Induced Deletion of the N-Cadherin Gene in the Heart Leads to Dissolution of the Intercalated Disc Structure

Igor Kostetskii,* Jifen Li,* Yanming Xiong, Rong Zhou, Victor A. Ferrari, Vickas V. Patel, Jeffery D. Molkentin, Glenn L. Radice

Abstract—The structural integrity of the heart is maintained by the end-to-end connection between the myocytes called the intercalated disc. The intercalated disc contains different junctional complexes that enable the myocardium to function as a syncytium. One of the junctional complexes, the zona adherens or adherens junction, consists of the cell adhesion molecule, N-cadherin, which mediates strong homophilic cell–cell adhesion via linkage to the actin cytoskeleton. To determine the function of N-cadherin in the working myocardium, we generated a conditional knockout containing loxP sites flanking exon 1 of the N-cadherin (Cdhh2) gene. Using a cardiac-specific tamoxifen-inducible Cre transgene, N-cadherin was deleted in the adult myocardium. Loss of N-cadherin resulted in disassembly of the intercalated disc structure, including adherens junctions and desmosomes. The mutant mice exhibited modest dilated cardiomyopathy and impaired cardiac function, with most animals dying within two months after tamoxifen administration. Decreased sarcomere length and increased Z-line thickness were observed in the mutant hearts consistent with loss of muscle tension because N-cadherin was no longer available to anchor myofilaments at the plasma membrane. Ambulatory electrocardiogram monitoring captured the abrupt onset of spontaneous ventricular tachycardia, confirming that the deaths were arrhythmic in nature. A significant decrease in the gap junction protein, connexin 43, was observed in the N-cadherin–depleted hearts. This animal model provides the first demonstration of the hierarchical relationship of the functional components of the intercalated disc in the working myocardium, thus establishing N-cadherin’s paramount importance in maintaining the structural integrity of the heart. (Circ Res. 2005;96:346-354.)

Key Words: cell adhesion • myocardium • adherens junction • desmosome • arrhythmia
found in extrajunctional sites where it colocalizes with α-actinin in the peripheral Z-disks of the sarcomeres. Complete loss of N-cadherin function in all murine tissues results in embryonic lethality at approximately mid-gestation, accompanied by multiple embryonic abnormalities including a severe cardiovascular defect. The primitive heart in the mutant embryo consists of loosely associated round cardiomyocytes surrounding the endocardium. Chimeric mice derived from N-cadherin–deficient embryonic stem (ES) cells demonstrated that N-cadherin-null cardiomyocytes were excluded from participating in the formation of the myocardial wall in the developing heart, further emphasizing the importance of N-cadherin in myocardial cell–cell interactions. Interestingly, myocyte adhesion and cardiac looping were restored in N-cadherin–null embryos expressing an epithelial cadherin, E-cadherin, specifically in the myocardium, demonstrating that these two classical cadherins are interchangeable during cardiac morphogenesis. However, cardiac-specific expression of E-cadherin in adult transgenic mice expressing endogenous N-cadherin led to dilated cardiomopathy indicating that ectopic cadherin expression in the intercalated disc is not compatible with normal cardiac function.

To specifically examine the function of N-cadherin in the working myocardium of the adult heart, we generated a N-cadherin conditional knockout (CKO) model using Cre-loxP technology. To overcome the requirement for N-cadherin in the embryonic heart, we used an inducible cardiac-specific Cre transgene consisting of α-myosin heavy chain (MHC) promoter expressing Cre recombinase flanked by mutant estrogen receptors (MerCreMer). Cardiac-specific deletion of N-cadherin was observed in the N-cadherin CKO mice after tamoxifen (Tam) administration. The intercalated disc structure was disassembled after removal of N-cadherin, resulting in abnormal morphology and decreased cardiac function. Myofibril organization was distorted in the N-cadherin CKO heart with decreased sarcomere length and wider, less dense Z-lines. The mutant animals had spontaneous ventricular arrhythmias and sudden cardiac death 2 months after cardiac-specific deletion of N-cadherin. This mouse model demonstrates the critical role of N-cadherin in maintaining the junctional complexes that constitute the intercalated disc structure.

Materials and Methods

Generation of the N-cadherin CKO Mice

To generate a targeting vector, a genomic library derived from a 129Sv mouse strain was screened with a probe corresponding to the first exon of the N-cadherin gene. Two genomic clones were characterized and used to generate the targeting vector illustrated in Figure 1A. The vector was linearized with Clal and electroporated into TL ES cells and colonies were selected with G418. Southern blot analysis was performed initially to detect all homologous recombination events (KpnI digest) and positive clones were subsequently screened for the presence of the distal loxP site using EcoRI digestion. The targeted ES cells were injected into blastocysts from C57Bl/6J mice. Mice were analyzed in a mixed 129Sv/C57Bl/6J background. Additional information concerning the characterization of these mice is available in the online data supplement at http://circres.ahajournals.org.

Results

Generation of the N-cadherin CKO mouse

To bypass the requirement for N-cadherin in the early embryo, we used Cre/loxP technology to inactivate the N-cadherin gene, specifically in the heart of adult mice. The targeting vector was designed to introduce loxP sites around exon 1, which contains the translational start site plus upstream transcriptional regulatory sequences. In addition to loxP sites, FLP recombination target (FRT) sequence from yeast Saccharomyces cerevisiae was used to allow excision of the neomycin selection cassette using FLP recombinase. The loxP/FRT/neomycin/FRT cassette was introduced into the first intron of the N-cadherin gene and a distal loxP site was introduced ≈2 kb upstream of the first exon. The targeting construct was electroporated into ES cells and ≈200 G418-resistant clones were examined by Southern blot analysis. We detected six clones that had undergone homologous recombination at the N-cadherin locus. Four of these six clones also contained the distal loxP site necessary for deleting exon 1 of the N-cadherin gene (Figure 1B). The targeted ES cells were introduced into blastocysts by standard methods to generate N-cadherin floxed (N-cadfllox/+) mice. The FRT flanked neomycin cassette was removed from the N-cadherin floxed allele by breeding to transgenic mice expressing the enhanced version of the site-specific recombinase FLP. The wild-type (WT) and N-cadherin floxed alleles were distinguished by polymerase chain reaction analysis followed by EcoRI digestion (Figure 1C). To confirm that Cre recombinase would mediate deletion of exon 1, N-cadfllox/+ mice were bred to protamine/Cre transgenic mice. The loss of the intervening sequence in the progeny was confirmed by polymerase chain analysis using primers flanking the two loxP sites (Figure 1C). The N-cadherin floxed allele did not affect expression of the endogenous gene, because WT and floxed animals expressed similar amounts of N-cadherin protein in heart and brain (Figure 1D).

Induced Deletion of the N-cadherin Gene in Heart

Based on the severity of the myocardial cell adhesion defect in N-cadherin–null embryos, we chose to use an inducible cardiac-specific Cre transgene to bypass the requirement of N-cadherin in the developing heart. Therefore, we mated our N-cadfllox/+ animals with the αMHC/MerCreMer transgenic mouse. The αMHC/MerCreMer mice mediate efficient recombination in the heart after Tam administration and, most importantly, show little activity without the drug. Homozygous N-cadfllox/lox mice were mated with αMHC/MerCreMer, N-cadnull/+, or N-cadnull/null mice in which the null allele was generated by either insertion of neo alone or βgeo. The resulting progeny αMHC/MerCreMer, N-cadnull/+, or N-cadnull/null animals were generated in the expected Mendelian frequency and appeared healthy and indistinguishable from their littermates. Six- to 10-week-old N-cadnull/fllox or N-cadnull/lox animals with the Cre transgene were administered Tam for 5 consecutive days. Controls included animals of the same genotype not given Tam and animals without the Cre transgene given Tam. In either case, no effect on N-cadherin expression was observed in the heart. To examine N-cadherin
depletion from the intercalated disc, immunohistochemistry was performed on N-cadherin CKO hearts 3 weeks after Tam administration. Representative images of ventricular myocardium are shown. The strong N-cadherin staining normally found in the intercalated disc was no longer present in the Tam-treated mice (Figure 2A and 2E). Cadherins and their cytoplasmic-binding partners, catenins, are coordinately regulated in cells; therefore, catenin expression and distribution were examined in the N-cadherin–depleted hearts. Consistent with N-cadherin being the only classical cadherin expressed in cardiomyocytes, β-catenin (Figure 2B and 2F), α-catenin (Figure 2C and 2G), and p120ctn (Figure 2D and 2H) were lost from the intercalated disc. Western analysis was performed on protein lysates from whole hearts to examine relative changes in total protein levels. N-cadherin was significantly decreased in the Tam-treated animal (84% versus controls; \( P < 0.05; n = 3 \)), consistent with its high expression in myocardium compared with nonmuscle cells in the heart (supplemental data; Figure 7).

The N-cadherin CKO hearts were elongated and flaccid and appeared to deflate after dissection compared with the normal firm control hearts. Histologic analysis of the N-cadherin depleted hearts 5 weeks after Tam demonstrated a modest dilation of both the ventricular and atrial chambers (Figure 3A and 3D). The overall cytoarchitecture of the mutant myocardium appeared relatively normal compared with WT (Figure 3B and 3E). However, enlarged hyperchromatic myocyte nuclei were observed in the mutant heart. The N-cadherin CKO animals displayed a moderate increase in heart weight/body weight ratio compared with WT littermates (+19% versus controls; \( P < 0.05; n = 6 \)). Fibrosis was detected in the mutant hearts by Masson trichrome stain (Figure 3C and 3F). The mutant animals died suddenly 2 months after Tam administration in the absence of overt signs of heart failure such as inactivity and edema.

**Loss of Intercalated Disc Structures in N-cadherin–Deficient Hearts**

To examine myocyte cell–cell interactions at the ultrastructural level, transmission electron microscopy was performed on the N-cadherin CKO hearts. Intercalated disc structures were readily visible in the WT hearts, with adherens junctions and desmosomes represented by submembranous electron dense material adjacent to intercellular space between the myocytes (Figure 4A). In contrast, intercalated disc structures were absent in the N-cadherin mutant hearts (Figure 4B). Furthermore, the lack of intercellular space between the myocytes made it difficult to discern where the myocytes made contacts with their neighbors. At higher magnification,
the sarcomeres appeared distorted and compressed compared with WT (Figure 4B and 4D) with decreased sarcomere length (1312.5 ± 122.3 versus 1686.0 ± 123.4 nm; P < 0.0001; CKO versus WT, respectively) and wider, less dense Z-lines (100.3 ± 27.0 versus 60.8 ± 8.6 nm; P < 0.0001; CKO versus WT, respectively). The sarcomere defects in N-cadherin CKO myocardium presumably reflect the lack of myofibril anchorage at the plasma membrane, resulting in a loss of myofibril tension.

To verify the loss of desmosomes in the N-cadherin CKO hearts, desmoplakin and plakoglobin, cytoplasmic desmosomal proteins were examined by immunohistochemistry. Similar to the N-cadherin/catenin complex, desmoplakin and plakoglobin staining were lost from the intercalated disc (Figure 5), consistent with the loss of electron dense desmosome structures in the transmission electron microscopy images of the N-cadherin CKO heart. Western blot analysis of heart lysates showed a reduction in plakoglobin, whereas desmoplakin levels remained unchanged in the mutant hearts (supplemental data).

Impaired Cardiac Function in N-cadherin CKO Mice

To assess cardiac function, cardiac-gated magnetic resonance imaging was performed on the mutant mice 5 weeks after Tam administration and compared with control mice (Figure 6A; supplemental data). Quantitative analysis of image data demonstrated significantly larger left ventricular (LV) end-diastolic volume and end-systolic volume in the cavity in the CKO group compared with WT (end-diastolic volume: 0.054 ± 0.003 versus 0.03 ± 0.004 cm³; end-systolic volume: 0.029 ± 0.002 versus 0.005 ± 0.002 cm³; P < 0.002; CKO versus WT, respectively), with similar LV stroke volumes (0.025 ± 0.002 cm³ for both groups; P = NS). LV end-diastolic short-axis cavity diameters were measured at the basal, mid-ventricular, and apical locations and demonstrated a
larger diameter for the mid-ventricular level only (3.54 ± 0.02 versus 2.84 ± 0.23 mm; *P* < 0.01; CKO versus WT, respectively), consistent with a more elongated rather than circumferential enlargement pattern. However, LV end-systolic diameters were markedly larger at every level for the CKO group, reflecting both reduced regional systolic wall thickening and overall ventricular function (basal: 2.71 ± 0.41 versus 0.65 ± 0.48 mm; mid-LV: 2.76 ± 0.16 versus 0.29 ± 0.5 mm; apical: 1.33 ± 0.57 versus 0.09 ± 0.2 mm; *P* < 0.02; CKO versus WT, respectively). Wall thicknesses were comparable between groups at all levels (0.71 ± 0.15 versus 0.82 ± 0.07 mm; *P* = NS; CKO versus WT, respectively) but trended toward thinner walls in CKO mice at the apex (0.47 ± 0.17 versus 0.71 ± 0.1 mm; *P* < 0.08; CKO versus WT, respectively). Heart rate was reduced in the CKO mice compared with WT (382 ± 67 versus 517 ± 42 bpm; *P* < 0.02; CKO versus WT, respectively). Both LV ejection fraction and cardiac output were significantly reduced in CKO mice (Figure 6B and 6C). These results are consistent with a decrease in force transmission attributable to disruption of the cadherin/catenin cytoskeletal linkage.

**Sudden Arrhythmic Death in N-cadherin CKO Mice**

To investigate the mechanism by which cardiac-restricted deletion of N-cadherin induces sudden cardiac death, miniaturized electrocardiogram telemetry transmitters were implanted in three N-cadherin CKO mice to record their cardiac rhythm. Each of these animals had been injected with tamoxifen ~6 weeks before the monitoring, and during the continuous recording period all of them remained in normal sinus rhythm with no ventricular ectopy until the terminal event. Within 1 week of monitoring, we were able to record the abrupt onset of ventricular tachyarrhythmia in two of the mice, coincident with sudden death (Figure 7A and 7B). In both of these events, the tachycardia was initiated by a ventricular premature depolarization inducing a regular tachyarrhythmia that subsequently degenerated into fibrillation. Sudden cardiac death is observed in cardiac-restricted connexin 43 (Cx43) CKO mice; therefore, we examined the level of Cx43 in the N-cadherin CKO mice. Western blot analysis demonstrated that Cx43 levels were significantly decreased (~60% versus controls; *P* < 0.001; *n* = 6) in the N-cadherin CKO animals (Figure 7C).

**Increased β1 Integrin Expression After Depletion of N-cadherin**

Many components of the intercalated disc were lost or significantly reduced in the N-cadherin CKO hearts including catenins, desmoplakin, and connexin 43, consistent with N-cadherin–mediated adhesion being critical for stabilization of junctional complexes at the plasma membrane. Cell–matrix interactions are also involved in maintaining muscle integrity; therefore, β1 integrin expression was examined in the N-cadherin CKO hearts. In contrast to other components of the intercalated disc examined above, β1 integrin expression was upregulated in N-cadherin–depleted myocardium, as shown by immunohistochemistry and confirmed by Western...
Discussion

Loss of N-cadherin from all murine tissues results in multiple developmental abnormalities, including a severe cardiovascular defect leading to embryonic lethality. To determine the function of N-cadherin in the adult myocardium, we generated a N-cadherin floxed allele to allow tissue-specific deletion of N-cadherin. Deleting N-cadherin specifically in the myocardium using a noninducible cardiac-specific MHC/Cre transgene resulted in embryonic lethality (Kostetskii and Radice, unpublished data). Therefore, an inducible cardiac-specific Cre transgene was necessary to overcome the requirement for N-cadherin in the embryonic myocardium. In this study, the N-cadherin CKO mice were bred with the αMHC/MerCreMer mice, allowing inducible deletion of the N-cadherin gene after administration of Tam.

The loss of recognizable intercalated disc structures along with the intercellular space normally separating the cardiomyocytes was quite surprising. To our knowledge, this is the first mutation with such a dramatic structural phenotype resulting in disassembly of different junctional complexes in the heart. The desmosomes provide structural support by interacting with the intermediate filaments (ie, desmin), whereas adherens junctions provide linkage to the actin cytoskeleton. Mutations in the desmosome-associated proteins, plakoglobin and desmplakin, cause arrhythmogenic right ventricular cardiomyopathy in humans. In addition, desmin mutations are associated with cardiac and skeletal myopathy. In this study, we demonstrate that desmosome stability is dependent on N-cadherin function in the adult myocardium.

Cardiac-specific loss of N-cadherin led to a modest dilated cardiomyopathy (DCM) phenotype before animals died of cardiac arrhythmic death. The lack of severe DCM phenotype in the mutant animals is somewhat unexpected. It is possible that N-cadherin CKO animals die too soon from sudden death (2 months) to observe compensation attributable to loss of force transduction across the plasma membrane. Alternatively, the cadherin/catenin complex may be an important mechanosensor in the compensatory process, hence myocytes may respond differently to the structural changes resulting from loss of N-cadherin compared with other cytoskeletal components. Interestingly, we observed an initial increase in the disease marker, atrial natriuretic factor (ANF), within 1 week after deleting N-cadherin from the myocardium; however, its expression decreased thereafter and remained relatively low until the animal’s sudden death (Li and Radice, unpublished data). Myofibril organization appeared relatively normal in the mutant hearts except for the compression of the sarcomeres, which is likely attributable to the loss of N-cadherin attachment sites. This result was consistent with...
our previous findings in embryonic cardiomyocytes lacking N-cadherin; however, it was not possible to assess myofibril alignment between the cardiomyocytes in the N-cadherin CKO hearts because the intercalated disc structure was missing. In future studies, it will be interesting to determine the expression and cellular distribution of myofibril-anchoring proteins in the N-cadherin mutant hearts because the intercalated disc is no longer present. For example, several cytoskeletal proteins of the LIM domain family, including the muscle-specific LIM protein, α-actinin–associated LIM domain protein, and nebulin-related anchoring protein are thought to mediate linkage of the myofibril to the cadherin/catenin complex. In the absence of N-cadherin, these anchoring proteins may facilitate myofibril linkage through the integrin-based adhesion system.

Naxos disease, a rare recessive form of arrhythmogenic right ventricular cardiomyopathy, is associated with a high incidence of arrhythmias and sudden cardiac death. This disease is associated with a two-nucleotide deletion in the gene of the desmosome-associated protein, plakoglobin, which causes a frame shift resulting in truncation of the C-terminal domain by 56 residues. Interestingly, Cx43 expression is affected in Naxos disease, suggesting that gap junction remodeling may contribute to the arrhythmogenic substrate and the increase risk of sudden death in these patients. Plakoglobin is also affected in N-cadherin CKO hearts and Cx43 expression is reduced, suggesting that plakoglobin may have an important role in gap junction stability.

The loss of mechanical coupling via both adherens junctions and desmosomes resulted in unique structural abnormalities. Although cardiac function was perturbed, based on magnetic resonance imaging and echocardiographic analysis (Ferrari and Radice, unpublished data), the mutant hearts did not exhibit the typical DCM morphology. Normally in DCM, the circumference of the left ventricular chamber in the short axis is enlarged; however, in our model enlargement was more pronounced in the long axis, giving the heart an elongated appearance. In addition, the mutant hearts appeared to deflate on removal from the animals, suggesting a loss of structural integrity.

How is it possible that the heart can continue to pump blood throughout the body without mechanical coupling mediated by adherens junctions and desmosomes normally found in the intercalated disc? One possibility is that a different cell adhesion system(s) is trying to compensate for loss of these junctional complexes. In addition to cell–cell, cell–extracellular matrix (ECM) interactions also are important determinants of myocyte cytoarchitecture providing structural integrity necessary for normal sarcomere organization. Because both N-cadherin and β1 integrin interact with the actin cytoskeleton and stabilize myofibrils, we speculate that β1 integrin may be trying to compensate for loss of...
N-cadherin. In addition, cadherin and integrin complexes share cytoskeletal components such as vinculin. Mutations were recently found in the muscle-specific isoform, metavinculin, which result in altered intercalated disc morphology in DCM patients. Cardiac-specific deletion of β1 integrin using the noninducible αMHC/Cre transgene was compatible with embryonic development and resulted in DCM in aged animals. In contrast, loss of N-cadherin in the embryonic heart using the same noninducible αMHC/Cre transgene resulted in embryonic lethal phenotype (Kostetskii and Radice, unpublished data), indicating that the cadherin-based adhesion system is more critical for maintaining the structural integrity of the myocardium compared with β1 integrin. In contrast to other intercalated disc components in the N-cadherin-depleted hearts, we observed increased expression of β1 integrin. The N-cadherin CKO animals exhibit a modest DCM phenotype relative to other animal models with increased β1 integrin expression; therefore, the upregulation of β1 integrin is unlikely to be in response to an adaptive hypertrophic stimuli. We speculate that increasing cell–ECM interactions may be a direct response to loss of N-cadherin linkage to the actin cytoskeleton, thus compensating for the loss of cell–cell adhesion. The increase in collagen deposition observed in the N-cadherin CKO hearts is consistent with this idea. Cardiac-restricted deletion of β1 integrin also results in myocardial fibrosis, albeit more severe than N-cadherin CKO mice, suggesting that perturbation of either actin-based adhesion complex stimulates a common signaling pathway that results in upregulation of ECM. An inducible cardiac-specific knockout of both N-cadherin and β1 integrin will be necessary to determine whether the integrin-based adhesion system is responsible for maintaining the myofibril organization and contractile ability in the N-cadherin–deficient heart. In addition to integrin-mediated ECM interactions, the dystrophin–glycoprotein complex may provide additional structural support in the absence of N-cadherin.

Given the severity of the cardiac phenotype in our animal model, it is possible that patients with idiopathic cardiomyopathy carry mutations in the N-cadherin gene. Complete loss of function is unlikely given N-cadherin’s requirement in the murine embryo, however reduction in N-cadherin levels or altered function may predispose individuals to cardiomyopathy. Examination of animals expressing half the normal levels of N-cadherin (ie, heterozygotes) under normal and stress-induced conditions may prove informative. In addition, it is also possible that dominant-negative mutations may arise, which affect cis (parallel) or trans (antiparallel) dimerization of N-cadherin, leading to a less strong cadherin zipper structure. These types of mutations may cause subtle conformational changes, which may not have a significant affect on N-cadherin–mediated cell adhesion in most tissues; however, it may have deleterious consequences for cardiac function attributable to the mechanical load on the junctional complex in the working myocardium.

In summary, we have generated a unique animal model that results in disassembly of the intercalated disc structure resulting in abnormal cardiac morphology and function. These findings have important implications for heart disease because the compensatory response may alter cadherin/catenin function, leading to changes in downstream cell adhesion and signaling pathways.

Acknowledgments

This study was supported in part by NIH grants HL57554, DK062748 (to G.R.), and HL074110 (to V.P.), and American Heart Association grants 9708294A and 0051086U (to G.R.). I.K. (0020169U) and J.L. (0425656U) were supported in part by post-doctoral fellowships from the PA/DE Affiliate of the American Heart Association. G.R. is an Established Investigator of the American Heart Association. We thank Patricia Labosky for ES cells, Jean Richa and the University of Pennsylvania Transgenic Core Facility, and Neelima Shah and the Biomedical Imaging Core Facility.

References


Induced Deletion of the N-Cadherin Gene in the Heart Leads to Dissolution of the Intercalated Disc Structure
Igor Kostetskii, Jifen Li, Yanming Xiong, Rong Zhou, Victor A. Ferrari, Vickas V. Patel, Jeffery D. Molkentin and Glenn L. Radice

Circ Res. 2005;96:346-354; originally published online January 20, 2005; doi: 10.1161/01.RES.0000156274.72390.2c
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2005 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/96/3/346

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2005/02/17/96.3.346.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
Material and Methods

**Generation of the N-cadherin CKO mice - continued**

The floxed mice were genotyped by PCR. The following primer pairs were used to detect the floxed allele: L07: 5’-TGC TGG TAG CAT TCC TAT GG-3’ and L08: 5’-TAC AAG TTT GGG TGA CAA GC-3’. The wild-type and floxed allele differ by only 40 bps, therefore the PCR product was digested with EcoRI to more easily distinguish the alleles. The following primer pairs were used to detect the deleted allele: L07: 5’-TGC TGG TAG CAT TCC TAT GG-3’ and NC23: 5’-GTA TGG CCA AGT AAT GGG GAC-3’.

To induce Cre-mediated recombination, adult αMHC/MerCreMer, N-cad^{null/flox} or N-cad^{flox/flox} mice were treated with Tamoxifen (Sigma) by intraperitoneal injection once a day for five consecutive days at a dosage of 2 mg/25 g mice per day. Tamoxifen (20mg/ml) was dissolved in corn oil (Sigma) by heating to 37°C for one hour.

**Western Immunoblotting**

Western blot analyses were performed with antibodies to N-cadherin (3B9, Zymed), β-catenin (5H10, Zymed), α-catenin (C2081, Sigma), p120^{ctn} (15D2, BD Transduction), Cx43 (71-0700, Zymed), plakoglobin (15, BD Biosciences), desmoplakin (AHP320, Serotec) and β1 integrin (18, BD Biosciences). For normalization of signals, blotting was also performed with anti-GAPDH (6C5, RDI) monoclonal antibodies, followed by blotting with alkaline phosphatase–conjugated secondary antibody, chemifluorescent processing (ECF, Amersham BioSciences). Densitometry of samples was performed via use of Image-Quant (Molecular Dynamics) software.
Immunofluorescence, Histology, and Transmission Electron Microscopy

Hearts were isolated from N-cadherin CKO mice and fixed in formalin. Indirect immunofluorescence was performed on paraffin sections of hearts as previously described. The sections were incubated with the primary antibodies described above. The secondary antibody was Cy3-conjugated goat anti-mouse or rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA). The sections were viewed and photographed with a confocal microscope.

For histological analysis, hearts were isolated at 5 weeks post Tam administration, fixed in formalin, processed for paraffin sectioning, and stained with hematoxylin and eosin. Ultrastructural analysis of the myocardium was performed by TEM as previously described.

MR Imaging and Image Analysis

The mouse was anesthetized during imaging with a 1% isofluorane/air mixture administered via a nose cone and its core temperature was maintained at 37±0.1°C. The surface electrocardiogram (ECG) was monitored and the R-wave used to synchronize MR data acquisition. All images were generated on 4.7T horizontal bore spectrometer using an elliptical surface coil (2.5 x 3.5 cm) for transmission and reception. ECG-gated cine images along the short axis of the heart were acquired using a fast gradient echo sequence as described using following parameters: repetition time = 12 ms; time of echo = 2.3 ms; field of view = 3.0 cm; matrix = 128 x128; slice thickness = 1.0 mm, Averages = 8. Global cardiac function (cardiac output and ejection fraction) was calculated from cine MR images as previously described. Data are presented as mean ± standard deviation (SD). For statistical analysis, one way ANOVA (Sigmastat, Point Richmond, CA) was used to compare the values from the N-cadherin CKO versus wild-type animals. P-value of < 0.05 was considered statistically significant.
Ambulatory ECG monitoring

Miniature telemetry transmitter devices (Data Science International) were implanted as previously described\(^4\). Once the mice fully recovered from the implantation procedure (48 to 72 hours) their electrocardiograms were recorded during regular physical activity for any signs of spontaneous ventricular ectopy or AV conduction disturbances and stored on the hard drive of a Windows-based PC for off-line analysis.

Statistics

Data are expressed as mean ± SEM. Comparisons between groups were performed with a 2-tailed Student \( t \) test using Microsoft Excel software. \( P < 0.05 \) was considered statistically significant.

References


Figure legends for supplemental data

Fig. 1. Western blot analysis of adherens junction proteins in N-cadherin CKO animals. Heart lysates from wild-type (+/+) and N-cad<sup>flox/-</sup> (F/-), Cre animals minus Tam or four weeks following Tam administration were immunoblotted for N-cadherin, β-catenin, α-catenin, and p120ctn. Beta-catenin and α-catenin showed reduced expression in total heart lysate compared to wild-type, however p120ctn levels appeared similar with control. Note the reduced catenin expression in the N-cadherin heterozygous heart (F/-, minus Tam). GAPDH signal shows loading of samples between lanes.

Fig. 2. Western blot analysis of desmosome proteins in N-cadherin CKO animals. Heart lysates from wild-type (+/+ and N-cad<sup>flox/-</sup> (F/-), Cre animals four weeks following Tam administration were immunoblotted for desmoplakin and plakoglobin. Plakoglobin showed reduced expression in total heart lysate compared to wild-type, whereas desmoplakin levels appeared similar with control. GAPDH signal shows loading of samples between lanes.

Online movies (4). MRI video images.
<table>
<thead>
<tr>
<th></th>
<th>N-cad</th>
<th>Cre</th>
<th>Tam</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td></td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>F/-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Protein</th>
<th>N-cadherin</th>
<th>β-catenin</th>
<th>α-catenin</th>
<th>p120</th>
<th>GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-cad +/+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-cad F/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-cad F/-</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The images show the expression levels of the proteins under different conditions.
<table>
<thead>
<tr>
<th></th>
<th>N-cad</th>
<th>Cre</th>
<th>Tam</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>+/+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F/-</td>
<td>F/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F/-</td>
<td>F/-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>F/-</td>
<td>F/-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**Desmoplakin**

**GAPDH**

**Plakoglobin**

**GAPDH**