Cadherin-mediated adhesion is essential for myofibril continuity across the plasma membrane but not for assembly of the contractile apparatus

Yang Luo and Glenn L. Radice*

Center for Research on Reproduction and Women's Health, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA

*Author for correspondence (e-mail: radice@mail.med.upenn.edu)

Accepted 23 December 2002
Journal of Cell Science 116, 1471-1479 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00339

Summary

The strong coordinated contraction of heart muscle is dependent on the correct alignment and connection of the myofibrils across the plasma membrane. Previous studies indicate that N-cadherin is involved in cardiac myocyte adhesion and myofibrillogenesis. To investigate whether N-cadherin is specifically required for normal myocyte structure and function, we cultured myocytes from wild-type, N-cadherin-null and mutant embryos expressing the epithelial cadherin E-cadherin. In contrast to previous studies in chicken using N-cadherin-perturbing antibodies, our in vitro studies with mouse cells demonstrate that N-cadherin is not required for myofibrillogenesis, but is critical for myofibril organization. That is, N-cadherin-deficient myocytes beat and myofibrils were well formed; however, alignment of the myofibrils through regions of cell-cell contact was lost, resulting in their random orientation. Gap junctions were perturbed in the N-cadherin-null myocytes. By contrast, focal contacts appeared normal in the mutant cells. Furthermore, E-cadherin restored normal cell morphology and behavior to the N-cadherin-deficient myocytes, including proper alignment of the myofibrils. We conclude that a different adhesive system, most probably integrin, is responsible for myofibrillogenesis in the N-cadherin-null myocytes.

Key words: N-cadherin, Cardiomyocyte, Myofibrillogenesis, Gap junctions, Focal contacts

Introduction

The coordinated interaction of individual myocytes with their neighbors and the extracellular environment is critical for the normal development and function of the working myocardium. Nascent myofibrils are thought to initiate assembly by interacting with the actin cytoskeleton at the plasma membrane (Lin et al., 1989). Adherens junctions and focal adhesions anchor the actin microfilament system to the membrane at points of cell-cell and cell-extracellular matrix (ECM) contact, respectively. The myofibril inserts into a submembranous plaque containing several cytoskeleton-associated proteins including vinculin (Geiger, 1979), which is shared between the two different junctional complexes (Lu et al., 1992). On the basis of the temporal and spatial distribution of sarcomeric proteins in cultured myocytes, different models have been proposed to explain how myofibrils assemble from the cell periphery (Dlugosz et al., 1984; Epstein and Fischman, 1991; Rhee et al., 1994); however the role of cell adhesion receptors in this process is less well understood.

The cadherin family of calcium-dependent cell adhesion molecules plays an important role in establishing and maintaining cell-cell interactions through its homotypic binding and adhesive specificities (Tepass et al., 2000). Cadherins are single-pass transmembrane proteins that interact intracellularly with a group of proteins called catenins, which mediate cadherin linkage to the actin cytoskeleton (Gumbiner, 2000). The catenin family consists of several members including αE-, αN-, αT-, β- and γ-catenin (plakoglobin), and p120ctn. Either β-catenin or plakoglobin binds directly to the C-terminal region of cadherin, and one of the α-catenin isoforms links the cadherin-catenin complex directly (Rimm et al., 1995) or indirectly (Knudsen et al., 1995; Watabe-Uchida et al., 1998) to the actin cytoskeleton. αT-catenin, a recently identified member of the catenin family, is highly expressed in heart, where it localizes to the intercalated disc (Janssens et al., 2001). The strength of adhesion is also modulated by p120ctn, which binds the juxtamembrane region of the cadherin cytoplasmic domain (Anastasiadis and Reynolds, 2000). Their adhesive specificity and cellular distribution during embryogenesis suggest an important role for cadherins in morphogenesis and maintenance of the tissue phenotype.

N-cadherin is expressed by the developing and mature myocardium, where it is found predominantly in the fascia adherens of the transverse region of intercalated disks and in the regions of close lateral contact between neighboring myocytes (Duband et al., 1988; Hatta et al., 1987; Kostin et al., 1999; Volk and Geiger, 1984; Volk and Geiger, 1986). It is also found in extrajunctional sites called costameres (Pardo et al., 1983) where it colocalizes with α-actinin in the peripheral Z-disks of the sarcomeres (Goncharova et al., 1992; Wu et al., 1999; Wu et al., 2002). Much of our knowledge on how N-cadherin might function in cardiomyocyte adhesion and myofibrillogenesis comes from in vitro studies employing antibodies that block specifically the function of N-cadherin.

In these investigations, blocking N-cadherin function decreased cell-cell contact between interacting myocytes as well as disrupted myofibril organization (Goncharova et al., 1992; Peralta Soler and Knudsen, 1994; Wu et al., 1999) and formation (Imanaka-Yoshida et al., 1998). A similar result was obtained with a dominant-negative N-cadherin construct microinjected into adult rat cardiomyocytes (Hertig et al., 1996b). Observations of interactions between living adult rat cardiomyocytes demonstrated recruitment of a N-cadherin/EGFP fusion protein to regions of initial cell-cell contact, which appeared to serve as insertion sites for stress-fiber-like actin-containing structures (Zuppinger et al., 2000). Consistent with a role for cadherin-mediated adhesion in gap junction formation (Frenzel and Johnson, 1996; Hertig et al., 1996b; Jongen et al., 1991; Meyer et al., 1992), the N-cadherin/EGFP fusion protein appeared before connexin 43 (Cx43) at newly established cell-cell contact sites between the myocytes. In the present study, we further demonstrate the importance of N-cadherin in maintaining proper cytoarchitecture of the cardiomyocyte required for normal myofibril contractility between the cells as well as for gap junction formation.

E-cadherin plays an important role in the maintenance of the epithelial phenotype and its downregulation is involved in tumor progression (Conacci-Sorrell et al., 2002). Mouse N- and E-cadherin show 49% amino acid similarity overall and in vitro studies indicate that N- and E-cadherin do not interact in either cis or trans (Miyatani et al., 1989; Shan et al., 2000). We recently demonstrated that cardiac-specific expression of E-cadherin could restore cell adhesion and looping morphogenesis in N-cadherin-null embryos (Luo et al., 2001). However, we did find that misexpression of E-cadherin in the adult myocardium led to severe cardiomyopathy in transgenic mice due to defects in the intercalated discs (Ferreira-Cornwell et al., 2002). In the present study, we examine E-cadherin-mediated myofibril organization in cultured N-cadherin-null myocytes.

Cell-ECM interactions are also important determinants of myocyte cytoarchitecture, providing structural integrity necessary for normal sarcomere organization (Ross and Borg, 2001). The integrin family of cell adhesion receptors mediates these interactions by binding to the substrata components, including collagens, fibronectins and laminins. Similar to N-cadherin, β1 integrin is expressed in the developing and mature myocardium (Carver et al., 1994; Terracio et al., 1991). In the presence of anti-β1 integrin antibodies, normal cell spreading and myofibril organization was perturbed in cultured neonatal rat cardiomyocytes (Hilenski et al., 1992). Myocytes derived from β1 integrin double-knockout embryonic stem (ES) cells exhibited altered in vitro differentiation and abnormal sarcomeric architecture (Fassler et al., 1996). Recently, the gene encoding β1 integrin was deleted specifically from ventricular myocytes, resulting in dilated cardiomyopathy in mice (Shai et al., 2002). In summary, over the past 10 years, numerous studies have indicated that both the integrin and cadherin adhesion systems are important for maintenance of myofibril structure.

The structural integrity of the nascent myocardium is severely perturbed in N-cadherin-null embryos, resulting in early lethality (Radice et al., 1997) and making it difficult to study the cellular characteristics of the mutant myocytes in vivo. In this study, we circumvented this problem by examining myocyte cultures derived from mutant and rescued (Luo et al., 2001) embryos. In contrast to previous in vitro studies, we found that N-cadherin is not required for myofibrillogenesis, but is essential for proper alignment of the myofibrils across the plasma membrane. In addition, an epithelial cadherin, E-cadherin, was capable of anchoring actin filaments into the membrane and thus restoring myofibril organization in the N-cadherin-null myocytes, demonstrating that these classical cadherins are interchangeable in this particular cellular context.

Materials and Methods

Mouse husbandry

The generation and genotyping of the N-cadherin-knockout (Radice et al., 1997) and α myosin heavy chain (αMHC)/E-cadherin-transgenic mice (Luo et al., 2001) were described previously. Briefly, a cDNA encoding human E-cadherin was cloned into an expression vector containing the αMHC promoter (Gulick et al., 1991). The cardiac-specific expression construct was used to generate transgenic mice (Luo et al., 2001). Tail DNA was obtained from the mice and subjected to PCR analysis using primers specific for the N-cadherin mutation and transgene. Heterozygous N-cadherin mice with or without the αMHC/E-cadherin transgene were interbred to generate litters of embryos for analysis.

Myocyte cultures and immunohistochemistry

Embryos were collected on either embryonic day (E)9.0 (mutant) or E10.5 (rescued), and the yolk sac from each embryo was harvested for genotyping by PCR analysis (Luo et al., 2001). In this study, 'rescued' embryos are defined as mutant embryos expressing the αMHC/E-cadherin transgene ($Ncad^{-/-}$; $Ecad^{+}$). Heart tubes were isolated, trypsinized and plated on fibronectin-coated coverslips in 24-well dishes. The myocytes were cultured in DMEM with high glucose and 15% FBS. Although the recovery of cardiac cells from the mutant embryos was variable, the remaining attached myocytes appeared normal as demonstrated by their well-formed myofibrils and ability to contract in the culture dish. After three days, the cultures were observed with a Nikon inverted microscope, and were photographed and fixed in freshly prepared 4% paraformaldehyde for 15 minutes at room temperature. The cells were washed with PBS, and treated with 0.5% Triton X-100/PBS for 10 minutes. The samples were washed in PBS before blocking with 5% skim milk/PBS for 30 minutes. Antibodies were diluted in 5% skim milk/PBS as follows: mouse monoclonal anti-N-cadherin, 1:100 (3B9; Zymed, South San Francisco, CA); mouse monoclonal anti-E-cadherin, 1:100 (4A2C7; Zymed); mouse monoclonal anti-sarcomeric α-actinin, 1:100 (EA-53, Sigma); mouse monoclonal anti-β-catenin, 1:100 (CAT-5H10; Zymed); rabbit polyclonal anti-Cx43, 1:100 (Zymed); mouse monoclonal anti-vinculin, 1:500 (Sigma); mouse monoclonal anti-p120ctn, 1:400 (#98, Transduction Laboratories, Lexington, KT); mouse monoclonal anti-desmosomal protein, 1:100 (ZK-31, Sigma); rat monoclonal anti-β1 integrin, 1:500 (Transduction Laboratories). Samples were incubated overnight at 4°C with primary antibodies. After washing in PBS, cultures were incubated with the appropriate secondary antibody, 1:100 (Jackson ImmunoResearch Laboratories, West Grove, PA): Cy3-anti-mouse IgG, FITC-anti-mouse IgG, Cy3-anti-rat IgG, Cy3-anti-rabbit IgG, or FITC-anti-rabbit IgG, for 1 hour at room temperature. The cultures were washed in PBS and mounted for analysis with a confocal microscope.

Results

Previous studies from our laboratory showed that cardiac-

specific expression of either N- or E-cadherin was sufficient to partially rescue N-cadherin-deficient embryos. Myocyte adhesion and cardiac looping morphogenesis was corrected in N-cadherin mutant embryos carrying either transgene (Luo et al., 2001). To our surprise, the epithelial cadherin appeared to replace N-cadherin functionally in the embryonic heart. In the present study, we sought to compare in more detail the cellular properties of wild-type, N-cadherin-null and rescued myocytes cultured in vitro. In this way, myocytes would not be exposed to the mechanical load associated with the working heart in vivo, allowing cell-cell and cell-ECM interactions to be examined under similar in vitro conditions.

E-cadherin restores normal morphology and adhesion in N-cadherin-null myocytes

As previously described (Luo et al., 2001), the human gene for E-cadherin expressed from the MHC promoter results in cardiac-specific expression in the primitive heart tube. Heterozygous N-cadherin mice with or without the αMHC/Ecad transgene were backcrossed to N-cadherin heterozygotes, and embryos recovered at E9 ($Ncad^{-/-}$) or E10 ($Ncad^{-/-}$; $Ecad^{+}$). Heart tubes were isolated, cells disassociated, and examined after 3 days in culture. The frequency of viable cultures obtained from the N-cadherin mutant embryos was low, probably due to the poor yield of myocytes from the developmentally delayed mutant embryos. Approximately 80% of the cells in culture were myocytes as determined by immunostaining for sarcomeric myosin (data not shown). Wild-type myocytes attached and spread extensively on fibronectin-coated cover slips and formed tightly adherent aggregates (Fig. 1A). By contrast, only a subset of N-cadherin-null myocytes attached and spread, forming small aggregates (Fig. 1B); however, many cells either never attached or initially attached and eventually rounded up and detached from the substrate (Fig. 1C). The mutant myocytes continued to beat, albeit weakly, demonstrating their viability. Consistent with this observation, we had previously shown no increase in apoptosis in mutant myocytes in vivo (Luo et al., 2001). Normal cell-cell contacts and morphology were restored in the N-cadherin-null myocytes expressing the αMHC/Ecad transgene (Fig. 1D). Similar to wild-type cultures, large aggregates of these myocytes were generated by E-cadherin-mediated adhesion.

Catenin expression is severely reduced in N-cadherin-deficient myocytes

Cadherins and catenins are coordinately regulated in cells. To investigate the expression and distribution of catenins in N-cadherin-null and rescued myocytes, immunohistochemistry was performed on the myocyte cultures. Consistent with N-cadherin being the primary cadherin present in myocytes, β-catenin and p120ctn were significantly reduced or absent at the cell membrane in the N-cadherin-null myocytes (Fig. 2B,E) compared with wild-type (Fig. 2A,D). E-cadherin expression restored catenin expression to normal levels in the mutant myocytes (Fig. 2C,F), verifying the co-regulation of these molecules.

N-cadherin-mediated adhesion is not required for myofibrillogenesis

Antibody perturbation experiments in chicken cardiomyocyte cultures suggested that N-cadherin was required for the formation (Imanaka-Yoshida et al., 1998) and maintenance of normal myofibril organization (Goncharova et al., 1992; Peralta Soler and Knudsen, 1994). Here, we examine myofibril structure in myocytes genetically null for N-cadherin. Cultures were stained with phalloidin (Fig. 3A,C), which binds F-actin in the I-bands, and with antibodies against sarcomeric α-actinin, a component of the Z-disk (Fig. 3B,D). In the absence of N-cadherin, the striated pattern of these myofibril components appeared normal, in contrast to predictions from previous experiments using N-cadherin function-blocking antibodies. Note the thicker-appearing myofibrils in the N-cadherin-deficient cells compared with wild-type, which might reflect the inability of the myocytes to remain well spread. We conclude that N-cadherin is not required for myofibrillogenesis, suggesting that other cell adhesion mechanisms (i.e. focal adhesions, Fig. 6) might be sufficient to promote myofibril formation in the absence of N-cadherin.

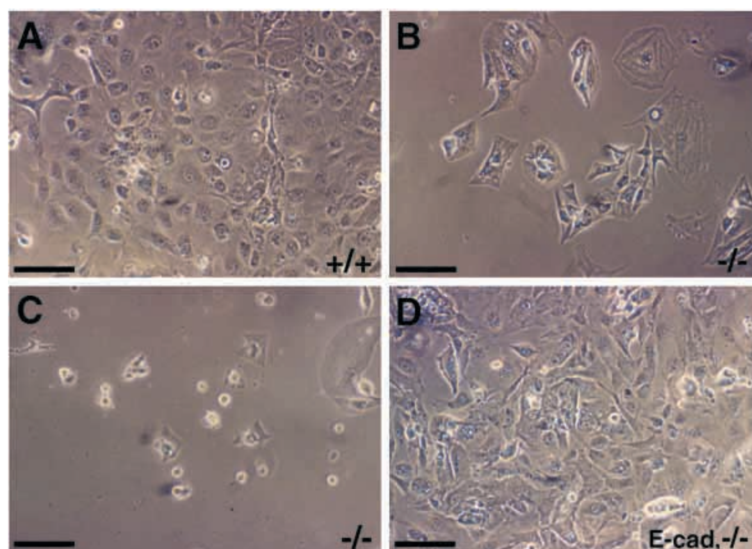

Fig. 1. In vitro culture of myocytes derived from N-cadherin-null and E-cadherin-rescued N-cadherin-null embryos. Wild-type myocytes formed tightly adherent aggregates (A) after 3 days in culture. In comparison, small, less-compacted aggregates of N-cadherin-deficient myocytes were observed (B). Although the cells were less adhesive, small aggregates of mutant myocytes were observed beating synchronously, albeit more weakly compared with wild-type aggregates. Many mutant cells had difficulty remaining attached to the substrate, causing them to round up (C). Introduction of the αMHC/Ecad transgene into the mutant background restored normal cell adhesion, resulting in large aggregates of strongly beating myocytes (D). Bar, 10 μm.

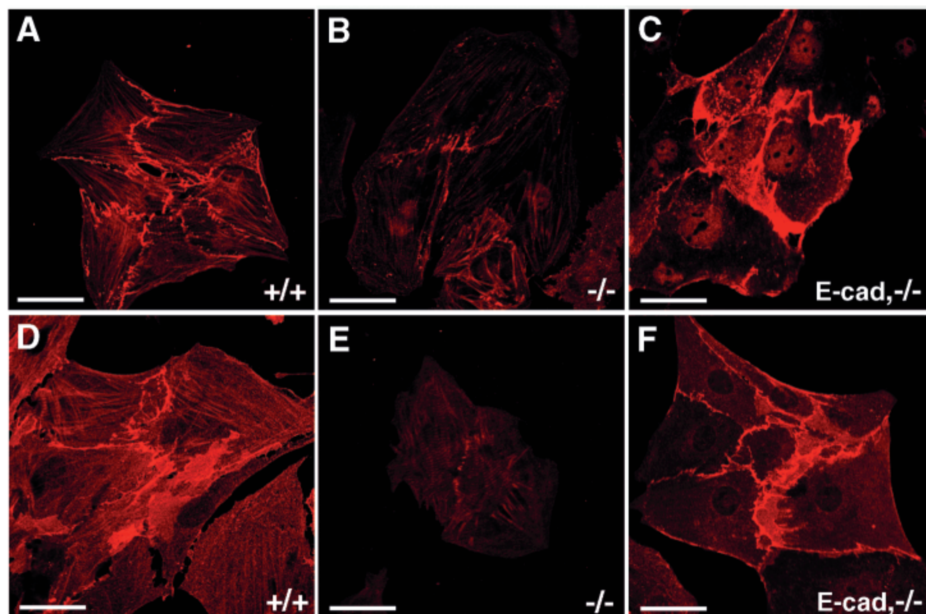

Fig. 2. Expression of β-catenin and p120ctn in transgenic myocytes. Myocyte cultures were examined by indirect immunofluorescence for expression of β-catenin (A-C) and p120ctn (D-F). Both catenins showed localization at cell-cell boundaries in wild-type cells (A,D). Expression of β-catenin (B) and p120ctn (E) were significantly reduced in the N-cadherin-null myocytes. Both catenins (C,F) were found localized to cell-cell contacts in the mutant myocytes expressing the αMHC/Ecad transgene. Bar, 50 μm.

Cadherin function is required for myofibril alignment between myocytes

For the myocardium to function normally, myofibrils must be aligned across the plasma membrane from one cell to the next, allowing efficient coordinated contraction of the muscle. Small aggregates of myocytes were examined for myofibril organization to determine why the N-cadherin-null aggregates appeared to contract more weakly compared with wild-type aggregates. The myocytes were double stained with phalloidin and cadherin, in the case of wild-type and rescued myocytes, and staining of a desmosomal protein was used to delineate the cell membrane in the mutant myocytes. The myofibrils of wild-type myocytes inserted into the plasma membrane, as demonstrated by the merged image of N-cadherin and F-actin (Fig. 4C). The myofibers appeared to run continuously from one cell to the next through regions of cadherin-mediated cell-cell contact. By contrast, although myofibrils appeared well formed in the mutant myocytes, they were not anchored at the cell membrane and their orientation was random with respect to their neighbors (Fig. 4E,F). E-cadherin was capable of anchoring the myofibers into the plasma membrane, thus restoring myofibril alignment between the rescued cells (Fig. 4I) and demonstrating that a non-muscle cadherin can substitute for N-cadherin in myocytes. As reported for N-cadherin (Goncharova et al., 1992), E-cadherin was also found localized to costameres as illustrated by its periodic striations at the dorsal surface of the rescued myocytes (Fig. 4J). In addition, the Z-disks were properly organized in the E-cadherin-rescued myocytes as demonstrated by sarcomeric α-actinin staining (data not shown). Taken together, we have shown using genetically modified myocytes that cadherin-mediated adhesion is required for myofibril organization between cells, but not formation within them.

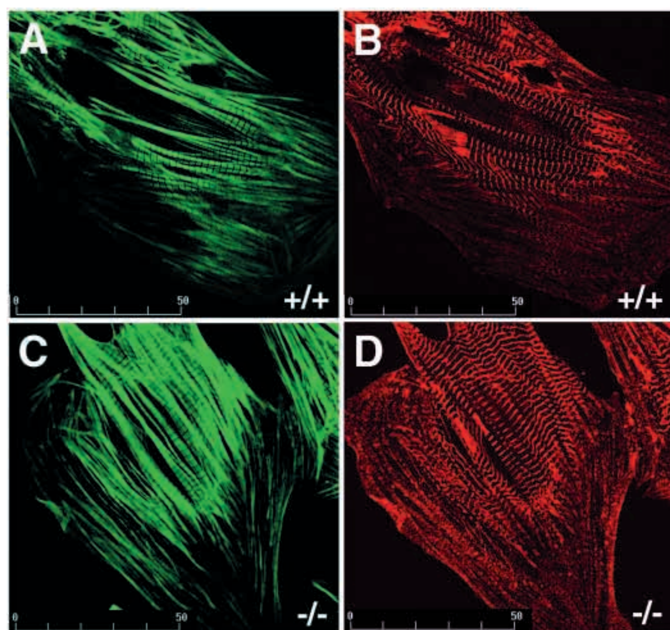

Fig. 3. Myofibrillogenesis in N-cadherin-null myocytes. Wild-type (A,B) and mutant (C,D) myocytes were double stained for F-actin (A,C) and sarcomeric α-actinin (B,D). Overall, myofibril structure appeared remarkably similar in these well-spread myocytes, as illustrated by the striated pattern of the myofibril components. Based on the phalloidin stain, it did appear that the mutant myofibrils were somewhat thicker or denser compared with the wild-type structures; however, no apparent difference was observed with α-actinin staining. Bar, 50 μm.

Gap junction protein Cx43 is downregulated in N-cadherin-null myocytes

On the basis of previous studies, it has been proposed that cell-cell contact mediated by N-cadherin is a prerequisite to gap junction formation in myocytes (Hertig et al., 1996a; Kostin et al., 1999; Zuppinger et al., 2000). Given the significant reduction of catenins in the N-cadherin-null cells (Fig. 2), we concluded that N-cadherin was the primary cadherin expressed by cardiomyocytes, therefore we decided to examine gap junction formation in these cells. To examine the presence of gap junctions in the myocytes, the cultures were

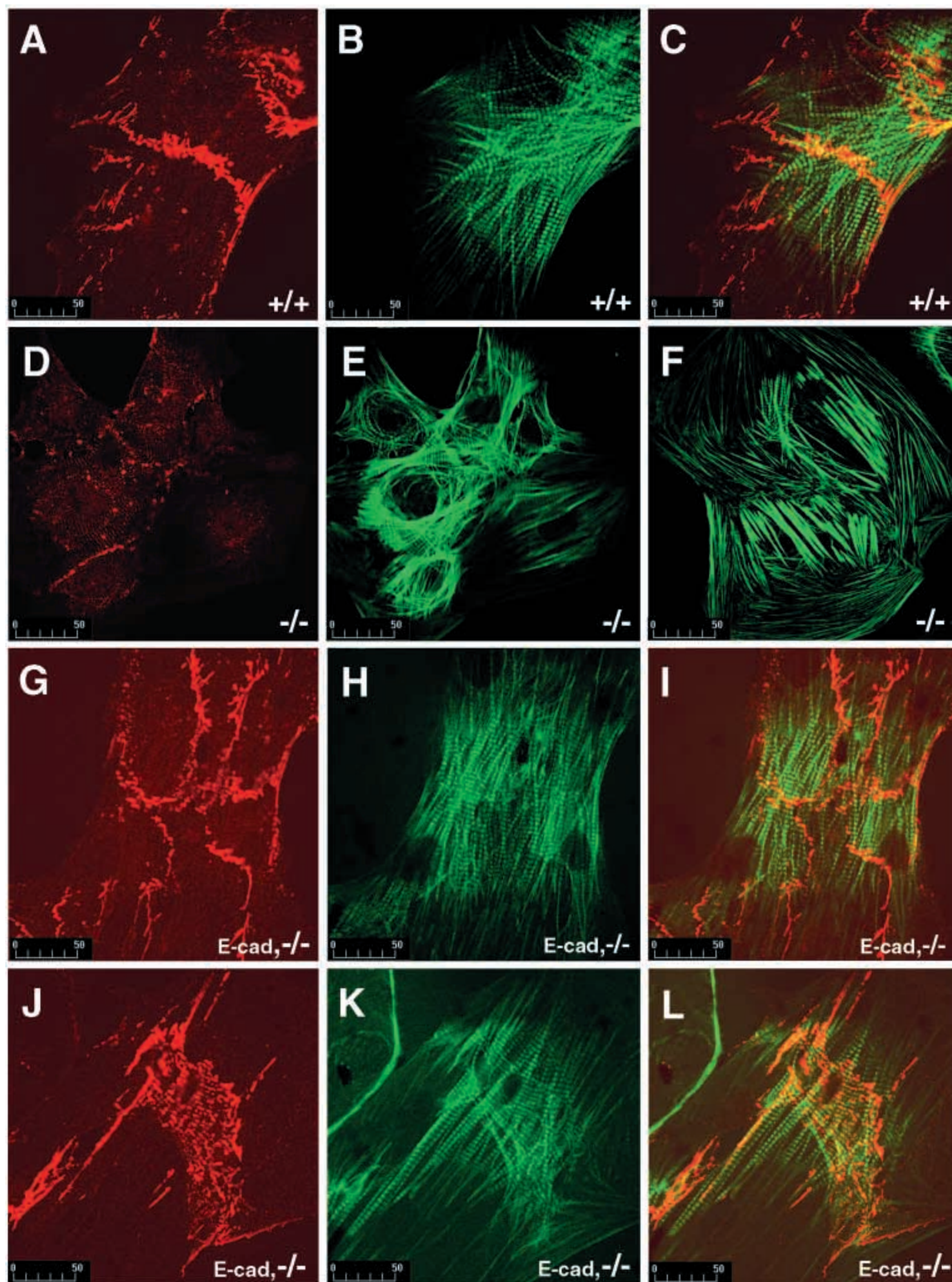

Fig. 4. Cadherin-mediated adhesion and myofibril alignment. Aggregates of wild-type (A-C), mutant (D-F) and rescued (G-L) myocytes were double stained for F-actin and either N-cadherin (A), desmosomal protein (D) or E-cadherin (G,J). In wild-type cells, myofibrils run in a linear fashion between neighboring myocytes with colocalization of their ends at regions of cell-cell contact as seen in the merged image (C). Myofibril disorganization is shown for mutant aggregates (E,F) from two independent experiments. In both cultures, the orientation of the myofibrils was random with respect to their neighboring cells (i.e. no continuity across the plasma membrane). E-cadherin-mediated cell adhesion restored proper alignment of the myofibrils between the N-cadherin-deficient myocytes (G-I) and E-cadherin localized to costameric structures on the dorsal surface of the myocyte (J). Bar, 50 μm.

immunostained with antibodies against Cx43. The characteristic punctate staining of Cx43 at regions of cell-cell contact was reduced in the N-cadherin-null myocytes (Fig. 5B) compared with wild-type cells (Fig. 5A). E-cadherin-mediated adhesion restored Cx43 to normal levels in the mutant myocytes (Fig. 5C). Although Cx43 was reduced, the ability of small aggregates of mutant myocytes to beat synchronously demonstrated that the cells remained electrically coupled.

Cell-ECM interactions appear normal in N-cadherin-deficient myocytes

Previous studies have indicated that ECM components play an important role in assembly and/or maintenance of myocyte cytoarchitecture (Hilenski et al., 1992; Imanaka-Yoshida et al., 1999; Shiraishi et al., 1997). In addition, it has been proposed that focal adhesions might serve as nucleation sites for the assembly of myofibrils (Lin et al., 1989). To examine cell-ECM interactions, cultures were double stained with β1 integrin and vinculin, and visualized on the ventral surface of the cell. Although mutant myocytes often appeared less well spread compared with wild-type, focal contacts looked normal as demonstrated by the colocalization of β1 integrin and vinculin in typical adhesion plaques (Fig. 6C,D). The focal contacts in E-cadherin-expressing mutant myocytes also appeared normal (Fig. 6E,F). The presence of focal adhesions suggests a possible explanation for the normal myofibrillogenesis observed in the N-cadherin-null myocytes since they may provide nucleation sites for myofibril assembly.

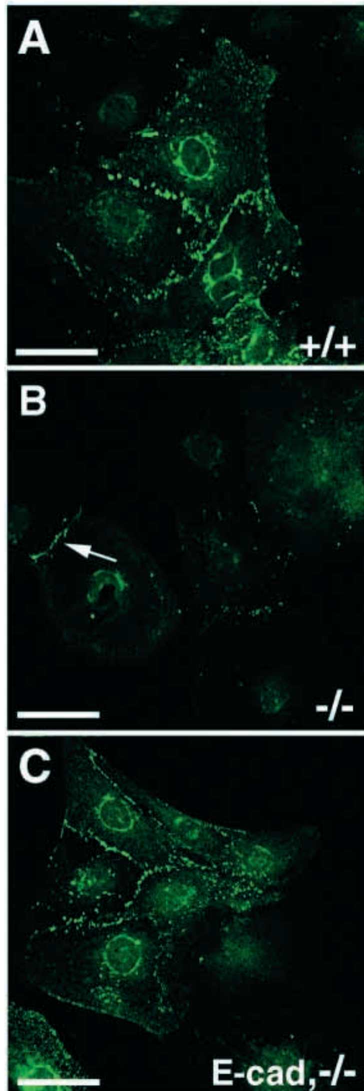

Fig. 5. Expression of Cx43 in transgenic myocytes. The presence of Cx43-containing gap junctions was determined by immunostaining wild-type (A), N-cadherin-null (B) and rescued (C) myocytes with a Cx43 antibody. A typical punctate pattern of Cx43 was observed in regions of cell-cell contact in the wild-type myocytes (A). Cx43 expression was reduced in N-cadherin-deficient myocytes (arrow, B). E-cadherin-mediated cell adhesion restored Cx43 expression to normal levels in the mutant myocytes (C). Bar, 50 μm.

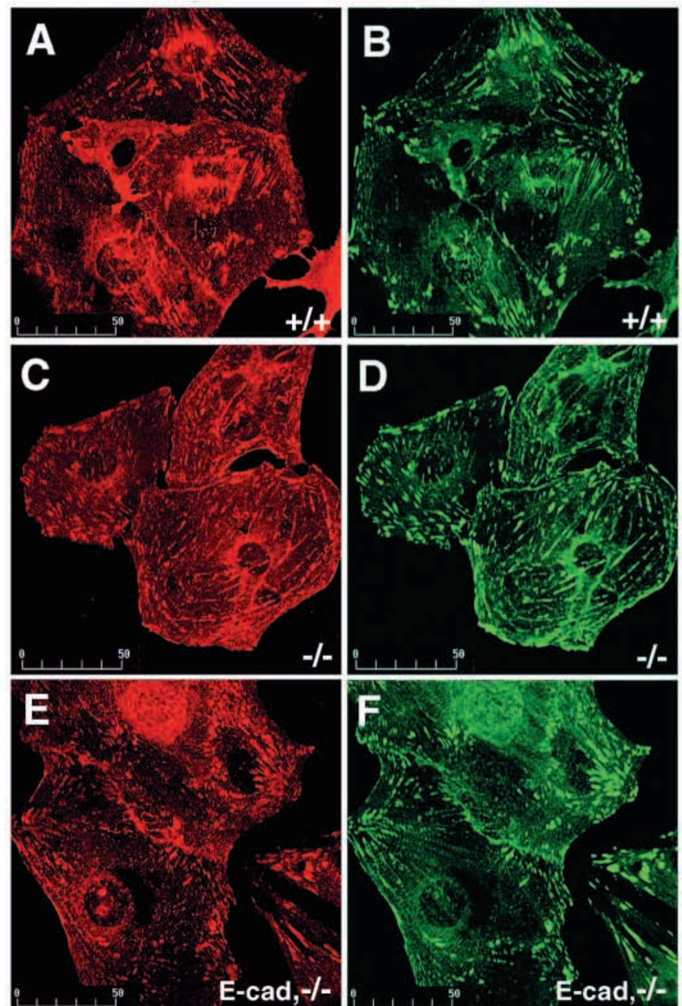

Fig. 6. Focal contact distribution in transgenic myocytes. Integrin-mediated cell-substrate adhesion is responsible for proper cell spreading and motility. Focal adhesions were visualized in wild-type (A,B), N-cadherin-null (C,D) and rescued (E,F) myocytes using antibodies against β1 integrin (A,C,E) and vinculin (B,D,F). Although the N-cadherin-deficient cells often appeared less well spread compared with wild-type, the distribution of integrin and vinculin was similar between the mutant (C,D) and wild-type (A,B) cells. Focal contacts in mutant cells expressing E-cadherin also appeared normal (E,F). Bar, 50 μm.

Discussion

In this study, we have examined cadherin function in cultured cardiomyocytes derived from wild-type, N-cadherin-deficient, and E-cadherin-rescued N-cadherin-null embryos. The results described here present the first genetic evidence that N-cadherin is not essential for myofibrillogenesis; however, it is required to anchor the myofibrils into the plasma membrane thus ensuring transmission of the contractile force from one cell to the next. Previous studies from several groups demonstrated that N-cadherin is important for myofibril organization. In vitro analysis using function-blocking antibodies (Goncharova et al., 1992; Imanaka-Yoshida et al., 1998; Peralta Soler and Knudsen, 1994; Wu et al., 1999) or dominant-negative cadherin molecules (Hertig et al., 1996b) indicated that N-cadherin plays an important role in the formation and maintenance of myofibril structure. These different findings can be explained by differences in the experimental systems. For example, in the dominant-negative experiment (Hertig et al., 1996b), a cDNA encoding a truncated N-cadherin molecule consisting of a large deletion of the extracellular domain was microinjected into adult rat cardiomyocytes. As the endogenous N-cadherin disappeared from the plasma membrane, the cells began to retract from one another. These myocytes exhibited severe disruption of myofibril structure. By contrast, the myofibril structure remained intact in our N-cadherin-null myocytes. In the case

of the microinjected myocytes, the myofibrils are dependent, at least in part, on the cadherin system for structural support since it is present in the wild-type cells. In the case of the N-cadherin-null myocytes, the cells have never seen N-cadherin and the myofibrils depend on an alternative cell adhesion system(s) for support. The fact that myofibrils assemble properly in N-cadherin-null myocytes is probably due to partially overlapping functions or compensation by other structural proteins (i.e. integrins). Compensation is more readily observed in genetic systems since the organism has the opportunity to respond by utilizing an alternative mechanism. By contrast, perturbing function of a protein being actively utilized by a cell is more likely to generate a dramatic phenotype as the organism will not have the opportunity to adjust and use another mechanism. We would predict that a conditional genetic knockout of N-cadherin would more closely resemble the myofibril phenotype observed in these other experimental systems. In contrast to the normal-appearing myofibrils in the N-cadherin-null myocytes, the cadherin/catenin complex is required for insertion of myofibrils into the plasma membrane at sites of cell-cell contact, which was not possible to ascertain from previous antibody-perturbation experiments.

We also examined N-cadherin-null myocytes expressing the αMHC/Ecad transgene (Luo et al., 2001). In addition to rescuing the cell adhesion defect, the epithelial cadherin E-cadherin also anchored myofibrils into the plasma membrane, demonstrating that N- and E-cadherin are functionally interchangeable in this system. This result is rather surprising given that E-cadherin is primarily expressed in epithelial cells and normally not found in muscle. By contrast, N-cadherin is found primarily in muscle and neuronal cell lineages. N- and E-cadherin show about 50% amino acid similarity overall (Miyatani et al., 1989), with the highest degree of conservation in the cytoplasmic domain (62%). It is interesting that the E-cadherin/catenin complex can support the insertion of myofibrils into the plasma membrane of N-cadherin-deficient myocytes, suggesting that the interaction of the cadherin/catenin complex with the myofibril is not cadherin-subtype specific. In support of our findings, it is interesting to note that, in *Drosophila*, in contrast to vertebrates, DE-cadherin is expressed in cardial cells instead of DN-cadherin (Iwai et al., 1997; Tepass et al., 1996).

Classical cadherins are defined by their ability to bind catenins at their cytoplasmic tail, thus linking the cadherin/catenin complex to the actin cytoskeleton. In contrast to skeletal muscle, which expresses multiple cadherin subtypes, cardiac muscle appears to depend on one classical cadherin, N-cadherin. Consistent with this idea, β-catenin and p120ctn staining was greatly reduced at regions of cell-cell contact in the N-cadherin-null myocytes. Furthermore, expression of the αMHC/E-cadherin transgene in mutant myocytes restored catenins to normal levels, demonstrating the coordinate regulation of the cadherin/catenin complex. On the basis of the significant reduction in the cadherin/catenin complex in the mutant myocytes, we proposed that the integrin-based cytoskeletal network provides the nucleation sites at the plasma membrane necessary for myofibril assembly.

In vitro studies using function-blocking antibodies have shown that cadherin-mediated adhesion is important for gap junction development (Frenzel and Johnson, 1996; Jongen et al., 1991; Meyer et al., 1992). Other studies, using adult rat cardiomyocytes (ARC), correlated the appearance of endogenous (Hertig et al., 1996b; Kostin et al., 1999) or a GFP-tagged N-cadherin (Zuppinger et al., 2000) at regions of cell-cell contact as a prerequisite for gap junction formation. Furthermore, introduction of a dominant-negative cadherin into ARC resulted in disruption of gap junctions (Hertig et al., 1996b). In the present study, we observed a reduction in gap junctions as depicted by loss of Cx43 cell-surface staining consistent with a role for the cadherin/catenin complex in channel formation. Although Cx43 expression is reduced in mutant myocytes, their ability to beat in synchrony demonstrates that gap junction communication remains operative at some level, at least sufficient to electrically couple the cells. Our studies indicate that gap junctions are reduced but still present in N-cadherin-deficient myocytes, suggesting that other cell adhesion molecules (i.e. N-CAM) are sufficient to bring cells in close apposition, a prerequisite for gap junction formation. Interestingly, we previously found no reduction in Cx43 gap junctions in N-cadherin-deficient neural crest cells, consistent with the expression of other cadherins in these cells (Xu et al., 2001). Nonetheless, there was a significant reduction in gap junction communication in the N-cadherin-null neural crest cells as determined by dye coupling between the cells. Therefore, the function of the remaining gap junctions in the N-cadherin-deficient myocytes remain to be determined in future studies.

Together, cadherins and integrins are thought to regulate cell adhesion during cell migration as demonstrated in neural crest cells (Monier-Gavelle and Duband, 1997) and myoblasts (Huttenlocher et al., 1998). In myocytes, in addition to its localization at adherens junctions, N-cadherin is found at costameres (i.e. attachment sites of peripheral myofibrils to the plasma membrane) similar to integrin. We found that E-cadherin also localized to costameric sites on the dorsal surface of the N-cadherin-deficient myocytes containing the αMHC/Ecad transgene. It is unclear how these two adhesion systems might cooperate to stabilize the contractile apparatus; however, our present studies and similar studies performed with myocytes mutant for β1 integrin suggest distinct roles for these adhesion systems. In contrast to N-cadherin-null cells, cardiomyocytes lacking β1 integrin displayed disorganized sarcomeric structures in culture (Fassler et al., 1996). In the same study, β1 integrin-null myocytes were capable of contributing to the developing heart of chimeric embryos; by contrast, N-cadherin-deficient myocytes were excluded from the myocardium (Kostetskii et al., 2001).

In our system, the distribution of focal adhesion plaques appeared similar in wild-type, mutant and rescued myocytes. Although focal contacts were present in the mutant myocytes, the cells often appeared less well spread compared with wild-type, suggesting that these structures may be less stable due to the lack of cadherin/cytoskeletal interactions. It is possible that loss of structural support from the cell periphery (i.e. less attachment sites for cortical actin) might cause retraction and subsequent compression of the myofibrils, resulting in their thicker appearance. Alternatively, the loss of N-cadherin-based costameres may perturb the cytoarchitecture, leading to subtle alterations in myofibril structure in the N-cadherin-null myocytes.

In summary, we provide genetic evidence that N-cadherin is not essential for myofibrillogenesis but that cadherin function is required for myofibril alignment between myocytes. The integrin-based focal adhesions are probably sufficient to provide nucleation sites for myofibril assembly, thus explaining the normal striated appearance of the myofibrils in the N-cadherin-null myocytes.

We thank Karen Knudsen, Joe Sanger and Jean Sanger for their comments on the manuscript; Melanie Lieberman for maintaining the animal colony; and the University of Pennsylvania Biomedical Imaging Core facility for use of the confocal microscope. Y.L. was supported in part by a postdoctoral fellowship from the PA/DE Affiliate of the American Heart Association (0225364U). This work was supported by the National Institutes of Health (HL57554).

References

Anastasiadis, P. Z. and Reynolds, A. B. (2000). The p120 catenin family: complex roles in adhesion, signaling and cancer. *J. Cell Sci.* **113**, 1319-1334.

Carver, W., Price, R. L., Raso, D. S., Terracio, L. and Borg, T. K. (1994). Distribution of beta-1 integrin in the developing rat heart. *J. Histochem. Cytochem.* **42**, 167-175.

Conacci-Sorrell, M., Zhurinsky, J. and Ben-Ze'ev, A. (2002). The cadherin-catenin adhesion system in signaling and cancer. *J. Clin. Invest.* **109**, 987-991.

Dlugosz, A. A., Antin, P. B., Nachmias, V. T. and Holtzer, H. (1984). The relationship between stress fiber-like structures and nascent myofibrils in cultured cardiac myocytes. *J. Cell Biol*. **99**, 2268-2278.

Duband, J. L., Volberg, T., Sabanay, I., Thiery, J. P. and Geiger, B. (1988). Spatial and temporal distribution of the adherens-junction-associated adhesion molecule A-CAM during avian embryogenesis. *Development* **103**, 325-344.

Epstein, H. F. and Fischman, D. A. (1991). Molecular analysis of protein assembly in muscle development. *Science* **251**, 1039-1044.

Fassler, R., Rohwedel, J., Maltsev, V., Bloch, W., Lentini, S., Guan, K., Gullberg, D., Hescheler, J., Addicks, K. and Wobus, A. M. (1996). Differentiation and integrity of cardiac muscle cells are impaired in the absence of beta 1 integrin. *J. Cell Sci.* **109**, 2989-2999.

Ferreira-Cornwell, M. C., Luo, Y., Narula, N., Lenox, J. M., Lieberman, M. and Radice, G. L. (2002). Remodeling the intercalated disc leads to cardiomyopathy in mice misexpressing cadherins in the heart. *J. Cell Sci*. **115**, 1623-1634.

Frenzel, E. M. and Johnson, R. G. (1996). Gap junction formation between cultured embryonic lens cells is inhibited by antibody to N-cadherin. *Dev. Biol*. **179**, 1-16.

Geiger, B. (1979). A 130K protein from chicken gizzard: its localization at the termini of microfilament bundles in cultured chicken cells. *Cell* **18**, 193-205.

Goncharova, E. J., Kam, Z. and Geiger, B. (1992). The involvement of adherens junction components in myofibrillogenesis in cultured cardiac myocytes. *Development* **114**, 173-183.

Gulick, J., Subramaniam, A., Neumann, J. and Robbins, J. (1991). Isolation and characterization of the mouse cardiac myosin heavy chain genes. *J. Biol. Chem.* **266**, 9180-9185.

Gumbiner, B. M. (2000). Regulation of cadherin adhesive activity. *J. Cell Biol.* **148**, 399-403.

Hatta, K., Takagi, S., Fujisawa, H. and Takeichi, M. (1987). Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. *Dev. Biol*. **120**, 215-227.

Hertig, C. M., Butz, S., Koch, S., Eppenberger-Eberhardt, M., Kemler, R. and Eppenberger, H. M. (1996a). N-cadherin in adult rat cardiomyocytes in culture. II. Spatio-temporal appearance of proteins involved in cell-cell contact and communication. Formation of two distinct N-cadherin/catenin complexes. *J. Cell Sci.* **109**, 11-20.

Hertig, C. M., Eppenberger-Eberhardt, M., Koch, S. and Eppenberger, H. M. (1996b). N-cadherin in adult rat cardiomyocytes in culture. I. Functional role of N-cadherin and impairment of cell-cell contact by a truncated N-cadherin mutant. *J. Cell Sci*. **109**, 1-10.

Hilenski, L. L., Ma, X. H., Vinson, N., Terracio, L. and Borg, T. K. (1992). The role of beta 1 integrin in spreading and myofibrillogenesis in neonatal rat cardiomyocytes in vitro. *Cell Motil. Cytoskeleton* **21**, 87-100.

Huttenlocher, A., Lakonishok, M., Kinder, M., Wu, S., Truong, T., Knudsen, K. A. and Horwitz, A. F. (1998). Integrin and cadherin synergy regulates contact inhibition of migration and motile activity. *J. Cell Biol*. **141**, 515-526.

Imanaka-Yoshida, K., Knudsen, K. A. and Linask, K. K. (1998). N-cadherin is required for the differentiation and initial myofibrillogenesis of chick cardiomyocytes. *Cell Motil. Cytoskeleton* **39**, 52-62.

Imanaka-Yoshida, K., Enomoto-Iwamoto, M., Yoshida, T. and Sakakura, T. (1999). Vinculin, talin, integrin α6β1 and laminin can serve as components of attachment complex mediating contraction force transmission from cardiomyocytes to extracellular matrix. *Cell Motil. Cytoskeleton* **42**, 1-11.

Iwai, Y., Usui, T., Hirano, S., Steward, R., Takeichi, M. and Uemura, T. (1997). Axon patterning requires DN-cadherin, a novel neuronal adhesion receptor, in the *Drosophila* embryonic CNS. *Neuron* **19**, 77-89.

Janssens, B., Goossens, S., Staes, K., Gilbert, B., van Hengel, J., Colpaert, C., Bruyneel, E., Mareel, M. and van Roy, F. (2001). αT-catenin: a novel tissue-specific beta-catenin-binding protein mediating strong cell-cell adhesion. *J. Cell Sci*. **114**, 3177-3188.

Jongen, W. M., Fitzgerald, D. J., Asamoto, M., Piccoli, C., Slaga, T. J., Gros, D., Takeichi, M. and Yamasaki, H. (1991). Regulation of connexin 43-mediated gap junctional intercellular communication by Ca^{2+} in mouse epidermal cells is controlled by E-cadherin. *J. Cell Biol*. **114**, 545-555.

Knudsen, K. A., Soler, A. P., Johnson, K. R. and Wheelock, M. J. (1995). Interaction of α-actinin with the cadherin/catenin cell-cell adhesion complex via α-catenin. *J. Cell Biol.* **130**, 67-77.

Kostetskii, I., Moore, R., Kemler, R. and Radice, G. L. (2001). Differential adhesion leads to segregation and exclusion of N-cadherin-deficient cells in chimeric embryos. *Dev. Biol.* **234**, 72-79.

Kostin, S., Hein, S., Bauer, E. P. and Schaper, J. (1999). Spatiotemporal development and distribution of intercellular junctions in adult rat cardiomyocytes in culture. *Circ. Res.* **85**, 154-167.

Lin, Z. X., Holtzer, S., Schultheiss, T., Murray, J., Masaki, T., Fischman, D. A. and Holtzer, H. (1989). Polygons and adhesion plaques and the disassembly and assembly of myofibrils in cardiac myocytes. *J. Cell Biol.* **108**, 2355-2367.

Lu, M. H., DiLullo, C., Schultheiss, T., Holtzer, S., Murray, J. M., Choi, J., Fischman, D. A. and Holtzer, H. (1992). The vinculin/sarcomeric-alpha-actinin/alpha-actin nexus in cultured cardiac myocytes. *J. Cell Biol.* **117**, 1007-1022.

Luo, Y., Ferreira-Cornwell, M., Baldwin, H., Kostetskii, I., Lenox, J., Lieberman, M. and Radice, G. (2001). Rescuing the N-cadherin knockout by cardiac-specific expression of N- or E-cadherin. *Development* **128**, 459-469.

Meyer, R. A., Laird, D. W., Revel, J. P. and Johnson, R. G. (1992). Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. *J. Cell Biol*. **119**, 179-189.

Miyatani, S., Shimamura, K., Hatta, M., Nagafuchi, A., Nose, A. and Matsunaga, M. (1989). Neural cadherin: role in selective cell-cell adhesion. *Science* **245**, 631-635.

Monier-Gavelle, F. and Duband, J. L. (1997). Cross talk between adhesion molecules: control of N-cadherin activity by intracellular signals elicited by β1 and β3 integrins in migrating neural crest cells. *J. Cell Biol*. **137**, 1663-1681.

Pardo, J. V., Siliciano, J. D. and Craig, S. W. (1983). Vinculin is a component of an extensive network of myofibril-sarcolemma attachment regions in cardiac muscle fibers. *J. Cell Biol.* **97**, 1081-1088.

Peralta Soler, A. and Knudsen, K. A. (1994). N-cadherin involvement in cardiac myocyte interaction and myofibrillogenesis. *Dev. Biol.* **162**, 9-17.

Radice, G. L., Rayburn, H., Matsunami, H., Knudsen, K. A., Takeichi, M. and Hynes, R. O. (1997). Developmental defects in mouse embryos lacking N-cadherin. *Dev. Biol.* **181**, 64-78.

Rhee, D., Sanger, J. M. and Sanger, J. W. (1994). The premyofibril: evidence for its role in myofibrillogenesis. *Cell Motil. Cytoskeleton* **28**, 1-24.

Rimm, D. L., Koslov, E. R., Kebriaei, P., Cianci, C. D. and Morrow, J. S. (1995). α1(E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex. *Proc. Natl. Acad. Sci. USA* **92**, 8813-8817.

Ross, R. S. and Borg, T. K. (2001). Integrins and the myocardium. *Circ. Res*. **88**, 1112-1119.

Shai, S. Y., Harpf, A. E., Babbitt, C. J., Jordan, M. C., Fishbein, M. C.,

Chen, J., Omura, M., Leil, T. A., Becker, K. D., Jiang, M. et al. (2002). Cardiac myocyte-specific excision of the β1 integrin gene results in myocardial fibrosis and cardiac failure. *Circ. Res*. **90**, 458-464.

Shan, W. S., Tanaka, H., Phillips, G. R., Arndt, K., Yoshida, M., Colman, D. R. and Shapiro, L. (2000). Functional cis-heterodimers of N- and R-cadherins. *J. Cell Biol.* **148**, 579-590.

Shiraishi, I., Simpson, D. G., Carver, W., Price, R., Hirozane, T., Terracio, L. and Borg, T. K. (1997). Vinculin is an essential component for normal myofibrillar arrangement in fetal mouse cardiac myocytes. *J. Mol. Cell. Cardiol*. **29**, 2041-2052.

Tepass, U., Gruszynski-DeFeo, E., Haag, T. A., Omatyar, L., Torok, T. and Hartenstein, V. (1996). shotgun encodes *Drosophila* E-cadherin and is preferentially required during cell rearrangement in the neuroectoderm and other morphogenetically active epithelia. *Genes Dev.* **10**, 672-685.

Tepass, U., Truong, K., Godt, D., Ikura, M. and Peifer, M. (2000). Cadherins in embryonic and neural morphogenesis. *Nat. Rev. Mol. Cell. Biol.* **1**, 91-100.

Terracio, L., Rubin, K., Gullberg, D., Balog, E., Carver, W., Jyring, R. and Borg, T. K. (1991). Expression of collagen binding integrins during cardiac development and hypertrophy. *Circ. Res*. **68**, 734-744.

Volk, T. and Geiger, B. (1984). A 135-kd membrane protein of intercellular adherens junctions. *EMBO J.* **3**, 2249-2260.

Volk, T. and Geiger, B. (1986). A-CAM: a 135-kD receptor of intercellular adherens junctions. I. Immunoelectron microscopic localization and biochemical studies. *J. Cell Biol*. **103**, 1441-1450.

Watabe-Uchida, M., Uchida, N., Imamura, Y., Nagafuchi, A., Fujimoto, K., Uemura, T., Vermeulen, S., van Roy, F., Adamson, E. D. and Takeichi, M. (1998). alpha-Catenin-vinculin interaction functions to organize the apical junctional complex in epithelial cells. *J. Cell Biol*. **142**, 847-857.

Wu, J.-C., Chung, T.-H., Tseng, Y.-Z. and Wang, S.-M. (1999). N-cadherin/catenin-based costameres in cultured chicken cardiomyocytes. *J. Cell. Biochem.* **75**, 93-104.

Wu, J.-C., Sung, H.-C., Chung, T.-H. and DePhilip, R. M. (2002). Role of N-cadherin- and integrin-based costameres in the development of rat cardiomyocytes. *J. Cell. Biochem.* **84**, 717-724.

Xu, X., Li, W. E., Huang, G. Y., Meyer, R., Chen, T., Luo, Y., Thomas, M. P., Radice, G. L. and Lo, C. W. (2001). Modulation of mouse neural crest cell motility by N-cadherin and connexin 43 gap junctions. *J. Cell Biol.* **154**, 217-230.

Zuppinger, C., Schaub, M. C. and Eppenberger, H. M. (2000). Dynamics of early contact formation in cultured adult rat cardiomyocytes studied by N-cadherin fused to green fluorescent protein. *J. Mol. Cell. Cardiol.* **32**, 539-555.