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Original article

Acute slowing of cardiac conduction in response to myofibroblast coupling to cardiomyocytes through N-cadherin

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ABSTRACT

The electrophysiological consequences of cardiomyocyte and myofibroblast interactions remain unclear, and the contribution of mechanical coupling between these two cell types is still poorly understood. In this study, we examined the time course and mechanisms by which addition of myofibroblasts activated by transforming growth factor-beta (TGF-β) influence the conduction velocity (CV) of neonatal rat ventricular cell monolayers. We observed that myofibroblasts affected CV within 30 min of contact and that these effects were temporally correlated with membrane deformation of cardiomyocytes by the myofibroblasts. Expression of dominant negative RhoA in the myofibroblasts impaired both myofibroblast contraction and myofibroblast-induced slowing of cardiac conduction, whereas overexpression of constitutive RhoA had little effect. To determine the importance of mechanical coupling between these cell types, we examined the expression of the two primary cadherins in the heart (N- and OB-cadherin) at cell–cell contacts formed between myofibroblasts and cardiomyocytes. Although OB-cadherin was frequently found at myofibroblast–myofibroblast contacts, very little expression was observed at myofibroblast–cardiomyocyte contacts. The myofibroblast-induced slowing of cardiac conduction was not prevented by silencing of OB-cadherin in the myofibroblasts, and could be reversed by inhibitors of mechanosensitive channels (gadolinium or streptomycin) and cellular contraction (blebbistatin). In contrast, N-cadherin expression was commonly observed at myofibroblast–cardiomyocyte contacts, and silencing of N-cadherin in myofibroblasts prevented the myofibroblast-dependent slowing of cardiac conduction. We propose that myofibroblasts can impair the electrophysiological function of cardiac tissue through the application of contractile force to the cardiomyocyte membrane via N-cadherin junctions.

1. Introduction

Cardiac fibroblasts are commonly known for their maintenance of the extracellular matrix (ECM), balancing synthesis and degradation of matrix components in the healthy myocardium and becoming key participants in the process of fibrotic remodeling post injury [1]. More recently, however, substantial evidence suggests that fibroblasts also play a more active role in the electrical activity of the heart through cell–cell contacts with cardiomyocytes [2,3]. After cardiac injury, such as myocardial infarction (MI), fibroblasts become activated by mechanical and biochemical signaling and undergo a phenotypic change to become α-smooth muscle actin (SMA)-positive cells. These contractile cells, known as myofibroblasts, increase the deposition of ECM and compact newly-formed scars in the injured myocardium [4]. Contractile forces produced by myofibroblasts expressing α-SMA stress fibers are regulated by phosphorylation of myosin light-chain (MLC) by Ca^{2+}-dependent myosin light chain kinase or by inhibition of MLC phosphatase by Rho/Rho-kinase (ROCK) [5]. Unlike in smooth or cardiac muscle cells, Rho/ROCK appears to be the main pathway that regulates myofibroblast contraction [5], and ROCK regulation (which is Ca-independent) is capable of producing large, sustained contraction [6].

In addition to exerting strong contractile forces to stabilize scar tissue, myofibroblasts persist in and around the scar following MI and influence neighboring cardiomyocytes through biochemical, mechanical and electrical interactions. We studied this interaction previously in vitro by examining heterocellular junctions between myofibroblasts and cardiomyocytes in co-culture, and observed that heterocellular mechanical adherens junctions were more prevalent than electrical gap junctions [7]. On a functional level, slowing of conduction in cardiac monolayers induced by the supplementation of myofibroblasts can be fully restored to control levels by applying mechanosensitive channel (MSC) blockers or contraction blockers [7], but only partially restored by knockdown of connexin43 (Cx43) in the myofibroblasts [7,8]. Corroborating these findings is the observation that pharmacological

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ablation of the protein responsible for exerting contractile forces, α-SMA, abolishes the arrhythmogenic effect of myofibroblasts on cardiomyocyte conduction, even if heterocellular electrotonic coupling is sustained [9]. Taken together, these studies suggest a novel mechanism by which myofibroblasts may impair cardiomyocyte electrophysiological function through the application of contractile force to the cardiomyocyte membrane and activation of MSCs [7].

Mechanical interactions between cardiomyocytes and myofibroblasts are a possible contributing factor for the high risk of arrhythmia following MI, given the increased sensitivity of cardiomyocytes [10] and fibroblasts [11] to mechanical perturbations post injury. The mechanical interactions can be investigated more closely by examining the components of the mechanical junctions that link these two cell types together. One class of proteins that constitute these intercellular adhesions is the cadherins, which are transmembrane receptors that provide attachment points for adjoining cells. Cells exert strong contractile forces through cadherins [12,13], and cadherins allow for bi-directional transmission of cytoskeletal tension between cells. There are two types of cadherins expressed in adherens junctions in the heart: OB-cadherin and N-cadherin. OB-cadherin has been shown to be the dominant cadherin in myofibroblasts associated with wound healing [14,15] and appears to be expressed at regions of focal contact between cardiac myofibroblasts both in vitro and in vivo [16]. N-cadherin is found in mechanical junctions between adjoining cardiomyocytes [17] and between adjoining myofibroblasts [14] and is upregulated in myofibroblasts during wound healing through transforming growth factor-β (TGF-β) overexpression [18]. Studies of postnatal cardiac development and of isolated adult cardiomyocytes in culture have shown that adherens junctions precede gap junction formation in the intercalated disc [19,20], possibly because the adherens junctions tether the plus ends of microtubules that traffic connexin43 hemichannels to the cell membrane [17]. Conditional knockout of the N-cadherin gene in the adult mouse heart reveals its active role in both structural and signaling components of cardiac cells, resulting in disruption of cardiomyocyte-intercalated disc structure, misaligned myofibrils, impaired mechanical function, and arrhythmia-induced sudden death [21].

At present, information on cadherin-based signaling in cardiac myofibroblasts and cardiac myocytes is very limited, with the exception of investigations of adhesion or development-based signaling [22]. On the other hand, several studies have investigated mechanotransduction at adherens junctions of other cell types. For instance, skin fibroblasts communicate intercellular mechanical signaling through tugging forces at adherens junctions that activate MSCs in neighboring cells [23,24], and these interactions can occur as quickly as 15 min [23]. Cadherins also directly impact cellular electrical activity by modulating voltage-gated mechanosensitive calcium [25] and potassium currents [26].

Currently, very little is known about the nature of myofibroblast–myocyte adherens junctions in the heart. The data presented here provide new information about mechanical coupling between myofibroblasts and cardiomyocytes by incorporating the use of myofibroblasts with genetically modified expression of cadherins or mutant RhoA proteins in an established co-culture in vitro model [7], defining the time scale of myofibroblast-induced conduction slowing, and visualizing the real time interactions between myofibroblasts and cardiomyocytes. Importantly, the acute dependence of conduction slowing at a syncytial level on such heterocellular mechanical junctions is demonstrated.

2. Methods

An expanded Materials and Methods section is available in the online data supplement. All animal experiments were performed in accordance with guidelines set by the Johns Hopkins Committee on Animal Care and Use and were in compliance with all federal and state laws and regulations. In brief, 20 mm diameter anisotropic monolayers of neonatal rat ventricular cells (NRVCs) were obtained by growing cells on parallel, 20 μm-wide fibronectin lines formed by microcontact printing. Cardiac fibroblasts were separately pre-treated with 5 ng/ml TGF-β for at least 48 h to promote the cardiac myofibroblast phenotype and then added onto patterned NRVC monolayers of 1 million cells at a concentration of 400,000 cells per monolayer. For time lapse experiments, monolayers were optically mapped with 10 μM voltage-sensitive dye, di-4-ANEPPS, at designated time points following myofibroblast supplementation (30 min, 1 h, 2 h, 3 h, 4 h, 8 h and 24 h). Activation maps were obtained at 2 Hz pacing during constant superfusion (with bath volume exchange approximately every 2 min) to determine myofibroblast impact on conduction velocity (CV) in the longitudinal (LCV) and transverse (TCV) directions. Dye transfer experiments, traction forces of myofibroblasts, and immunocytochemistry of adherens junction formation between myofibroblasts were also visualized on the same time scale. Live cell imaging was performed to characterize the interactions between myofibroblasts and cardiomyocytes in real time. Fibroblasts were also transduced with OB-cadherin or N-cadherin shRNA lentiviral particles containing a puromycin resistance gene. Two days later, cells stably expressing shRNA were selected with puromycin, and OB-cadherin or N-cadherin knockdown was confirmed using Western blots. As a negative control, fibroblasts were transduced with shRNA lentiviral particles encoding a scrambled shRNA sequence. The transduced fibroblasts were treated with 5 ng/ml TGF-β for 48 h prior to addition to initiate differentiation towards myofibroblasts; 400,000 myofibroblasts were added onto control NRVC monolayers for subsequent electrophysiological analysis. Then, an excitation–contraction uncoupler (blebbistatin) or MSC blocker (gadolinium or streptomycin) was superfused over the monolayer to determine its impact on LCV and TCV. Fibroblasts were also transduced for 24 h with lentiviruses encoding activated RhoA (RhoA-V14) or dominant negative RhoA (RhoA-N19) IRES-GFP cassettes under control of the tetracycline-inducible TRE3G promoter. For the RhoA experiments, cells were co-transduced with a lentivirus harboring the Tet-ON transactivator (tet3g) and a neomycin selection cassette. As a negative control, fibroblasts were transduced with lentiviruses that encoded doxycycline-inducible AcGFP only. The transduced fibroblasts were selected by adding neomycin, treated with 5 ng/ml TGF-β for 48 h, and added onto control NRVC monolayers (total number of 400,000 cells/monolayer) for subsequent electrophysiological analysis. Induction of RhoA mutants in myofibroblast-supplemented monolayers was achieved by the addition of 1 μM of the tetracycline analog, doxycycline, 5 h after myofibroblast supplementation. Electrophysiological analysis was conducted 20–24 h later. Additionally, adjoining pairs of myofibroblasts and cardiomyocytes were characterized by immunostaining for troponin I, SMA, N-cadherin (or OB-cadherin), and DAPI. All data are expressed as mean ± (standard error of mean). Wilcoxon signed rank tests were performed for paired data, such as fibrotic monolayers before and after treatment, and ANOVA with TukeyHSD tests were performed for multiple comparisons to determine statistically significant differences ($p < 0.05$).

3. Results

3.1. Supplemented myofibroblasts dramatically slow cardiomyocyte monolayer conduction velocity as early as 30 min

Myofibroblasts were added on top of anisotropic NRVC monolayers to determine the incubation time required for the previously reported slowing effect on CV [7] to occur. Both LCV and TCV decreased by about 50% after just 30 min and remained low for 24 h (Figs. 1A–B). Visualization of the pan-cadherin staining in co-culture monolayers following optical mapping confirmed increased expression levels at 1 h after supplementation (Figs. 2A–B), consistent with myofibroblast attachment to the cardiomyocytes. Pure myofibroblast cultures also demonstrated positive pan-cadherin staining at homocellular junctions within 1 h (Supplementary Fig. 1).

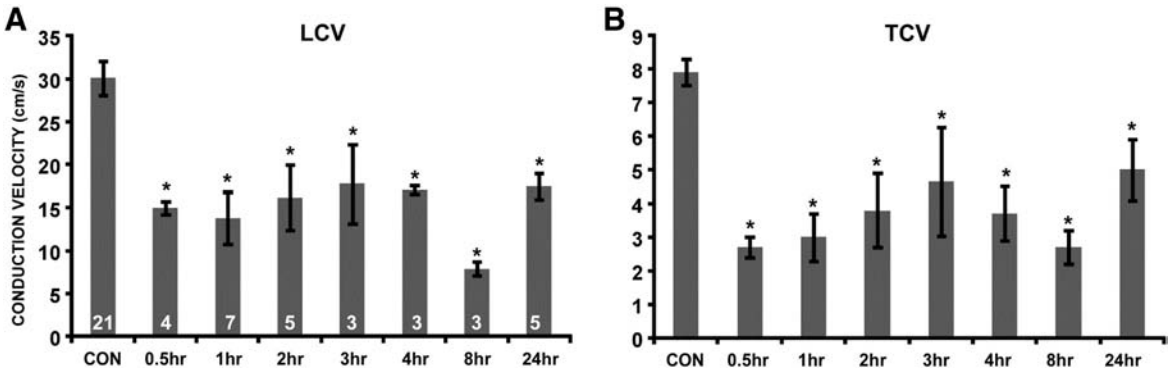

Fig. 1. Time scale of conduction velocity suppression following myofibroblast supplementation. Longitudinal conduction velocity (LCV, A) and transverse conduction velocity (TCV, B) were measured in cell monolayers prior to (control) and at various times (0.5, 1, 2, 3, 4, 8 and 24 h) after addition of myofibroblasts. Numbers in white are values of n. *$P < 0.05$ signifies difference from control by ANOVA/TukeyHSD.

3.2. Dye transfer between myofibroblasts and cardiomyocytes occurs only several hours after observed initial slowing of CV

Myofibroblasts loaded with calcein and DiI were added onto cardiomyocyte monolayers and imaged at 30 min, 1 h and then every hour for the next 6 h to observe potential dye transfer. Although there was significant slowing of CV 30 min after myofibroblast addition, moderate dye transfer was only observed between cardiomyocytes and myofibroblasts after a minimum of 5 h of contact ($n = 5$ trials, Figs. 3A–B), suggesting the absence of functional gap junctions during this early time period. However, cardiomyocytes loaded with calcein and added onto cardiomyocyte monolayers revealed dye transfer within 30 min of contact (Figs. 3C–D).

3.3. Myofibroblasts are capable of exerting strong contractile forces within 30 min

Myofibroblasts are capable of exerting considerable force within 30 min of seeding onto deformable posts (Fig. 3E). While cell area remains relatively stable over time, total cell strain energy continues to increase, indicating an increase in cell contractile force.

3.4. Myofibroblasts are motile cells that can actively deform the cardiomyocyte membrane

To further investigate our hypothesis that myofibroblasts are capable of applying tension to myocytes through adherens junctions, we performed live cell imaging to visualize interactions between the two cell types. We found that cardiac myofibroblasts are extremely motile cells that constantly interact with the cardiomyocytes as they migrate (Supplementary Movies). It appears that heterocellular connections are formed and broken repeatedly, and that these interactions result in deformation of the cardiomyocyte membrane (Fig. 4A, Supplementary Movie 1). At times, the heterocellular interactions could deform the myocyte membrane to a breaking point, which ultimately resulted in myocyte rounding (Fig. 4B, Supplementary Movie 2). Imaging of 20 separate cardiomyocytes (selected as those instances wherever isolated cells with adjoining myofibroblasts could be found and clearly imaged) showed that 100% of the cells were physically affected by the presence of myofibroblasts; 65% of the cells in contact with myofibroblasts were deformed and in the other 35%, the cells became completely rounded after undergoing membrane deformation.

3.5. RhoA activity is necessary for myofibroblast-induced slowing of cardiac conduction

Because treatment with blebbistatin, a contraction inhibitor, restored the slowed CV to near control levels in myofibroblast supplemented monolayers [7], we investigated the effect of dominant negative RhoA (RhoA-N19) expression in myofibroblasts. Significant slowing of conduction occurred with non-activated RhoA-N19 or GFP (control), but failed to occur with RhoA-N19 activated by doxycycline (Figs. 5A, B). Increase in RhoA-N19 expression level was confirmed by Western blot (Fig. 5E), and suppression of contractile force was confirmed by micropost force arrays (126 ± 20 fJ, $n = 15$) (Fig. 5F), which is much less than the average values of 183 to 278 fJ ($n = 129$)

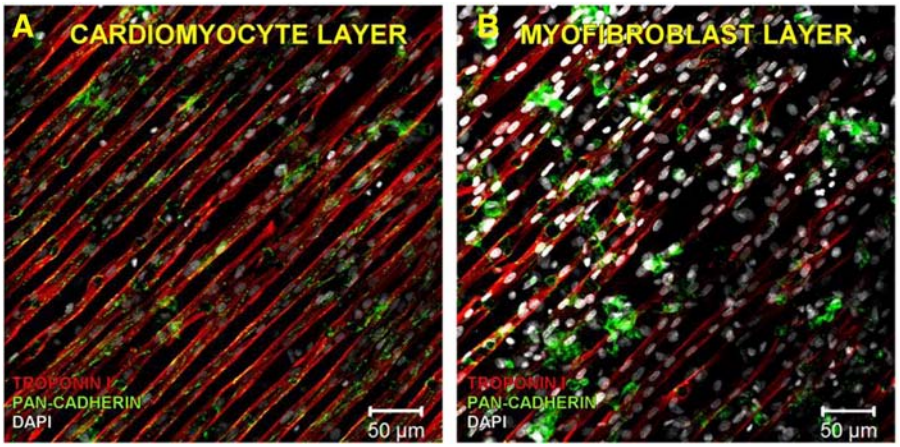

Fig. 2. Heterocellular adherens junctions form rapidly. Cardiomyocytes in red are mechanically coupled through pan-cadherin (green) prior to myofibroblast addition (A). One hour after supplementation, myofibroblasts, whose DAPI-stained nuclei are in the plane of focus, have attached to the myocyte layer and there is greatly increased expression of pan-cadherin (green) (B). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

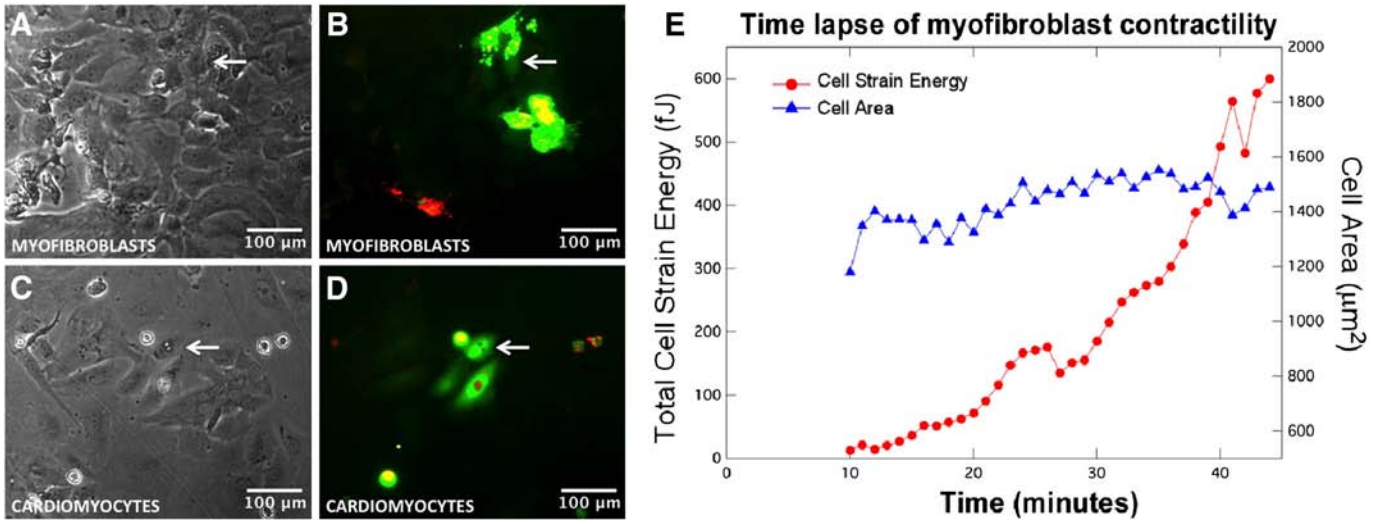

Fig. 3. Myofibroblasts show minimal dye transfer to cardiomyocytes after 5 h even though they begin exerting forces within 20 min of contact. DiI labeled myofibroblasts (red) begin to show limited dye transfer of calcein-AM (green) to neighboring cardiomyocytes as seen in the phase image (A) after 5 h of contact (denoted by arrows in A and B and green dye transfer in B). Alternatively, cardiomyocytes are able to transfer calcein-AM (green) to adjacent cardiomyocytes (indicated by arrows in C and D and green dye transfer in D) 30 min after addition. Time lapse of myofibroblast contractile ability confirms that although no dye transfer is observed with an hour of contact, myofibroblasts are capable of exerting contractile forces as early as 20 min post addition (E). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

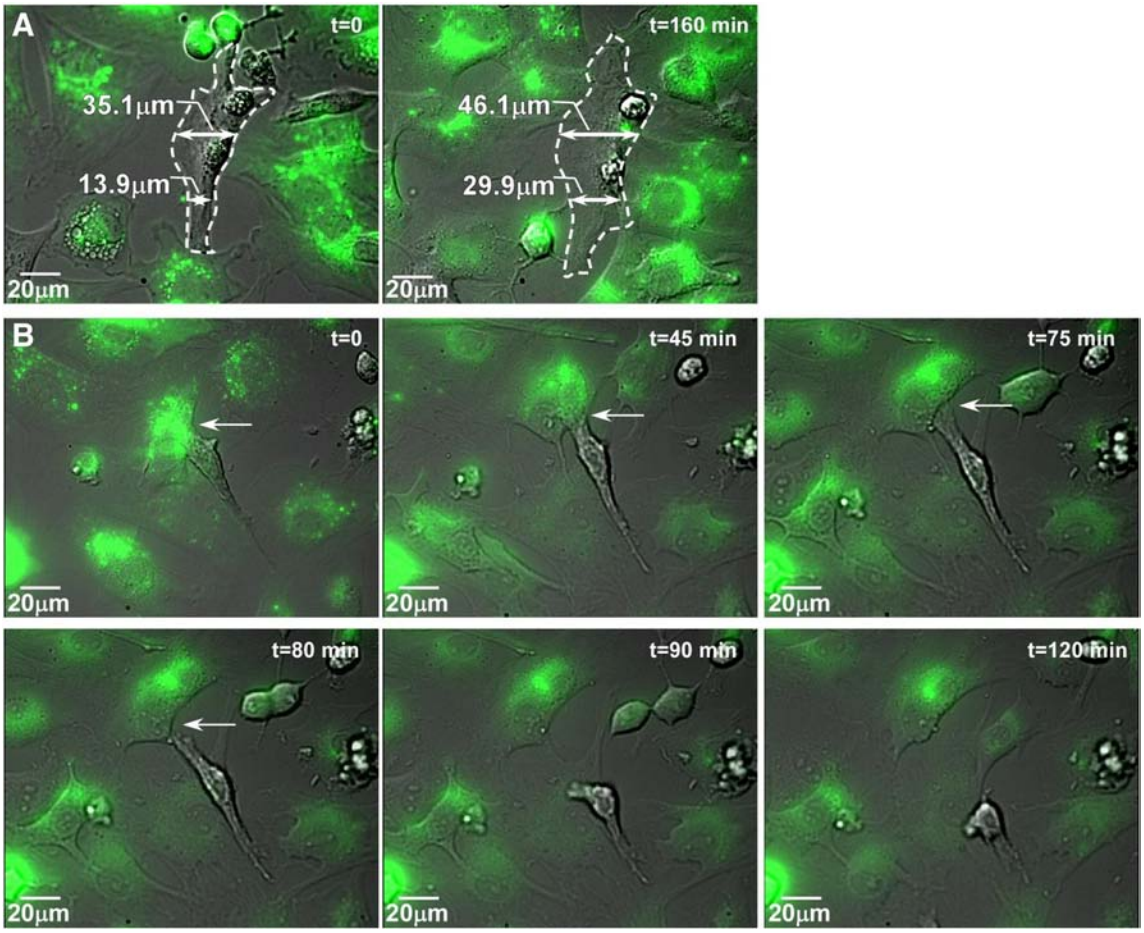

Fig. 4. Still frames from live cell time lapse experiments show that myofibroblasts interact repeatedly with cardiomyocytes. As a result, all cardiomyocytes experienced membrane deformation and at times, complete cell rounding. At time 0 in panel A, the myocyte is surrounded by myofibroblasts (labeled with green DiO staining). After 160 min, the myocyte membrane is significantly elongated in the two regions indicated by the white arrows. In panel B, the time series shows that a labeled myofibroblast interacts with a cardiomyocyte at time 0. After 45 min, the myocyte begins to extend at the heterocellular contact site (area indicated by white arrow) and by 80 min, the contact is beginning to break. After 90 min, the heterocellular contact has broken and by 120 min the myocyte has completely rounded.

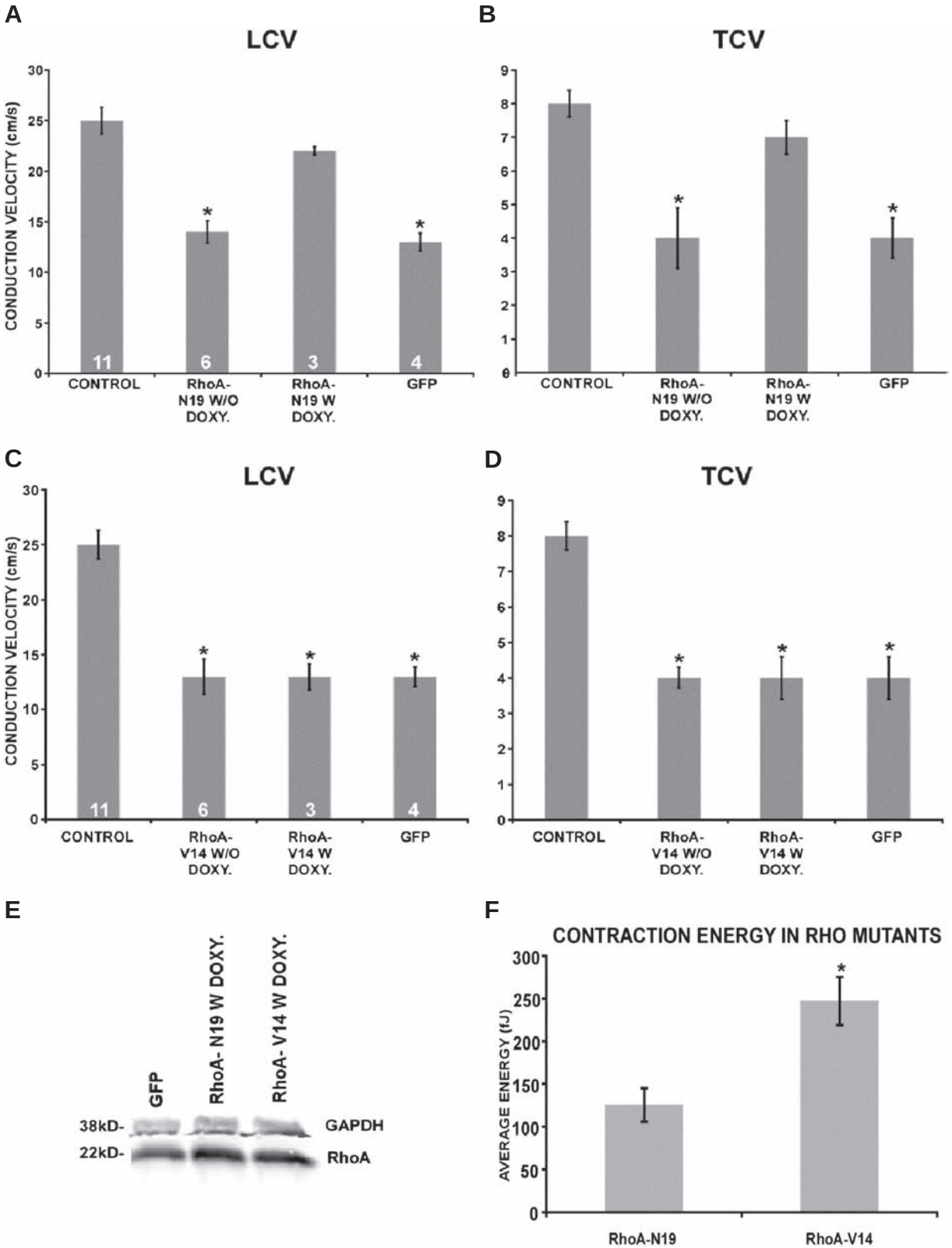

Fig. 5. Dominant negative RhoA transduced myofibroblasts do not produce the characteristic slowing effect on NRVC CV while constitutively active RhoA transduced myofibroblasts are indistinguishable from control myofibroblasts. NRVC monolayers supplemented with myofibroblasts transduced with lentivirus GFP (GFP) or NRVC monolayers supplemented with RhoA-N19 myofibroblasts without doxycyline treatment have decreased CV in comparison with control monolayers. Monolayers supplemented with myofibroblasts transduced with dominant negative RhoA (RhoA-N19) that was activated with doxycyline had LCV (A) and TCV (B) indistinguishable from control monolayers. NRVC monolayers supplemented with myofibroblasts transduced with lentivirus GFP (GFP) have decreased CV in comparison with control monolayers. Monolayers supplemented with myofibroblasts transduced with constitutively active RhoA (RhoA-V14) and activated with doxycyline do not have significantly different LCV (C) or TCV (D) compared with NRVC monolayers supplemented with Rhoa-V14 myofibroblasts without doxycyline treatment. Myofibroblasts transduced with either RhoA-N19 or RhoA-V14 and treated with doxycycline have significantly higher expression of RhoA when compared to control myofibroblasts, as indicated by Western blot (E). *$P < 0.05$ signifies difference from control by ANOVA/TukeyHSD. Constitutively active RhoA myofibroblasts exert significantly higher contractile forces than dominant negative RhoA transduced myofibroblasts (F). Contractile forces were measured 18–24 h after myofibroblasts were added to the posts. *$P < 0.01$ signifies difference from RhoA-N19 by Student's *t*-test. All CV measurements were performed 24 h after the addition of transduced myofibroblasts to NRVC monolayers.

that we previously reported for control myofibroblasts [7]. Both non-activated and activated RhoA (RhoA-V14) produced similar levels of slowing comparable to GFP control (Figs. 5C, D), even though the increase in RhoA-V14 expression was confirmed by Western blot (Fig. 5E) and an increase in contractile force was measured (247 ± 28 fJ, $n = 15$) (Fig. 5F).

3.6. N-cadherin plays a critical role in mechanical coupling whereas OB-cadherin does not

N-cadherin or OB-cadherin was imaged to determine the identity of the dominant cadherin present between the two cell types. Although OB-cadherin was expressed abundantly between neighboring myofibroblasts (Fig. 6A), it was not frequently visualized between the two cell types (Fig. 6B). In contrast, N-cadherin was commonly found at the junctions between myofibroblasts and cardiomyocytes (Figs. 6C–F). It also appeared that myofibroblasts have the capacity to physically deform the cardiomyocyte membrane through N-cadherin coupling (denoted by arrows in Figs. 6E and F and in Supplementary Fig. 2).

Depressed conduction was observed in NRVC monolayers supplemented with myofibroblasts transduced with either scrambled shRNA or OB-cadherin shRNA, but not with N-cadherin shRNA (Fig. 7A). Knockdown of OB-cadherin and N-cadherin was confirmed by Western

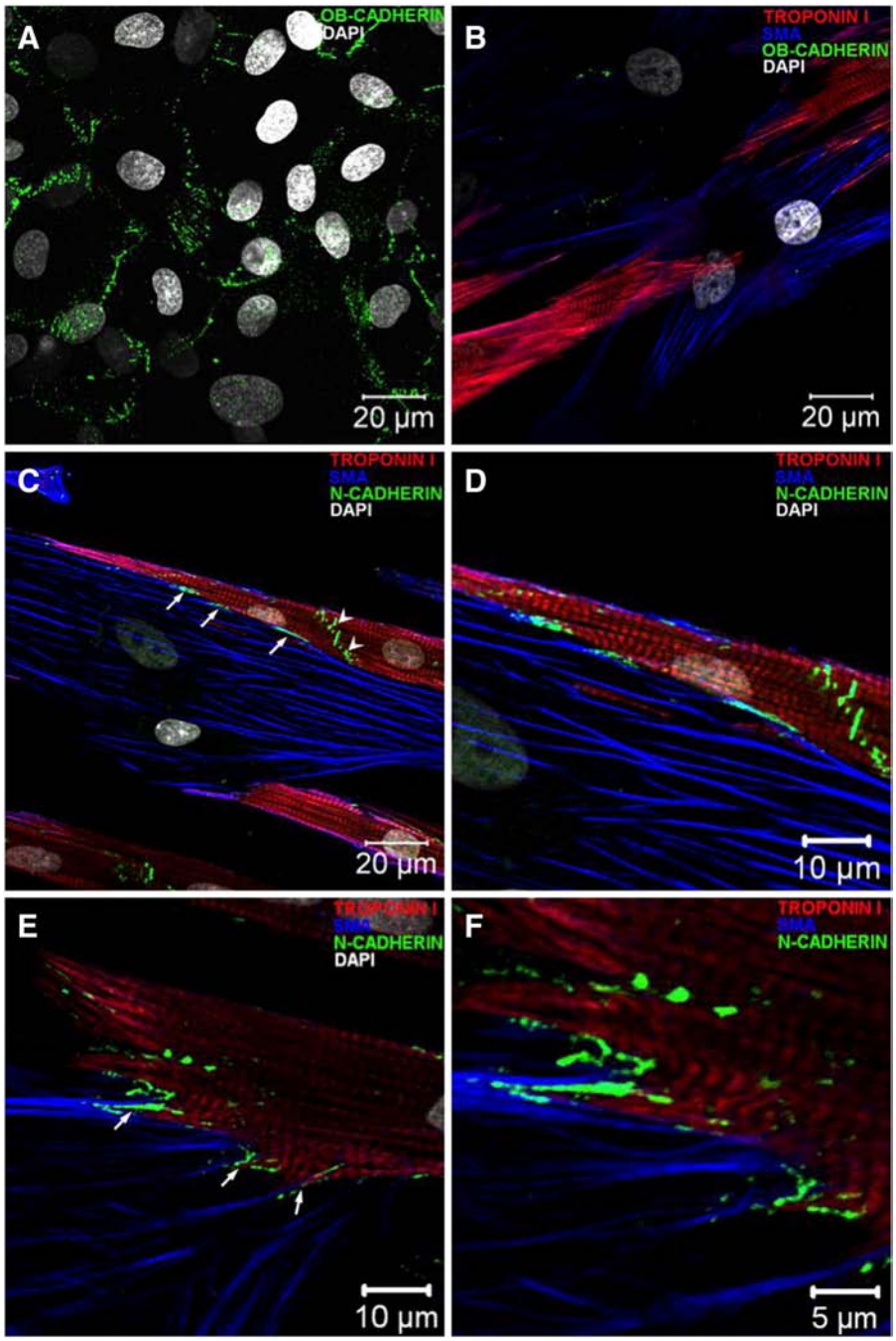

Fig. 6. Cadherin expression at homocellular and heterocellular myofibroblast junctions. Purified TGF-β treated myofibroblasts express OB-cadherin at homocellular junctions (A). Frequently, no OB-cadherin expression was observed between myofibroblasts (blue) and cardiomyocytes (red, B). Heterocellular junctions between a cardiomyocyte (red) and myofibroblast (blue) at both the lateral side (C) and end (E) of the myocyte show expression of N-cadherin (green) at the sites of contact between the myofibroblast and cardiomyocyte. Arrows indicate specific areas of N-cadherin contact between myofibroblasts and cardiomyocytes (C, E) while arrow heads indicate areas of N-cadherin at homocellular junctions between neighboring cardiomyocytes (C). Magnified images of the heterocellular junctions at the lateral sides (D) and ends (F) of the myocytes more clearly show N-cadherin expression.

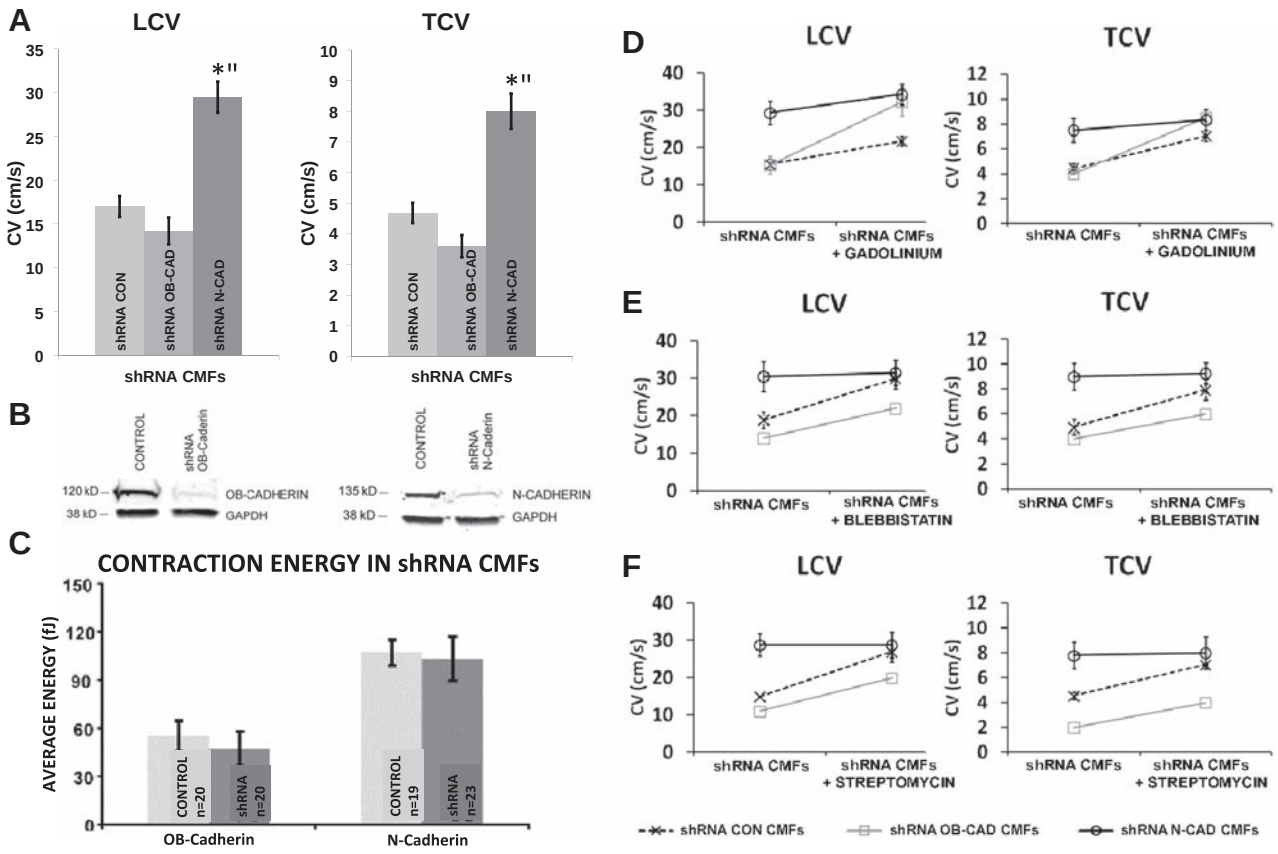

Fig. 7. Summary LCV and TCV graphs for NRVC monolayers supplemented with myofibroblasts transduced with shRNA control (scrambled) lentivirus (shRNA CON CMFs), shRNA OB-cadherin lentivirus (shRNA OB-CAD CMFs) or shRNA N-cadherin lentivirus (shRNA N-CAD CMFs). Pre-silencing of OB-cadherin in myofibroblasts prior to addition to the NRVC monolayer did not prevent the characteristic slowing of CV observed when myofibroblasts transduced with shRNA control particles were used, whereas pre-silencing myofibroblasts with N-cadherin shRNA did inhibit the conduction slowing (A). Western blots show protein expression of OB-cadherin ($n = 3$) in OB-cadherin shRNA-transduced myofibroblasts, and of N-cadherin ($n = 3$) in N-cadherin shRNA-transduced myofibroblasts, was significantly reduced compared with control myofibroblasts (B). Neither shRNA OB-cadherin transduced myofibroblasts, nor N-cadherin transduced myofibroblasts, had significantly different contraction forces than control myofibroblasts in the same batch of cells, confirming that reduced expression of cadherin has little impact on forces produced by myofibroblasts (C). Treatment with gadolinium (D), blebbistatin (E), or streptomycin (F) recovered CV in NRVC monolayers supplemented with control or OB-cadherin shRNA-transduced myofibroblasts, but had little effect on CV of monolayers supplemented with N-cadherin shRNA-transduced myofibroblasts. * denotes significance of $P < 0.01$.

blot (Fig. 7B). Neither the transduction of OB-cadherin shRNA, nor transduction of N-cadherin shRNA had an effect on the contractile forces produced by myofibroblasts (Fig. 7C). The slowed CV in monolayers supplemented with control shRNA or OB-cadherin shRNA myofibroblasts could be restored to near control levels following treatment with gadolinium (Fig. 7D), blebbistatin (Fig. 7E), or streptomycin (Fig. 7F), similar to our previous results using non-transduced myofibroblasts [7]. Moreover, treatment with blebbistatin, gadolinium or streptomycin had no significant effect on CV of monolayers supplemented with shRNA N-cadherin myofibroblasts (Figs. 7D–F). When all the treatments (gadolinium, blebbistatin, and streptomycin) were considered together, LCV and TCV increased significantly ($P < 0.01$) for control myofibroblasts (from 17 ± 1.2 cm/s to 26.4 ± 1.8 cm/s and 4.7 ± 0.3 cm/s to 7.5 ± 0.4 cm/s, respectively, $n = 13$) and OB-cadherin shRNA-transduced myofibroblasts (from 14.2 ± 1.4 cm/s to 27.8 ± 3.5 cm/s and 3.6 ± 0.4 cm/s to 7.1 ± 0.9 cm/s, respectively, $n = 5$), but not for N-cadherin shRNA-transduced myofibroblasts (from 29.4 ± 1.8 cm/s to 31.7 ± 1.8 cm/s and 8.0 ± 0.6 cm/s to 8.5 ± 0.6 cm/s, respectively, $n = 15$).

4. Discussion

Throughout the body, fibroblasts transition into contractile myofibroblasts in order to initiate the wound healing process after an injury is sustained. In the heart, because of the limited regenerative capability of cardiomyocytes, wound healing concludes with loss of cardiac muscle and formation of a stable scar through myofibroblast contraction, ultimately leading to a fibrotic, arrhythmogenic substrate [27]. Myofibroblasts also utilize contraction as a means to communicate mechanically and electrically, and as adherens junctions mechanically couple SMA stress fibers of adjoining myofibroblasts, these proteins provide a pathway for transmission of contractile force and subsequent mechanoelectric coupling through MSC activation in coupled cells [24]. Further, exogenous forces applied to adherens junctions of fibroblasts, either through anti-N-cadherin antibody-coated magnetic beads or through cell–cell adhesions with other cells loaded with magnetic beads, activate MSCs and increase actin polymerization in the fibroblasts [23]. Although there is an abundance of information relating to cell–matrix adhesions and their ability to respond to matrix mechanics and subsequently modify cell functions [28], information on cell-to-cell adhesions in the heart is relatively limited. Although cell–matrix and cell–cell adhesions are structurally and functionally distinct mechanosensitive systems, the extent to which they act through different or common signaling pathways is largely unknown [29,30]. Our previous work demonstrating the prevalence of heterocellular adherence junctions and conduction slowing in myofibroblast-supplemented cardiac monolayers suggested that adherens junctions between myofibroblasts and cardiomyocytes may play a role in electrical disturbances of the heart by communicating mechanical tugging forces that activate depolarizing mechanosensitive channels in the myocyte, raise the resting potential, inactivate sodium channels, and slow conduction [7]. Although not addressed in this study, the mechanical tugging forces

might alternatively activate depolarizing mechanosensitive channels in the myofibroblast [11], which, if electrically coupled to the myocyte [3] could depolarize the myocyte and slow conduction. Thus, heterocellular coupling via adherens junctions was ultimately the focus of this study.

N-cadherin junction formation precedes connexin43 junction formation in both in vitro [20] and in vivo studies [21], and adherens junctions begin forming as early as 1 min between neighboring fibroblasts [31]. Therefore, we hypothesized that myofibroblast-induced slowing of conduction in cardiomyocyte monolayers may occur soon (within minutes) after the introduction of myofibroblasts, when cells become coupled through adherens junctions. Indeed, we found that conduction slowing occurred as early as 30 min after myofibroblast supplementation (Fig. 1) and that cadherin was concomitantly expressed at the junctions over the same time interval (Fig. 2). Our time-lapse micropost array experiments confirmed that myofibroblasts are capable of exerting strong, contractile forces within this time scale (Fig. 3E). Further, we did not visualize dye transfer between myofibroblasts and cardiomyocytes during this early time interval (Figs. 3A–B), although it did occur between neighboring cardiomyocytes within 30 min of contact (Figs. 3C–D). These results corroborate our previous findings that SMA-positive myofibroblasts had little Cx43 expression but enhanced pan-cadherin expression [7]. Even the electrical coupling of cardiomyocyte cell sheets with fibroblast cell sheets, which have the advantage of both cell types already expressing junctional proteins with neighboring cells, requires at least 2 h of heterocellular contact before dye transfer can be observed [32].

Complementing our previous finding that treatment of myofibroblast-supplemented NRVC monolayers with the contraction inhibitor, blebbistatin, restores depressed conduction [7], expression of a dominant negative RhoA protein, which reduced the myofibroblasts' ability to produce contractile forces (Fig. 5F), prevented myofibroblast-induced slowing of cardiac conduction (Figs. 5A–B). However, overexpression of activated RhoA in supplemented myofibroblasts did not slow NRVC conduction further (Fig. 5C–D), even though contractile forces were significantly increased in myofibroblasts (Fig. 5F), suggesting that the contraction of non-transduced myofibroblasts provides enough force to saturate the slowing response of the NRVC monolayers. This is not surprising since extensive cardiomyocyte deformation frequently occurs during contact with non-transduced myofibroblasts (Figs. 4, 6).

Considerable deformation of the cardiomyocyte membrane upon heterocellular contact was often observed in both our time-lapse live cell imaging (Fig. 4, Supplementary Movies) and N-cadherin heterocellular junctional staining (Figs. 6E, F Supplementary Fig. 2), as well as in previous studies of guinea pig cardiomyocytes pulled upon by contacting fibroblasts [33]. In contrast, cardiomyocytes interacting with other cardiomyocytes did not experience significant membrane displacement even though intercellular coupling was also through N-cadherin junctions (Fig. 6C). This may be a consequence of the scaffolding complex formed at the adherens junction between N-cadherin, plakoglobin, p120, catenin and ZO-1 that is mechanically anchored to the actin cytoskeleton [34], which we speculate is absent in newly formed heterocellular myocyte-myofibroblast junctions. In our co-culture experiments, cardiomyocytes have been in culture for 6 days prior to myofibroblast supplementation, which previous work has shown is long enough for neighboring cardiomyocytes to form mature intercalated disks that serve as anchoring points for the actin cytoskeleton [17]. We believe the transient nature of heterocellular adherens junctions is more likely to resemble the initial cadherin contacts observed between cardiomyocytes, which lack these mature structures [17].

Myofibroblast sensitivity to mechanical perturbations increases following myocardial infarction [11], indicating that mechanical interactions could play a larger role than suggested here in terms of myofibroblast-induced conduction abnormalities. Although OB-cadherin is the isoform shown to be important in subcutaneous skin wound healing [14,15] and is clearly expressed at junctions between myofibroblasts (Fig. 6A), cardiomyocyte adherens junctions primarily express N-cadherin [35], and ultimately, very little OB-cadherin was observed between contacting myofibroblasts and cardiomyocytes (Fig. 6B). The sparse OB-cadherin expression aligns with previous studies in CHO cells showing that N-cadherin forms homodimers with higher affinity than N/E-cadherin heterodimers [36]. Moreover, when heterocellular adherens junctions between fibroblasts (expressing N-cadherin) and epithelial cells (expressing E-cadherin) were observed, the junctions had an irregular organization compared with homocellular junctions [37], indicating that heterotypic adherens junctions may initiate alternative signaling pathways, as downstream signaling is directly related to cadherin structure in cardiomyocyte adherens junctions [38].

Given that pre-silencing of OB-cadherin in myofibroblasts prior to their addition to the NRVC monolayers did not prevent the characteristic myofibroblast-induced slowing of conduction or its recovery with contraction or MSC blockers (Fig. 7A), it appears that functional heterotypic OB-N cadherin junctions between myofibroblasts and myocytes were not present. This data is inconsistent with previous research indicating that blocking antibodies against OB-cadherin inhibit myofibroblast/myocyte aggregation [16]. On the other hand, N-cadherin, which is expressed in both myocytes [35] and myofibroblasts [14], was found at the heterocellular junctions (Figs. 6C–F); these findings align with data showing that blocking antibodies against N-cadherin inhibit myofibroblast/myocyte aggregation [16]. Pre-silencing of N-cadherin in the myofibroblasts prevented their ability to slow of conduction (Fig. 7C), indicating a primary role for this isoform of cadherin in heterocellular interactions.

A limitation of our approach is the use of an in vitro system, which may significantly affect cellular functional and distribution of adherens junctions. The interaction between myofibroblasts and cardiomyocytes in vivo is a topic of debate [39], and it remains to be seen to what extent mechanical adherens junctions affect the electrophysiology of cardiomyocytes in healthy and diseased myocardium.

5. Conclusions

Our data demonstrate that impaired conduction in an in vitro fibrotic model is mechanically dependent on heterocellular contact between myofibroblasts and cardiomyocytes. Furthermore, our findings suggest that mechanical coupling between myofibroblasts and cardiomyocytes occurs through N-cadherin adherens junctions, and through this coupling, myofibroblasts can exert electrophysiological influence on cardiomyocytes within 30 min of contact and substantially deform the membrane of isolated cardiomyocytes during this time. Finally, we propose that myofibroblasts may impair cardiomyocyte electrophysiological function through the application of contractile force to the cardiomyocyte membrane via N-cadherin junctions.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2013.12.025.

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Disclosures

The authors have no potential conflicts of interest to disclose.

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Glossary

NRVCs: neonatal rat ventricular cells
CV: conduction velocity
LCV: longitudinal CV
TCV: transverse CV