional Gel Electrophoresis

Protein lysates from neonatal cardiac fibroblasts were isolated, immunoprecipitated with 1611 antibody, and subjected to 2D gel electrophoresis using the Bio-Rad Protean IEF system (Bio-Rad Laboratories, Hercules, CA, USA). After separation by isoelectric point (pI), the sample was separated by molecular weight. The gel was silver stained, photographed, and spots picked for matrix-assisted laser desorption/ionization-mass spectrometry (MALDIMS). Isolated protein spots were sent out for identification (Prot-Tech, Inc., Eagleville, PA, USA).

Immunofluorescent Staining and Confocal Microscopy

Whole heart sections were cut, fixed in 4% paraformaldehyde for 20 min at room temperature, washed in 5% bovine serum albumin (BSA) in PBS, and blocked with 10% goat serum in 5% BSA for 1 h. Sections were incubated with primary antibodies overnight at 4° C. Sections were washed with 1% BSA/PBS and incubated for 45 min at 37° C with secondary antibodies. Sections were washed with 1% BSA/PBS and cover-slipped using DABCO (Sigma). Dried slides were imaged using a Supercontinuum Confocal Leica TCS SP5 X microscope (Leica Microsystems, Wetzlar, Germany).

Interleukin-6 ELISA

Media samples were collected from co-cultures of wild-type cardiac fibroblasts and myocytes or wild-type cardiac fibroblasts and ECs that were untreated or treated with 1611 antibody. Media were also collected from co-cultures of cardiac fibroblasts and myocytes or cardiac fibroblasts and ECs where DSP was knocked down in the fibroblasts. Culture media were sampled at the indicated time points to examine IL-6 expression using a rat IL-6 ELISA kit (Thermo Scientific, Waltham, MA, USA)as described by the manufacturer.

Transmission Electron Microscopy

Wild-type adult murine hearts (12 weeks old) were isolated following cervical dislocation, rinsed in ice-cold PBS, “bread-loafed,” and fixed in 4% glutaraldehyde. Mid-wall sections were cut into 1–2 mm cubes and prepared for electron microscopy as previously described (Nakagawa et al., 1997). Samples were examined on a JEOL 200CX TEM (JEOL, Tokyo, Japan) at 160 kV.

Statistical Analysis

Data obtained from all analyses were measured for significance using Student’s t-test or ANOVA. Results were considered significant when $p \leq 0.05$.

Results

Polyclonal Antibody 1611 Recognizes DSP Specifically in Fibroblasts

Transmission electron microscopy was performed on normal 12 week old mouse hearts to examine cardiac cell-cell interactions (Figs. 1A–1F). TEM studies demonstrated tight cell-cell interactions between cardiac fibroblasts and myocytes, ECs and myocytes, and fibroblasts and ECs (Figs. 1A–1C, respectively). Higher magnification of each image clearly shows electron dense regions and intermingling of plasma membranes (Figs. 1D–1F). We next investigated the reciprocal spatial distribution of DSP and cells marked by 1611 in whole heart sections using confocal microscopy. A whole heart section stained with 1611 antibody demonstrates that 1611 specifically recognizes cardiac fibroblasts (Figs. 2A– 2D). Higher magnification demonstrates clear cell-cell interactions between myocytes and fibroblasts. In addition, we demonstrate that 1611 specifically stains fibroblasts, as it demarks the same population identified using vimentin (Supplementary Fig. 1).

In heart sections stained with 1611 antibody and anti-DSP antibody, we observed co-localization of signals (Figs. 2E–2I). While not a complete overlay of expression, these studies demonstrate that our 1611 antibody recognizes DSP or possibly a protein complex involving DSP.

Desmoplakin is Recognized by the Polyclonal Antibody 1611

To identify the proteins recognized by our 1611 polyclonal antibody, we first chose to examine the 2D gel electrophoresis profile of protein lysates from cardiac fibroblasts that were subjected to IP using our 1611 antibody. 2D gel electrophoresis was performed on the immunoprecipitated samples, and the gel was silver stained to visualize proteins (Fig. 3). Figure 3 is a representative 2D gel showing the location of the five distinct protein spots that were immunoprecipitated by 1611 antibody. To identify the proteins, we picked the spots and subjected them to MALDIMS analyses, which identified one of the major protein spots as DSP. The protein spots were identified as containing DSP I, DSP II, plakoglobin, obscurin, and tropomyosin 2.

To confirm that 1611 antibody did indeed recognize DSP, we performed IP studies on protein lysates from myocytes, cardiac fibroblasts, or whole heart using our 1611 antibody or using anti-DSP antibody (Fig. 4). Subsequently, we performed western blot analyses using either 1611 antibody or anti-DSP antibody (Figs. 4A, 4B). When protein lysates were immunoprecipitated with 1611 antibody and then western blotted with anti-DSP antibody, we observed a single protein band in the cardiac fibroblast, while two protein bands were observed in whole heart samples; however, no bands were observed in the myocyte sample (Fig. 4A). When the 1611 immunoprecipitated lysates were blotted with 1611 antibody, we observed multiple bands ranging in size from 60 kDa to over 250 kDa (Fig. 4A). This was expected because when we performed 2D gel electrophoresis (Fig. 3), we observed multiple protein spots recognized by 1611 antibody. When protein lysates were immunoprecipitated with anti-DSP antibody, we were able to detect a single protein band in cardiac fibroblast and whole heart samples western blotted with 1611 antibody and two distinct protein bands when anti-DSP antibody was used (Fig. 4B). These data demonstrate that DSP is a key protein recognized by our 1611 antibody.

Desmoplakin is Important for Cardiac Cell-Cell Interactions

Several studies have unveiled that DSP plays a key role in endothelial cell-cell interactions, as well as tube formation (Schmelz & Franke, 1993; Schmelz et al., 1994; Gallicano et al., 2001). Therefore, to better define the nature of these cell contacts, 3D *in vitro* cell-cell interaction assays were used to define adhesion between myocytes and fibroblasts, fibroblasts and fibroblasts, and fibroblasts and ECs (Fig. 5). To examine the role of DSP in cell-cell interactions, we performed siRNA knockdown of DSP in cardiac fibroblasts. Over a 48-h time period, we were able to knockdown *dsp* expression by approximately 85%, while control siRNA showed little effect on *dsp* expression (Fig. 5A). Control IgG-treated cardiac fibroblasts attached to myocytes over an 8-h time period, with adhesion being measured at 2, 4, and 8 h; however, attachment of fibroblasts to myocytes was significantly blocked by addition of 1611 antibody or knockdown of *dsp* (Fig. 5B). Strong interactions were also observed between cardiac fibroblasts, and 1611 antibody or *dsp* loss effectively inhibited these interactions (Fig. 5C). Furthermore, we observed cell-cell interactions between cardiac fibroblasts and ECs, but as with the other cardiac cell-cell interactions, treatment with 1611 antibody or *dsp* knockdown resulted in significant disruption of these interactions (Fig. 5D).

Disruption of Cell-Cell Interactions Affects Cytokine Production

Previous studies have shown that cytokine and growth factor secretion is altered when myocyte-fibroblast interactions are disrupted (Banerjee et al., 2006; Bowers et al., 2010).

Therefore, to determine whether the secretion of IL-6 could be altered by blocking cell-cell interactions, myocytes or ECs were co-cultured with cardiac fibroblasts that had previously been incubated with control IgG, 1611 antibody, or had *dsp* expression knocked down. As previously demonstrated by our laboratory (Banerjee et al., 2006; Bowers et al., 2010), the addition of 1611 antibody to myocyte-fibroblast co-cultures significantly reduced the concentration of IL-6 secreted into the media (Fig. 6A). In addition, knockdown of *dsp* in cardiac fibroblasts resulted in reduced IL-6 expression in myocyte-fibroblast co-cultures (Fig. 6A). Finally, similar results were observed in co-cultures of cardiac fibroblasts and ECs treated with 1611 antibody or having reduced *dsp* expression (Fig. 6B).

Discussion

The results from the current study demonstrate the importance of cell-cell interactions in the heart and help to define the key players in these interactions. Cell-cell junctions, especially desmosomes, display a range of morphology from simple contacts to complex, IF interactions with numerous proteins. DSP has been considered as a cytoplasmic marker of epithelial desmosomes and by morphological criteria; ECs do not have desmosomes and do not express desmosomal components such as desmogleins and desmocollins (Schmelz & Franke, 1993; Schmelz et al., 1994). In this study, we used an in-house polyclonal antibody to identify and investigate the function of DSP in cardiac cell-cell interactions. We were able to demonstrate that our 1611 antibody can detect DSP in cardiac fibroblasts. We also showed that 1611 antibody recognizes multiple proteins, as demonstrated by 2D gel electrophoresis and western blot analyses. 1611 may be recognizing multiple individual proteins, as it is a polyclonal antibody, or it may be recognizing a protein complex. Future studies will further define the intricacies of these interactions. In addition, we demonstrate that we can disrupt cell-cell interactions between myocytes and fibroblasts, fibroblast interactions among themselves, and fibroblasts and EC interactions with our 1611 antibody. As previously shown by our laboratory, interactions between cardiac fibro-blasts and myocytes are a necessary component of *in vitro* cytokine secretion (Banerjee et al., 2006; Bowers et al., 2010). What is important to note from these previous studies is that disruption of myocyte-fibroblast interactions through the use of connexin 43 (Cx43) blocking antibody depleted IL-6 secretion (Bowers et al., 2010). Additional studies by our lab and others have also demonstrated that reduction or loss of *dsp* results in reduced Cx43 expression (Hames et al., 2008; Asimaki et al., 2009 and data not shown), which could be the mechanism through which DSP is altering cell-cell interactions.

TEM and confocal microscopy demonstrated fibroblast-myocyte, myocyte-EC, and fibroblast-EC interactions. It is clear from these images that these cells are intimately associated with each other both *in vivo* and *in vitro*, and can communicate with each other as demonstrated by the changes in IL-6 secretion in our co-culture assays. Since it is beneficial for cardiac cells to be responsive to different signals, such as chemical, mechanical, and electrical, diverse avenues of communication are necessary to properly differentiate these signals. The current study demonstrates that DSP clearly plays a role in integration of these signals.

Little is known concerning the signaling relationships between cardiac fibroblasts, myocytes, and ECs in either normal or injured tissues. Our previous studies have demonstrated that multiple molecules are most likely involved in these cell-cell interactions; however, these studies are the first to demonstrate that DSP could also be involved. These data reveal that DSP plays a role in myocyte-fibroblast, fibroblast-fibroblast, and fibroblast-EC interactions, and it may be a critical player in these interactions during the developmental process, as well as during cardiac remodeling. However, it is important to emphasize that the contacts we are observing in the heart, especially the myocyte-fibroblast

and fibroblast-EC are most likely the simple cell contact type and not the complex. Furthermore, there may be additional differences in the factors that are involved when examining homotypic versus heterotypic interactions and future directions will continue to address these questions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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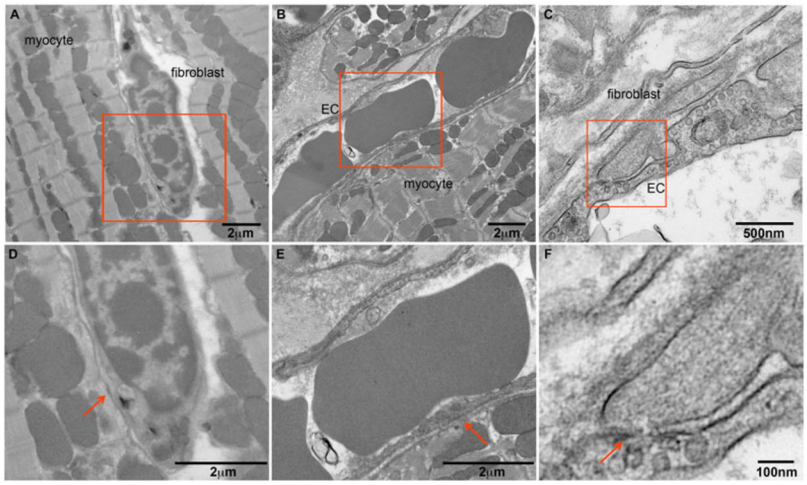


Figure 1.

Tight interactions between various cells of the heart. TEM of murine left ventricle demonstrating cell-cell interactions between (**A**) myocytes and fibroblasts, (**B**) myocytes and ECs, and (**C**) fibroblasts and ECs. High magnification of inset is shown in panels **D**, **E**, and **F**. Red arrows indicate regions of tight cell-cell interactions. Scale bars are shown in individual panels.

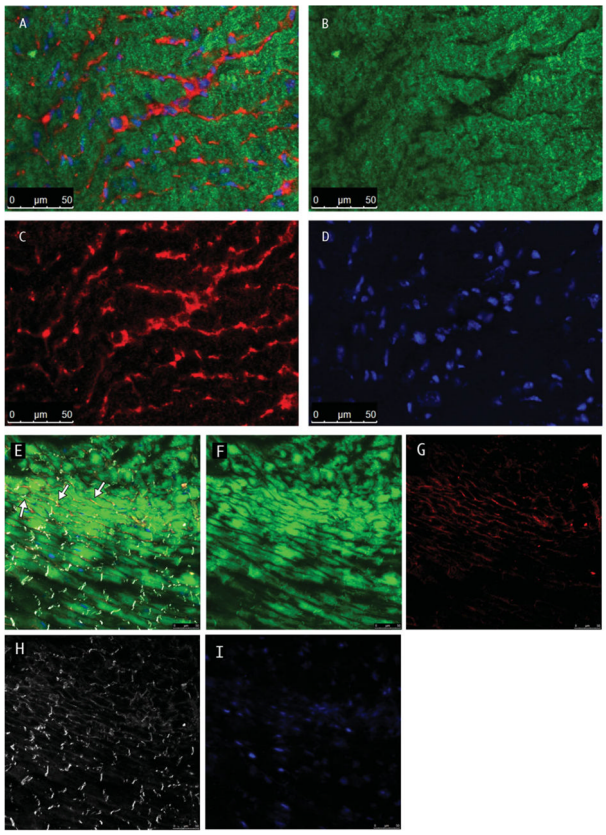
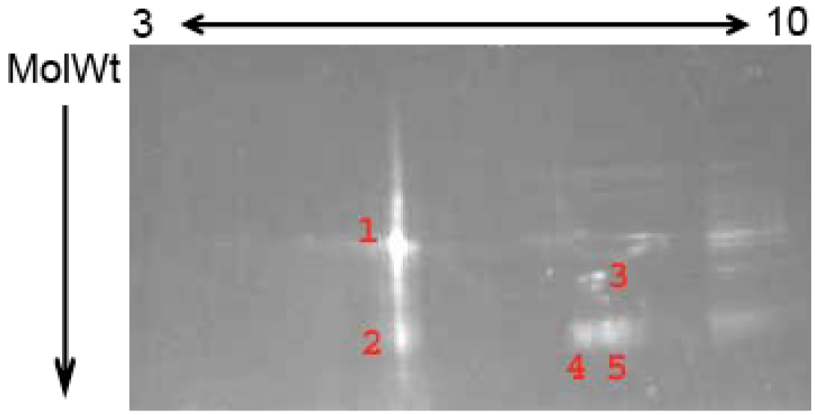


Figure 2.
Desmoplakin staining co-localizes with 1611 antibody staining. **A–D:** Confocal micrograph of murine left ventricle showing 1611 staining (red) of cardiac fibroblasts, myocytes with phalloidin (green), and nuclei with DAPI (blue). **E–I:** Confocal micrograph demonstrating partial co-localization of 1611 (red) with DSP (white) in the left ventricle of the mouse heart. The section was also stained with phalloidin (green) and DAPI (blue). Scale bars are shown in individual panels.



Spot Number	Protein Identity
1	DSP I
2	Obscurin
3	DSP II
4	Tropomyosin 2
5	Plakoglobin

Figure 3.
Two-dimensional electrophoresis profile of cardiac fibro-blast proteins recognized by 1611 antibody. Cardiac fibroblast protein lysates were immunoprecipitated with 1611 antibody and subjected to 2D gel electrophoresis. Samples were focused on 7 cm IPG strips (pH 3–10) and separated in 4–20% SDS-PAGE gels. The proteins were visualized with silver stain and subjected to MALDI-MS analysis. The protein spots were identified as DSP I, DSP II, plakoglobin, obscurin, and tropomyosin 2 as indicated in the table. $N = 5$.

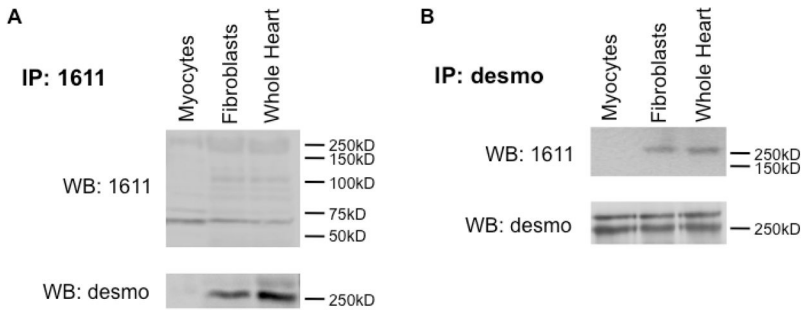


Figure 4.

IP analyses demonstrating that 1611 antibody recognizes DSP. **A:** Protein lysates from myocytes, cardiac fibroblasts, or whole heart were immunoprecipitated with 1611 antibody and then subjected to western blot analyses with either 1611 antibody (upper panel) or anti-DSP antibody (lower panel). **B:** Protein lysates from myocytes, cardiac fibroblasts, or whole heart were immunoprecipitated with anti-DSP antibody and then subjected to western blot analyses with either 1611 antibody (upper panel) or anti-DSP antibody (lower panel). $N = 3$.

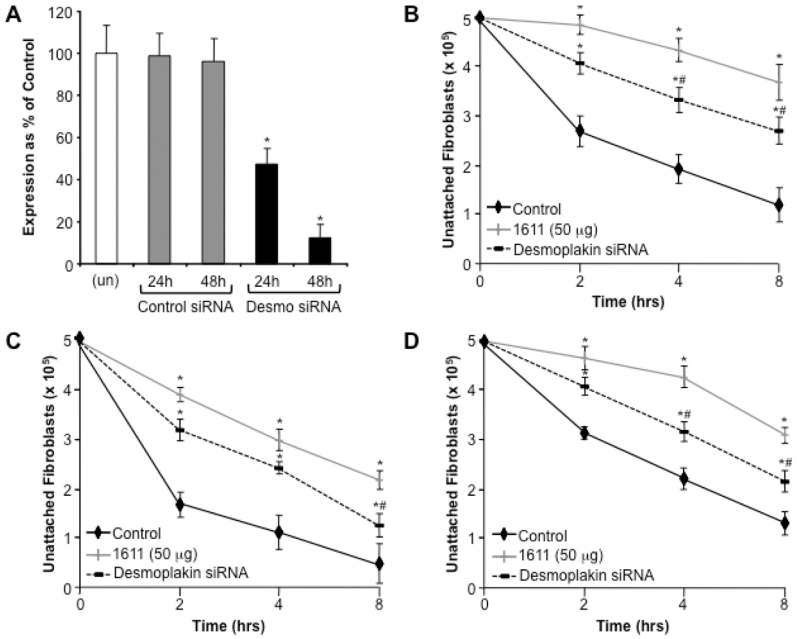


Figure 5.

Knockdown of DSP expression decreases cell-cell interactions between cardiac fibroblasts, myocytes, and ECs. **A:** Cardiac fibroblasts were left untreated or transfected with control or DSP siRNA (10 nM). Either at 24 or 48 h, the cells were collected and examined for DSP expression. Significant knockdown of DSP was observed at both 24 and 48 h ($p < 0.05$). Fibroblasts were collected at 48 h and used in cell-cell interaction assays. The effects of DSP knockdown or treatment with 1611 antibody were examined on cell-cell interactions between (**B**) myocytes and fibroblasts, (**C**) fibro-blasts and fibroblasts, or (**D**) ECs and fibroblasts. 50 μg of 1611 antibody were used in these studies. Cell-cell interactions were blocked in all three cultures treated with 1611 antibody ($p < 0.05$). DSP knockdown in fibroblasts also inhibited cell-cell interactions ($p < 0.05$). $N = 3$. * indicates a significant difference between experimental sample (1611 treated or DSP knockdown cells) and control cells. # indicates a significant difference between the two experimental groups (1611 treated versus DSP knockdown cells).

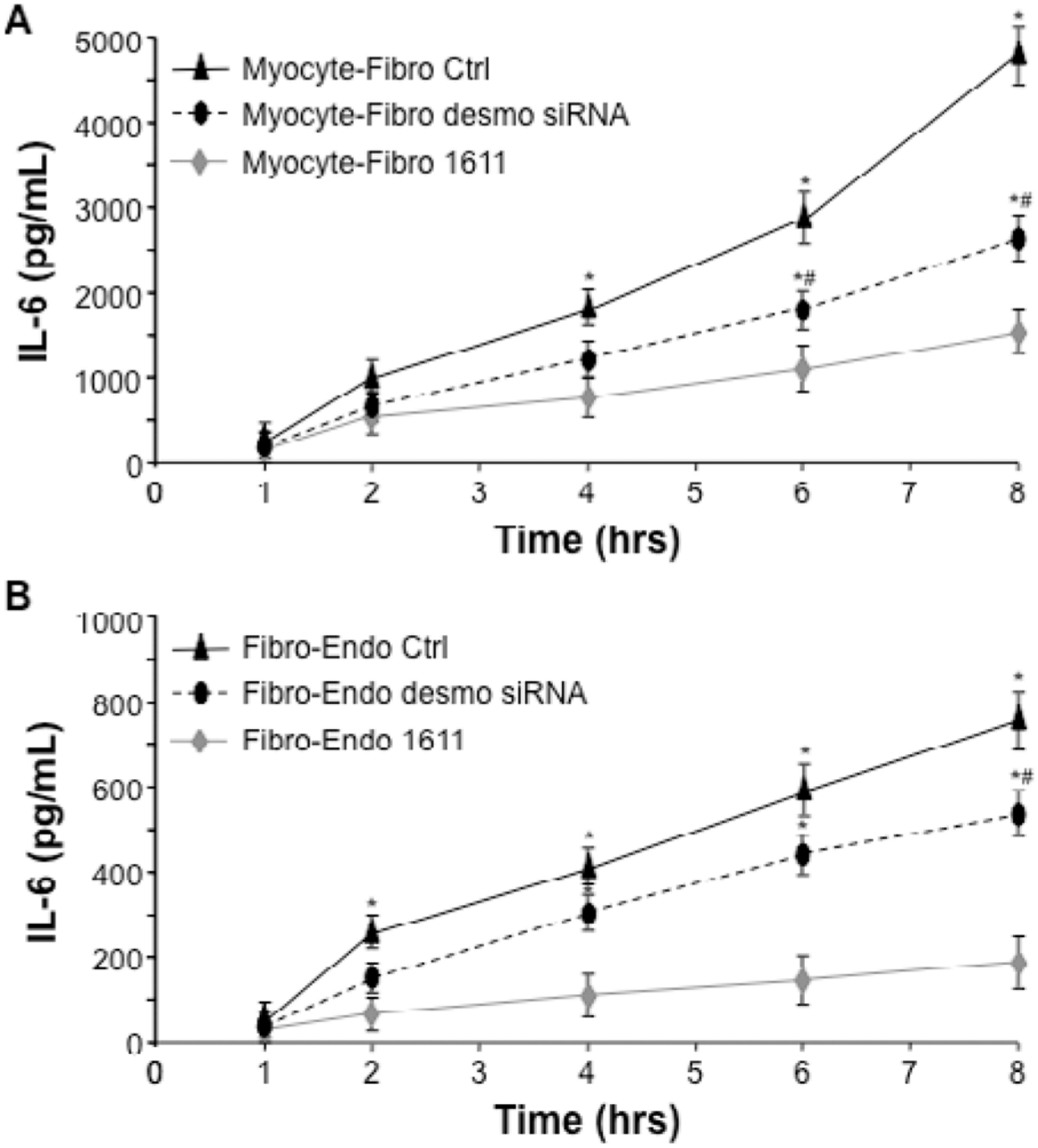


Figure 6.

Disruption of cell-cell interactions results in reduced IL-6 expression. Cardiac fibroblasts were left untreated, treated with 1611 antibody (50 mg), or treated with DSP siRNA (10 nM). Fibroblasts were then co-cultured with either (**A**) myocytes or (**B**) ECs, and media were collected at indicated time points. Disruption of cell-cell interactions with either 1611 antibody ($p < 0.05$) or with DSP knockdown ($p < 0.05$)resulted in reduced IL-6 secretion. $N = 3$.