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Rescuing the N-cadherin knockout by cardiac-specific expression of N- or E-cadherin

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SUMMARY

Cell-cell adhesion mediated by some members of the cadherin family is essential for embryonic survival. The N-cadherin-null embryo dies during mid-gestation, with multiple developmental defects. We show that N-cadherin-null embryos expressing cadherins using muscle-specific promoters, α- or β-myosin heavy chain, are partially rescued. Somewhat surprisingly, either N-cadherin or E-cadherin was effective in rescuing the embryos. The rescued embryos exhibited an increased number of somites, branchial arches and the presence of forelimb buds; however, in contrast, brain development was severely impaired. In rescued animals, the aberrant yolk sac morphology seen in N-cadherin-null embryos was corrected, demonstrating that this phenotype was secondary to the cardiac defect. Dye injection studies and analysis of chimeric animals that have both wild-type and N-cadherin-null cells support the conclusion that obstruction of the cardiac outflow tract represents a major defect that is likely to be the primary cause of pericardial swelling seen in null embryos. Although rescued embryos were more developed than null embryos, they were smaller than wild-type embryos, even though the integrity of the cardiovascular system appeared normal. The smaller size of rescued embryos may be due, at least in part, to increased apoptosis observed in tissues not rescued by transgene expression, indicating that N-cadherin-mediated cell adhesion provides an essential survival signal for embryonic cells. Our data provide in vivo evidence that cadherin adhesion is essential for cell survival and for normal heart development. Our data also show that E-cadherin can functionally substitute for N-cadherin during cardiogenesis, suggesting a critical role for cadherin-mediated cell-cell adhesion, but not cadherin family member-specific signaling, at the looping stage of heart development.

Key words: Cell adhesion, Cardiac morphogenesis, Outflow tract, Apoptosis, Mouse, N-cadherin

INTRODUCTION

The correct spatial organization of cells is critical for normal tissue architecture and function. Perturbation of normal cell-cell interactions leads to structural problems during embryogenesis as well as progression of the malignant phenotype. The cadherin family of calcium-dependent cell-adhesion molecules plays an important role in establishing and maintaining cell-cell interactions through their homotypic binding and adhesive specificities (Gumbiner, 1996; Takeichi, 1995). The ability of cadherins to promote strong cell-cell adhesion is dependent on their interaction with a group of proteins called catenins, which mediate cadherin linkage to the actin cytoskeleton (Kemler, 1993). The cytoplasmic domain of classic cadherins contains at least two distinct protein-binding sites; a C-terminal region for β- or γ-catenin (plakoglobin) and a juxtamembrane region for p120ctn binding (Anastasiadis and Reynolds, 2000). These cytoplasmic binding proteins regulate the adhesive activity of the cadherin. Deletion of the cytoplasmic domain of E-cadherin results in a dramatic reduction in cadherin-mediated cell adhesion (Nagafuchi and Takeichi, 1988).

Members of the cadherin family have distinct spatial and temporal patterns of expression during embryonic development and in the adult (Takeichi, 1988). Changes in cadherin expression often are associated with changes in cellular morphology and tissue architecture. At gastrulation, E-cadherin is downregulated in the primitive streak as cells undergo an epithelial-mesenchymal transition leading to expression of N-cadherin in the migrating mesoderm (Duband et al., 1988; Hatta et al., 1987). During neurulation, a similar change in expression occurs in the developing neuroepithelium when E-cadherin is lost during the invagination of the neural tube and replaced by N-cadherin. In the developing heart, N-cadherin is the only classical cadherin known to be expressed in the myocardium with VE-

cadherin found in the endocardium. N-cadherin has been implicated in several aspects of cardiac development including sorting out of the precardiac mesoderm (Linask et al., 1997), establishment of left-right asymmetry (Garcia-Castro et al., 2000), cardiac looping morphogenesis (Shiraishi et al., 1993), and trabeculation of the myocardial wall (Ong et al., 1998).

The function of different cadherin subtypes depends on the cellular context. E-cadherin is expressed in most epithelia throughout the body. In contrast, N-cadherin expression is more restricted to specific cell types such as muscle and neurons. Recent data suggests that tumor cells upregulate different cadherins during cancer progression. Ectopic expression of N-cadherin in squamous cell carcinoma cell lines (Islam et al., 1996) and breast cancer cells (Hazan et al., 2000; Nieman et al., 1999) leads to increased invasiveness in vitro and in vivo. The mechanism by which N-cadherin influences cell morphology and behavior is not well understood. However, recent evidence suggests that it may facilitate lateral dimerization of growth factor receptors such as fibroblast growth factor receptor (FGFR) thus stimulating signaling pathways leading to changes in cell behavior (Nieman et al., 1999).

Besides providing structural integrity to cells, recent evidence suggests that cadherins may provide specific signals to cells influencing their differentiation potential. Embryonic stem (ES) cells lacking both copies of the gene for E-cadherin display a disaggregated phenotype (Larue et al., 1996). This phenotype can be rescued by constitutively expressing either E-, N-, or R-cadherin. The differentiation ability of these different ES cell lines was examined by generating benign teratomas in mice. The teratomas derived from the E-cadherin-expressing ES cells consisted almost exclusively of epithelia. In contrast, the teratomas derived from N-cadherin- and R-cadherin-expressing ES cells exhibited a very different phenotype. In the case of N-cadherin, neuroepithelium and cartilage were prevalent (Larue et al., 1996), whereas expression of R-cadherin led to striated muscle and epithelia (Rosenberg et al., 1997). Furthermore, loss of E-cadherin resulted in the expression of the transcription factor, T-brachyury, demonstrating that E-cadherin can influence gene expression (Larue et al., 1996). In the embryo, T-brachyury expression commences after E-cadherin expression is downregulated in the primitive streak mesoderm, suggesting that the downregulation of E-cadherin is important in this process.

Anchorage of cells to the extracellular matrix through integrins, as well as cell-cell interactions mediated by cadherins, are thought to play an important role in cell survival. Recent data indicate that VE-cadherin plays a critical role in endothelial cell survival (Carmeliet et al., 1999; Herren et al., 1998). Endothelial cells mutant for VE-cadherin undergo increased apoptosis and do not respond to the endothelial survival factor, VEGF-A, even though mutant cells express the VEGF-A receptor, VEGFR-2, on their cell surface (Carmeliet et al., 1999). However, bFGF was capable of rescuing the VE-cadherin mutant cells from death. Co-immunoprecipitation experiments revealed an association between VE-cadherin, β-catenin, PI3-kinase and VEGFR-2 in wild-type, but not mutant cells. Furthermore, β-catenin and PI3-kinase are thought to interact to stimulate cell survival through the VEGFR-2 pathway. The role of N-cadherin in programmed cell death is less well understood. However, evidence suggests that N-cadherin is involved in granulosa cell survival (Makrigiannakis et al., 1999; Peluso et al., 1996) and that this effect may be mediated through the FGFR (Trolice et al., 1997).

The use of gene targeting technology in mice has allowed the generation of null mutations in many genes, some of which are required for early embryonic development. However, the phenotypic analysis of embryonic lethal mutations is often difficult to interpret when the mutated gene is normally expressed in multiple cell lineages of the early post-implantation embryo. Consistent with its expression pattern, embryos lacking N-cadherin exhibit several developmental abnormalities, including malformed somites and yolk sac, undulated neural tube, and a severe cardiovascular defect, which is the apparent cause of death (Radice et al., 1997). Although it was clear that N-cadherin is critical for myocyte adhesion, the early lethality made it difficult to examine the function of N-cadherin in other cell lineages in the embryo. Hence, the rationale for the present experiments was twofold: (1) to restore cadherin-mediated adhesion in the heart of N-cadherin-null embryos; and (2) to examine development of the heart, as well as the remaining N-cadherin-null tissues. The data presented indicate the following: N-cadherin can rescue the heart defect; epithelial cadherin (E-cadherin) can substitute for N-cadherin at the looping stage of cardiac development; the yolk sac phenotype is secondary to the cardiovascular defect; outflow tract formation is dependent on cadherin-mediated adhesion; and, finally, we provide the first in vivo evidence that N-cadherin-mediated adhesion is required for embryonic cell survival.

MATERIALS AND METHODS

Transgenic mice

Transgenic mice expressing cadherins in muscle were generated as follows. A cDNA encoding chicken N-cadherin, *Eco*RV-*Sma*I fragment, was modified with either *Sal*I or *Spe*I linkers (plasmid provided by M. Takeichi, Kyoto University, Kyoto, Japan). The cDNA was cloned into either the *Sal*I or *Spe*I site of the expression vector containing either the αMHC or βMHC promoter, respectively (plasmids provided by J. Robbins, The Children Hospital Research Foundation, Cincinnati, OH). A cDNA encoding human E-cadherin, *Hin*dIII-*Hin*dIII fragment, was cloned into the *Hin*dIII site of the expression vector containing the αMHC promoter (plasmid provided by K. Johnson, University of Toledo, Toledo, OH). The cadherin vectors were linearized with either *Not*I alone (αMHC) or *Sfi*I and *Not*I (βMHC), purified and injected into fertilized oocytes derived from SJL/C57BL/6J mice, and two-cell embryos were transferred into the oviducts of pseudopregnant CD-1 mice. Transgenic mice were identified by either Southern blot using a cDNA probe or by PCR using the following primer sets: αMHC/Ncad, 5′ GTC-CACATTCTTCAGGATTC 3′ and 5′ CATGTCTTCACAAGTTGC 3′; αMHC/Ecad, 5′ GTCCACATTCTTCAGGATTC 3′ and 5′ GCTTCTAGTTAGTCAGTCGAC 3′; βMHC/Ncad, 5′ TCTCC-AGCTCTCCTACAGGC 3′ and 5′ CATGTCTTCACAAGTTGC 3′.

Immunohistochemistry and histological analysis

Embryos were fixed overnight in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4. After rinsing in PBS, the embryos were dehydrated and embedded in paraffin

wax according to standard procedures. Sections (6 μm) were cut and mounted on Superfrost slides (Fisher). The slides were deparaffinized in xylene and rehydrated through an alcohol series, and then heated in 1× Antigen Unmasking Solution (Vector Laboratories, Burlingame, CA) in a microwave oven (350 watts) for 10 minutes to unmask the epitope. The sections were washed in PBS, then 0.2% glycine/PBS, and again 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, So. San Francisco, CA); mouse monoclonal anti-E-cadherin, 1:100 (HECD-1; Zymed, So. San Francisco, CA), and mouse monoclonal anti-sarcomeric myosin, 1:250 (MF20, Developmental Studies Hybridoma Bank, University of Iowa). Samples were incubated overnight at 4°C with primary antibodies. After washing in PBS, the sections were incubated with Cy3-conjugated goat anti-mouse (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour at room temperature. In the case of MF20, peroxidase staining was performed with the VectastainABC kit (Vector Laboratories, Burlingame, CA) according to manufacturer's instructions. The sections were washed in PBS, and mounted for viewing and photography with either a fluorescence or bright field microscope.

For histological analysis, embryos were isolated at different stages of gestation and fixed in Bouin's at 4°C overnight, processed for paraffin sectioning, and stained with Hematoxylin and Eosin.

Ink injections

For gross examination of the cardiovascular system, the ventricle of embryonic day (E) 9.5 embryos was injected with Pelikan India ink diluted 50% in PBS. Prior to injection embryos were kept in DMEM culture media at 37°C to maintain the heartbeat. Embryos were fixed in 4% paraformaldehyde at 4°C overnight, processed through a series of methanol/PBS to 100% methanol, and cleared for several hours in two vol benzyl benzoate and 1 vol benzyl alcohol. Embryos were photographed under a dissecting microscope.

Chimera analysis

ES cell lines were isolated from embryos derived from N-cadherin$^{+/-}$ intercross matings as previously described (Moore et al., 1999). ES cell lines expressing the bacterial β-galactosidase (*lacZ*) gene driven off the constitutive EF1α promoter were injected into blastocysts from C57BL/6J mice as previously described (I. K., R. M., R. K. and G. R., unpublished observations). Chimeric embryos were recovered, fixed in 0.2% glutaraldehyde, and stained for β-galactosidase activity as previously described (Ciruna et al., 1997). After whole-mount β-galactosidase staining, chimeric embryos were postfixed in 4% paraformaldehyde and photographed under a dissecting microscope. The embryos were then dehydrated through an ethanol series and embedded in paraffin according to standard procedures. Paraffin blocks were sectioned at 6 μm, mounted on glass slides, dewaxed and counterstained with Nuclear Fast Red (Vector Laboratories, Burlingame, CA).

Programmed cell death

Whole-mount apoptosis analysis of E8.5 and E9.5 embryos was performed using the Apoptag Fluorescein In Situ Apoptosis Detection kit (Intergen, Purchase, NY). Embryos were fixed in 4% paraformaldehyde at 4°C overnight, washed in PBS/1% Triton X-100, and transferred to equilibration buffer for 30 minutes at room temperature. The embryos were incubated with terminal deoxynucleotidyl transferase (TdT) enzyme for 3 hours at 37°C, rinsed with stop/wash buffer and incubated with anti-digoxigenin-fluorescein overnight in the dark at room temperature. The embryos were washed in PBS/1% Triton X-100 and incubated with propidium iodide for 30 minutes at room temperature. The embryos were cleared in 80% glycerol and mounted for analysis with a confocal microscope. Apoptosis was confirmed by transmission electron microscopy (TEM) analysis, according to standard protocols.

RESULTS

Generation of transgenic mice expressing different cadherin subtypes in the heart

To determine the effects of cardiac-specific rescue of the cardiovascular defect in N-cadherin-null embryos, three transgenic strains were produced to breed with the N-cadherin mutant mice. The well-characterized mouse α-myosin heavy chain (MHC) promoter was used to express either the gene for human E-cadherin (αMHC/Ecad) or chicken N-cadherin (αMHC/Ncad) specifically in the heart. A 5.5 kb genomic fragment containing the promoter and first three noncoding exons of the gene for αMHC was used to express the cadherin genes in a cardiac-specific manner (Fig. 1A). In addition, a 5.6 kb genomic fragment containing the promoter and first four noncoding exons of the gene for βMHC also was used to express chicken N-cadherin (βMHC/Ncad) in the heart (Fig. 1A). Both αMHC and βMHC promoters are initially active in the early heart tube; however, after chamber specification their expression becomes restricted to the atria and ventricles, respectively. The genes for human E-cadherin and chicken N-cadherin were chosen for these experiments because both homologs are well conserved across species (90% and 92% amino acid similarity, respectively), and species-specific antibodies are available to distinguish exogenous and endogenous proteins. Furthermore, mouse N-cadherin can bind to chicken N-cadherin in a cell aggregation assay confirming its functional similarity (Miyatani et al., 1989). In contrast, N-cadherin does not interact with either mouse E- or P-cadherin (Miyatani et al., 1989). Since cadherin-mediated adhesion occurs primarily in a homophilic and homotypic manner, i.e. cadherins on one cell interact with like molecules on an adjacent cell, we predicted that using cadherin transgenes from different species would not affect their adhesive function in mice. The epithelial cadherin, E-cadherin, was chosen for the rescue experiment to determine if N-cadherin is specifically required in the myocardium during early cardiac development.

Several founder lines were obtained for both the αMHC/Ncad (5/49) and αMHC/Ecad (8/38) constructs, whereas only two βMHC/Ncad lines (2/68) were obtained. We chose two of the highest expressing αMHC/Ecad and αMHC/Ncad lines and the only βMHC/Ncad line that expressed exogenous protein (low expression) for the rescue experiments. The second βMHC/Ncad line did not transmit the transgene to its progeny. Expression from the βMHC promoter is restricted to the embryo; however, the αMHC promoter is active postnatally in both atria and ventricles. Because of this postnatal activity, many of the αMHC/Ecad and αMHC/Ncad transgenic animals develop cardiac hypertrophy and die (M. C. F.-C., Y. L. and G. R., unpublished observations). The surviving transgenic males appear healthy and fertile, whereas transgenic females have difficulty during pregnancy. Therefore, the αMHC/Ecad and αMHC/Ncad transgenes are transmitted through the male germline. Animals containing the βMHC/Ncad transgene had no problem transmitting the transgene to their progeny.

N- or E-cadherin restores myocyte adhesion and cardiac looping morphogenesis in N-cadherin-null embryos

The three different transgenic lines expressing cadherin in the heart were crossed to heterozygous N-cadherin mice, which

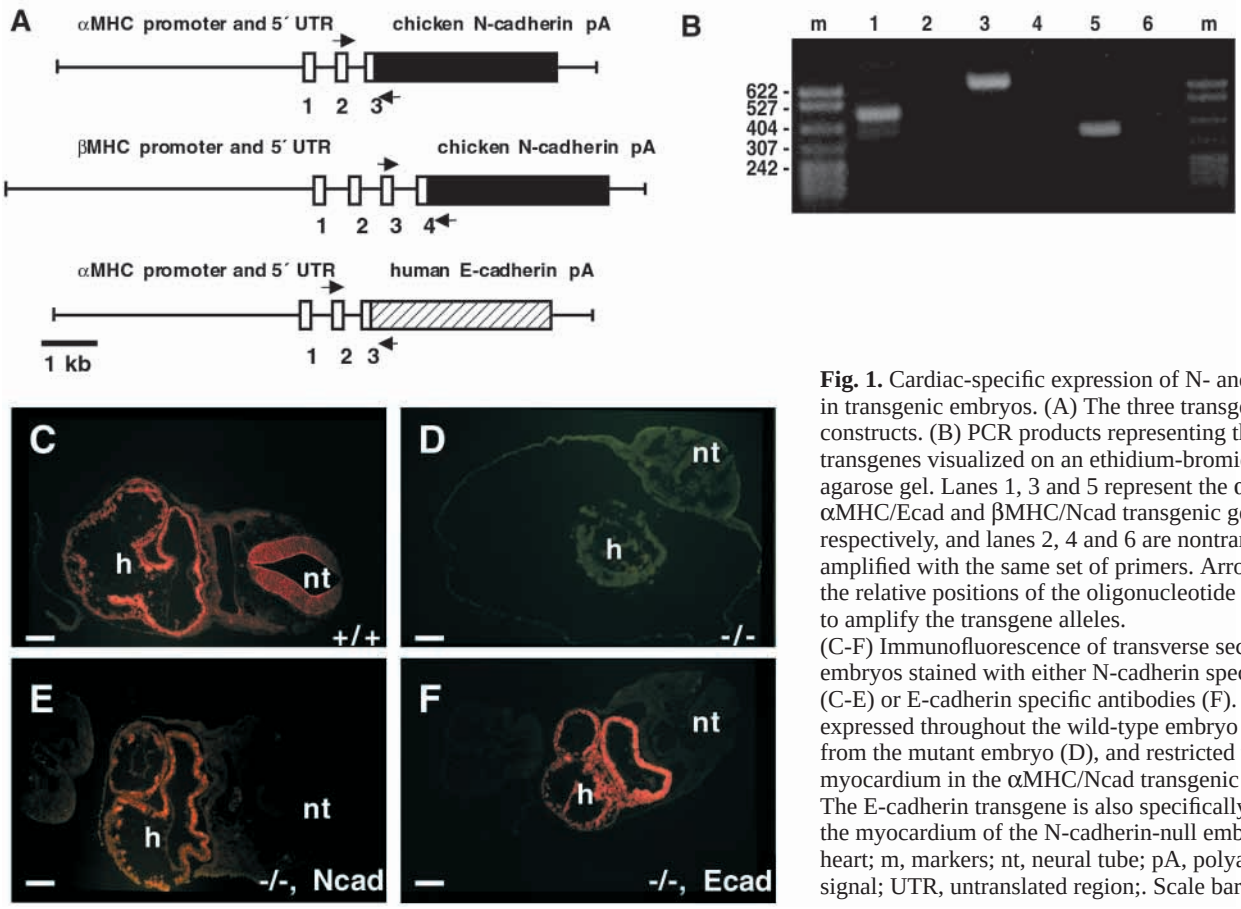


Fig. 1. Cardiac-specific expression of N- and E-cadherin in transgenic embryos. (A) The three transgenic constructs. (B) PCR products representing the different transgenes visualized on an ethidium-bromide-stained agarose gel. Lanes 1, 3 and 5 represent the αMHC/Ncad, αMHC/Ecad and βMHC/Ncad transgenic genomic DNA, respectively, and lanes 2, 4 and 6 are nontransgenic DNA amplified with the same set of primers. Arrows represent the relative positions of the oligonucleotide primers used to amplify the transgene alleles.
(C-F) Immunofluorescence of transverse sections of embryos stained with either N-cadherin specific antibodies (C-E) or E-cadherin specific antibodies (F). N-cadherin is expressed throughout the wild-type embryo (C), absent from the mutant embryo (D), and restricted to the myocardium in the αMHC/Ncad transgenic embryo (E). The E-cadherin transgene is also specifically expressed in the myocardium of the N-cadherin-null embryo (F). h, heart; m, markers; nt, neural tube; pA, polyadenylation signal; UTR, untranslated region;. Scale bars: 100 μm.

were identified by genotyping as previously described (Radice et al., 1997). The presence of the transgenes was demonstrated by PCR analysis using allele-specific oligonucleotides (Fig. 1B). Heterozygous N-cadherin mice carrying the transgene were backcrossed to N-cadherin heterozygotes, and litters were examined beginning at E9.5 when the N-cadherin-null phenotype is most apparent (Radice et al., 1997).

To verify that transgene expression was restricted to the myocardium, immunohistochemistry was performed on E9.5 embryos. In wild-type embryos, endogenous N-cadherin expression was observed in somites, notochord, neural tube, and myocardium (Fig. 1C and data not shown). In contrast, N-cadherin-null embryos carrying the different transgenes exhibited no endogenous N-cadherin expression in the myocardium, but showed specific exogenous cadherin expression as detected by antibodies to either chicken N-cadherin (Fig. 1E) or human E-cadherin (Fig. 1F). The restricted expression pattern of the transgenes is consistent with previous studies using these well-characterized promoters (Rindt et al., 1993; Subramaniam et al., 1991).

The morphology of the embryos was studied by examining whole-mount embryos. All non-transgenic N-cadherin-null embryos displayed the characteristic severe cardiovascular defect reported previously (Radice et al., 1997), including the distended pericardial cavity (Fig. 2). In striking contrast, all N-cadherin-null embryos carrying either of the three different

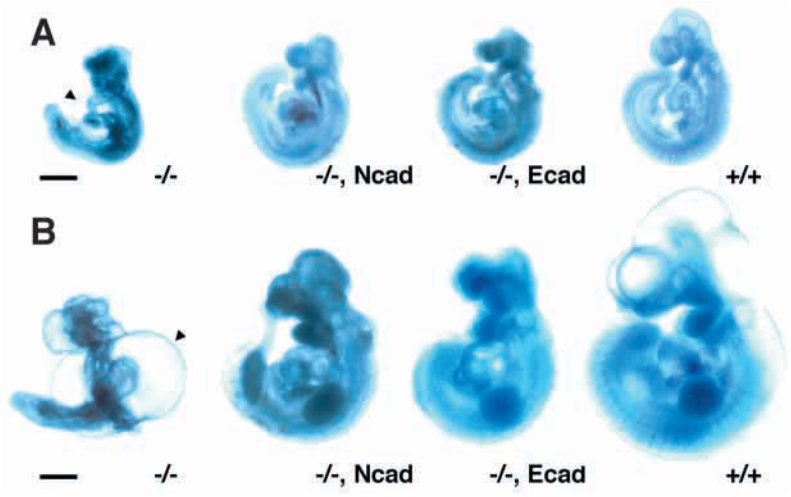


Fig. 2. Whole mounts of N-cadherin-null (–/–), cardiac-specific rescued (–/–, Ncad and –/–, Ecad) and wild-type (+/+) embryos. A lateral view of E9.5 (A) and E10.5 (B) embryos. The N-cadherin-null embryos display an enlarged pericardial cavity (arrowheads) with a malformed heart tube inside. In contrast, N-cadherin-null embryos carrying either the N- or E-cadherin transgene exhibit normal cardiac development. The cardiac rescued embryos were developmentally more advanced compared with the N-cadherin-null embryos without the transgene. However, brain formation was severely impaired. Scale bars: 500 μm.

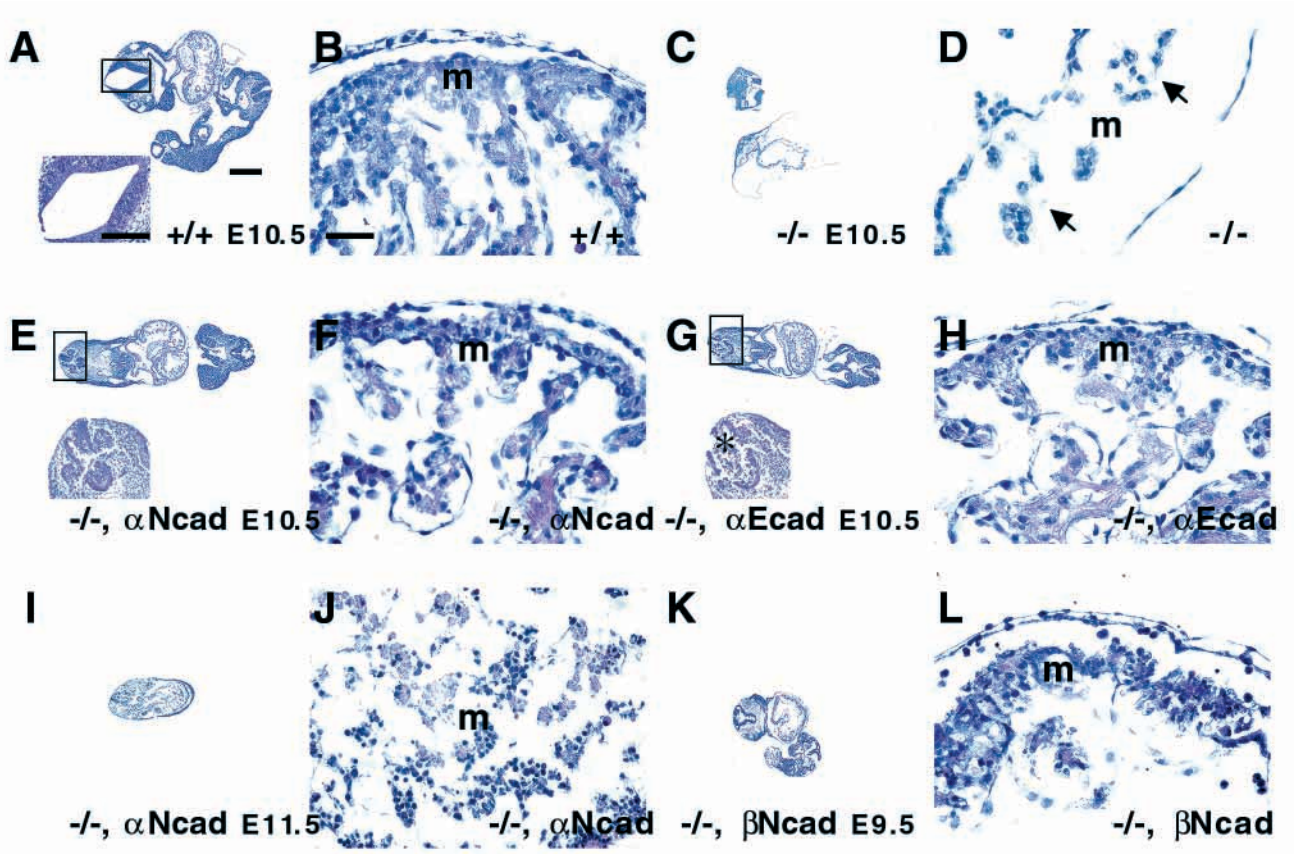


Fig. 3. Histological analysis of rescued N-cadherin-null embryos. Transverse sections through the thoracic region of wild-type (A,B), mutant (C,D), Ncad rescued (E,F) and Ecad rescued (G,H) embryos at E10.5. Note that the cell adhesion defect (D, arrows) was corrected in the myocardium of the rescued embryos and that the morphology of the E-cadherin rescued myocardium was remarkably similar to the N-cadherin expressing myocardium. The structural integrity of the neural tube was severely compromised in the cardiac rescued embryos consistent with the lack of transgene expression in that tissue (insets in A,E,G). Many dissociated cells (asterisk in G) were observed in the lumen of the malformed neural tube. The rescued embryos deteriorated around E11-E12 (I,J), presumably because the αMHC promoter is no longer active throughout the heart tube at this stage. The myocytes lost their adhesiveness and were found dispersed throughout the heart region (J). An intermediate rescue phenotype was observed in mutants carrying the low-expressing βMHC/Ncad transgene at E9.5 (K,L). m, myocardium. Scale bar in A, 500 μm for A,C,E,G,I,K; in the inset in A, 200 μm for A,E,G insets; in B, 50 μm for B,D,F,H,J,L.

transgenes (αMHC/Ncad, αMHC/Ecad, βMHC/Ncad) exhibited normal heart development at this stage with no signs of cardial effusion normally observed in the N-cadherin-null embryos (Fig. 2). The ability of the low expressing βMHC/Ncad line to rescue the cardiac defect was variable, therefore only the αMHC/Ncad embryo is shown in comparison with the αMHC/Ecad embryo. Surprisingly, E-cadherin corrects the pericardial swelling, and restores normal cardiac looping morphogenesis in the mutants as well as N-cadherin does, even though E-cadherin normally is not expressed in muscle cells.

The transgenic rescue of the cardiovascular defect

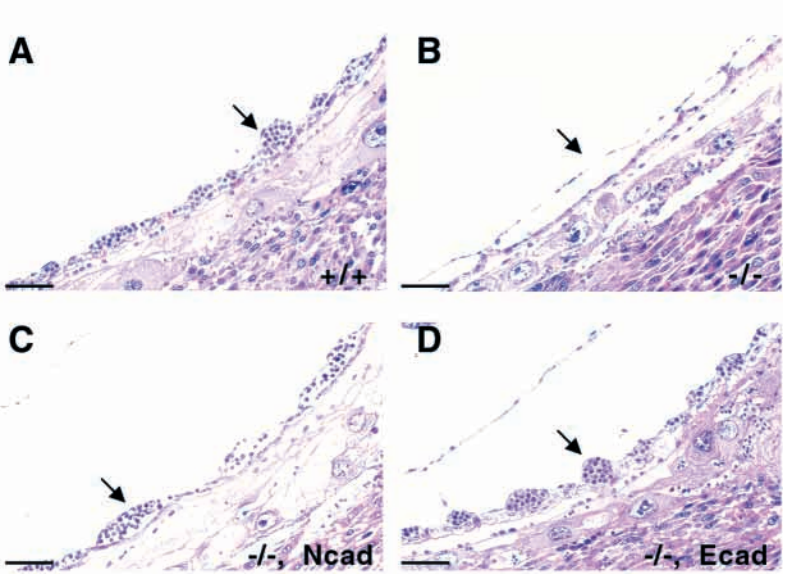


Fig. 4. Histological analysis of the yolk sac at E9.5. Transverse sections of wild-type (A), mutant (B) and rescued (C,D) embryos revealed normal yolk sac morphology (arrows) in the rescued embryos in the absence of transgene expression in the yolk sac, indicating the yolk-sac phenotype was secondary to the cardiovascular defect. Scale bars: 100 μm.

permitted the N-cadherin-null embryos to develop further compared with the mutant embryos without the transgene (Fig. 2). The anterior-posterior axis was further developed, as evidenced by an increased number of somites, branchial arches and the presence of a forelimb bud, which was never observed previously in the mutants. The most striking phenotype of the rescued embryos was the inability of the anterior neuropore to close and the subsequent collapse of the neural folds. Hence, the cardiac-specific rescued embryos reveal a requirement for N-cadherin during early brain development.

Histological analysis of E10.5 embryos indicated that the cell adhesion defect seen in the heart of N-cadherin-null embryos (Fig. 3D) was corrected in the transgenic mutant embryos. The morphology of the E-cadherin rescued myocardium appeared remarkably similar to that of the N-cadherin-expressing myocardium of both N-cadherin rescued and wild-type animals (Fig. 3B,F,H). The myocytes displayed normal fusiform morphology and trabeculation behavior, even when the tissue was maintained by an epithelial cadherin (Fig. 3H). N-cadherin-null embryos carrying the low-expressing βMHC/Ncad transgene displayed an intermediate cell-adhesion defect in the myocardium at E9.5 (Fig. 3L), and by E10.5 exhibited pericardial swelling to a similar extent as E9.5 N-cadherin-null embryos without the transgene (data not shown). Hence, low-level expression of N-cadherin was not sufficient to maintain myocyte adhesion under increased contractile stress. Consistent with the restricted expression of the transgene in the myocardium, the somites, and particularly the neural tube, continued to be malformed in the transgenic mutant embryos. Many dissociated cells were observed in the lumen of the malformed neural tube (Fig. 3G, inset). Chamber-specific downregulation of the αMHC promoter takes place in the ventricle at E10.5. Mutant embryos carrying the cadherin transgenes deteriorated around E11-E12, presumably due to the noticeable loss of cadherin-mediated adhesion in the myocardium (Fig. 3J).

Yolk-sac vascular defect in N-cadherin-null embryos is secondary to the cardiac defect

Before the placenta is functional, the embryo is critically dependent on the yolk sac for nutrition, hematopoiesis and maintenance of the vitelloembryonic blood circulation. Abnormal yolk-sac morphology is frequently associated with mutations affecting early cardiac development (i.e. the genes for Nkx2.5, Mef2c, Cx45). However, it is often difficult to determine if these phenotypes are due to a primary or secondary effect of the mutations. N-cadherin-null embryos always display a pale, nonvascularized yolk sac. In comparison, wild-type littermates have large vessels containing blood in the yolk sac. The pale appearance and paucity of vasculature in the mutant yolk sac was corrected by the cardiac-specific expression of a cadherin transgene (not shown). In the E9.5 N-cadherin-null embryo, the mesodermal and endodermal cell layers of the yolk sac were separated, showing fewer points of contact between the two cell layers compared with the wild-type yolk sac (Fig. 4B). In addition, there were far fewer primitive blood cells between the cell layers. In contrast, the wild-type and rescued N-cadherin-null yolk sacs looked remarkably similar, with many blood cells between the well-organized cell layers (Fig. 4A,C,D). To confirm that the transgene was cardiac specific and not expressed in the yolk sac, western blot analysis was performed on yolk sacs isolated from transgenic conceptuses. Neither exogenous N- or E-cadherin was expressed in the yolk sac, consistent with the cardiac-specific expression of the promoter (data not shown). Thus, transgenic expression of either N- or E-cadherin in the myocardium rescued the yolk sac defect associated with the N-cadherin-null phenotype. We conclude that the yolk sac vascular defect associated with the N-cadherin mutation is secondary to the cardiac defect.

Cadherin function is necessary to maintain the structural integrity of the outflow tract

The cardiovascular system of N-cadherin-null and rescued embryos was examined by injecting ink into the hearts of living E9.5 embryos. Regardless of the pressure applied during the injection, the ink remained in the heart of the N-cadherin-null embryos (n=9, Fig. 5B) and appeared to stop abruptly at the outflow tract. In contrast, the ink flowed freely out of the wild-type heart, thereby marking the vasculature including the aortic arch arteries, dorsal aorta and cranial vessels (Fig. 5A). The ink showed a similar distribution pattern in the N- and E-cadherin rescued embryos as wild type, indicating that restoration of cell adhesion in the ventricular myocardium and outflow tract was sufficient to restore normal integrity of the vasculature (Fig. 5C,D). Closer examination of heart morphology (Fig. 6) indicated that the structural integrity of the outflow tract was restored to normal in rescued embryos, consistent with the findings from the dye injection studies.

To further investigate the requirement for N-cadherin in heart development, we generated chimeric embryos derived from N-cadherin-null ES cells. Chimera analysis has proved to be a powerful method to complement gene knockout studies in mice. We observed that embryonic cells derived from N-cadherin-null ES cells exhibit dramatic cell-sorting behavior when intermixed with wild-type cells in chimeric embryos (I. K., R. M., R. K. and G. R., unpublished observations). The N-cadherin-null cells sort out from wild-type cells and form distinct aggregates in several tissues, including somites, neural tube and heart. Whole-mount β-galactosidase staining of two chimeric embryos derived from N-cadherin-null ES cells revealed very different phenotypes (Fig. 7A,B). Whereas both embryos exhibited high overall contribution of N-cadherin-null cells (i.e. *lacZ* positive), one embryo displayed an unique distribution of N-cadherin-null cells in the outflow tract. This particular embryo also exhibited the enlarged pericardial cavity seen in the constitutive N-cadherin-null embryos. In comparison, the other chimera had little contribution of N-cadherin-null cells to the outflow tract, and its heart appeared normal. Histological sections through the outflow tract indicated the absence of *lacZ*-positive cells in the morphologically normal heart, whereas the chimeric animal with the greatly distended pericardial cavity had many *lacZ*-positive cells obstructing the lumen of the outflow tract (Fig. 7C,D). The outflow tract was immunostained with the muscle-specific MF20 antibody, verifying its muscle identity. Owing to quenching of the peroxidase staining by the X-gal precipitate, it was difficult to visualize the *lacZ*-positive cells stained for MF20; however, as reported elsewhere, we have demonstrated that N-cadherin-null cells in the heart lumen are indeed MF20 positive (I. K., R. M., R. K. and G. R., unpublished observations). Together, these data indicate that the major defect in the heart of the N-cadherin-null embryos is the collapse of the outflow tract leading to severe cardial effusion and ballooning of the pericardial sac.

N-cadherin-mediated cell-cell adhesion is required for cell survival

Although the rescued mutant embryos developed further than the N-cadherin-null embryos, they were smaller and developmentally delayed compared with their wild-type littermates. Since N-cadherin is widely expressed throughout the embryo at this stage, we investigated whether loss of N-cadherin had other effects on the embryo. Two parameters that might explain the small size, developmental delay and eventual demise of the rescued embryos are changes in cell proliferation rate and cell death. We examined the cell proliferation rate of N-cadherin-null, rescued and wild-type embryos at E8.5 and E9.5, using BrdU labeling in utero. No significant overall difference in BrdU incorporation was observed among the different types of embryo (data not shown). To examine programmed cell death, or apoptosis, we performed whole-mount TUNEL analysis on E8.5 and E9.5 embryos followed by confocal microscopy. At E8.5, before the N-cadherin-null phenotype is apparent, there was already an increase in apoptosis in the neural folds of mutant embryos compared with wild-type littermates (Fig. 8A,B). This observation was confirmed by TEM, which showed a significant increase in apoptotic cells in the mutants compared with the wild-type littermates as characterized by condensation of heterochromatin and progressive degeneration of residual nuclear and cytoplasmic structures (Fig. 9). At E9.5, we also found an increase in apoptosis in the collapsed neural folds and somites of mutants carrying the transgene compared with wild-type embryos (Fig. 8C-F). The cell survival effect appeared tissue specific, since no significant increase in apoptosis was observed in the N-cadherin-null myocardium compared with wild type (Fig. 8G,H). The absence of TUNEL-positive cells in the early heart tube has been reported previously for wild-type embryos (Zhao and Rivkees, 2000). The increase in cell death in noncardiac tissues of the transgenic mutant embryos may explain, at least partly, the smaller size, developmental delay, and eventual death of the transgenic mutant embryos compared with their wild-type littermates.

DISCUSSION

N-cadherin-deficient embryos exhibit several abnormalities including a severe cardiovascular defect, the apparent cause of their early death. It is often difficult to ascertain which phenotypes are primary or secondary to a mutation when the gene is normally widely expressed in the early embryo, as is the case for N-cadherin. By restoring cadherin-mediated adhesion in the myocardium, partially rescued embryos were able to develop further than the N-cadherin-null embryos, allowing closer examination of the role of N-cadherin in early development. In this report, we make the following points: (1) cardiac-specific expression of either N- or E-cadherin is sufficient to rescue the myocardial defect; (2) brain development is severely impaired in the rescued embryos; (3) yolk-sac phenotype is secondary to the cardiovascular defect; (4) outflow tract formation is dependent on cadherin-mediated adhesion; and (5) N-cadherin-mediated cell-cell adhesion is required for cell survival.

Several studies have shown that ectopic expression of cadherins can provide specific information to cells that affect their differentiation and behavior. For example, E-cadherin-deficient ES cells constitutively expressing N-cadherin preferentially differentiate into neuroepithelium and cartilage, whereas the same cells expressing E-cadherin form epithelia almost exclusively (Larue et al., 1996). Furthermore, ectopic expression of E-cadherin in retinal pigment epithelial (RPE) cell lines affects the distribution of polarized proteins such as Na^+/K^+-ATPase and the expression of cytoskeletal proteins (Marrs et al., 1995). In addition, ectopic expression of N-cadherin in mammary epithelial tumor cell lines leads to increased motility and migration (Hazan et al., 2000; Nieman, et al., 1999). In contrast to the above studies, N- and E-cadherin can also substitute for one another under certain conditions, suggesting that cadherin-specific signals are not always dominant over cellular phenotype. In a study by Redfield et al., aggregation dependent muscle differentiation in the BHK cell line is induced by either N- or E-cadherin-mediated adhesion (Redfield et al., 1997). Taken together, these studies show that cadherin function is dependent on cellular context.

Epithelial cells are normally cuboidal with distinct polarity. In comparison, muscle cells are fusiform with no apparent structural polarity. The tissue-specific expression of cadherin subtypes is thought to regulate morphology, behavior and function of different cell types. We show that the epithelial cadherin, E-cadherin, can functionally substitute for N-cadherin during early cardiac morphogenesis, demonstrating that these two classical cadherins are interchangeable as cell adhesion molecules in myocytes, at least at the particular stage of myocardial development studied here. The E-cadherin-expressing myocytes exhibit normal myocyte behavior, including the ability to migrate toward the endocardium and form trabeculae, a process that normally involves N-cadherin function (Ong et al., 1998). This result was surprising given the distinct tissue specificity of these different cadherin subtypes. E-cadherin is the predominant classical cadherin of all embryonic and adult epithelia, whereas N-cadherin is more restricted to specific cell types, including muscle and neurons. Mouse N- and E-cadherin show 49% amino acid similarity overall, with the highest degree of conservation in the cytoplasmic domain (62%). However, there is precedence for E-cadherin performing a cell adhesion function in a contractile structure. The expression pattern of DE- and DN-cadherin in *Drosophila* is very similar to that of vertebrates, including the E- to N-cadherin switch during gastrulation; however, there is one exception to this conserved expression pattern (Iwai et al., 1997; Tepass et al., 1996). In the *Drosophila* heart, DE-cadherin is expressed in cardial cells instead of DN-cadherin. Hence, early in the evolution of the simple heart tube, DE-cadherin performed the cell adhesion function in the heart instead of DN-cadherin.

The tissue-specific rescue of the myocardial defect allowed investigation into the function of N-cadherin in other tissues not rescued by transgene expression. The rescued embryo developed further than the mutant without the transgene, demonstrating that the cardiovascular defect was the primary cause of the early lethality. A partial correction of the cell adhesion defect was observed in the myocardium of mutant embryos that carry the low expressing βMHC/Ncad transgene, demonstrating a dosage effect of cell adhesive activity. The most striking phenotype of the rescued embryo was the lack of brain development, indicating a requirement for N-cadherin in

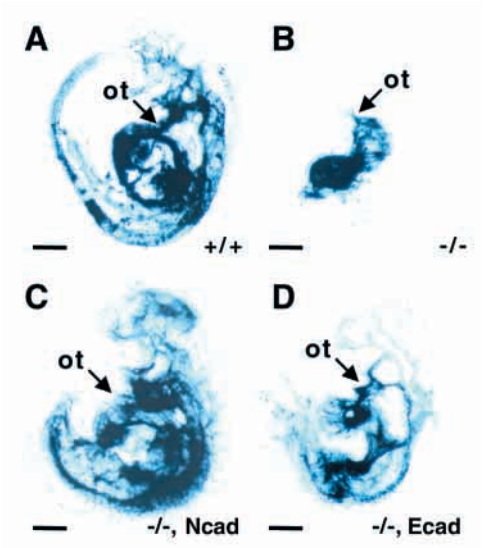


Fig. 5. Examination of the cardiovascular system by dye injection. Lateral views of whole-mount E9.5 embryos injected with India ink into the right ventricle. The overall distribution of the ink was similar in wild-type (A) and rescued embryos (C,D). In contrast, the ink remained in the heart of the N-cadherin-null embryo (B) and stopped abruptly at the outflow tract (ot). Scale bars: 250 μm.

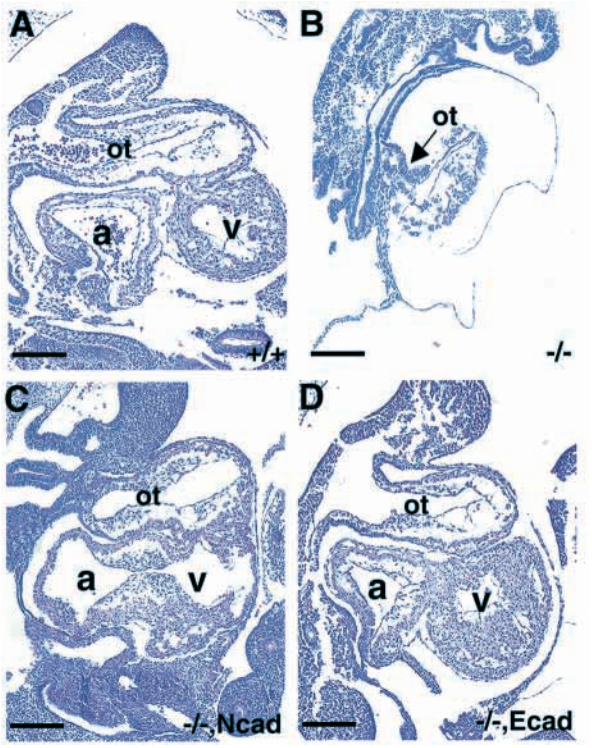
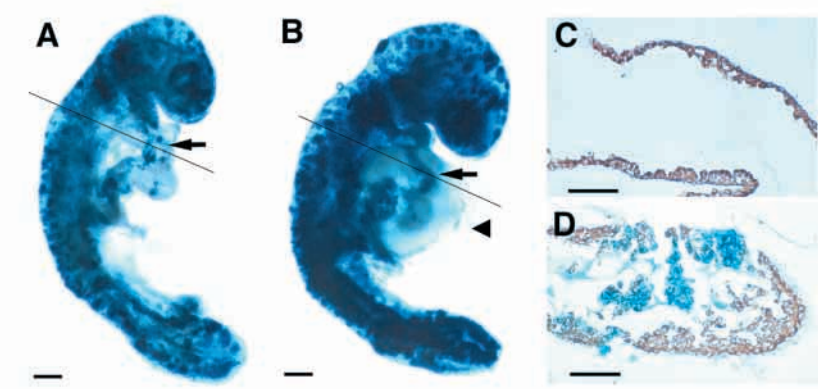


Fig. 6. Histological analysis of heart formation. Sagittal sections through the heart of wild-type (A), rescued (C,D) E10.5 embryos and E9.5 mutant embryo (B). The outflow tract (ot) appears collapsed in the N-cadherin-null embryo (B), in contrast, normal cardiac morphology is restored in the rescued embryos (C,D) compared with wild-type (A). Note that the morphology of the E-cadherin rescued heart (C) looks remarkably similar to wild type (A). a, atrium; v, ventricle. Scale bars: 150 μm.

this process. It was not possible to observe this phenotype in the constitutive N-cadherin-null embryo as embryonic development arrested around the neural fold stage, owing to the severe cardiovascular defect. Several mutations affecting early cardiac development (i.e. of the genes for Nkx2.5, Mef2c, Cx45) have abnormal yolk sac morphology (Bi et al., 1999; Kruger et al., 2000; Lin et al., 1998; Tanaka et al., 1999); however, it has been unclear whether the yolk-sac defect was secondary to the cardiovascular defect. The yolk-sac phenotype observed in the N-cadherin-null embryo was corrected in the rescued embryo even though the yolk sac did not express the transgene, indicating that the phenotype is secondary to the myocardial defect. We conclude that the yolk-sac defect in the N-cadherin-null embryo is caused by abnormal hemodynamics triggered by loss of cadherin-mediated adhesion in the myocardium.

Owing to the severity of the cardiovascular defect in the N-cadherin-null embryos it was difficult to determine which region of the heart was most affected by loss of N-cadherin, since it is expressed throughout the myocardium. For example, is a particular region of the N-cadherin-null heart responsible for the severe pericardial swelling? Dye injection experiments showed that αMHC driven expression of either N- or E-cadherin in the myocardium was sufficient to restore normal cardiovascular integrity. Furthermore, the normal appearing dye distribution pattern in the rescued N-cadherin-null vasculature is consistent with the major role of VE-cadherin in

Fig. 7. Whole-mount and histological analysis of X-gal-stained chimeric embryos. Embryos derived from blastocysts injected with N-cadherin-null ES cells were stained for *lacZ* expression (β-galactosidase) to discriminate ES cell-derived cells (blue) from host cells. The overall contribution of N-cadherin-null cells was high in both embryos. However, the embryo (B) with specific contribution to the outflow tract (arrow) exhibited pericardial swelling (arrowhead) similar to the N-cadherin-null embryos, whereas the embryo (A) with lesser contribution to the outflow tract appeared normal. Histological sections of the two chimeras showed a large number of *lacZ*-positive cells in the outflow tract of the abnormal heart (D), compared with the unobstructed outflow tract in the heart of the apparently normal chimeric animal (C). Scale bars: 250 μm in A,B; 50 μm in C,D.



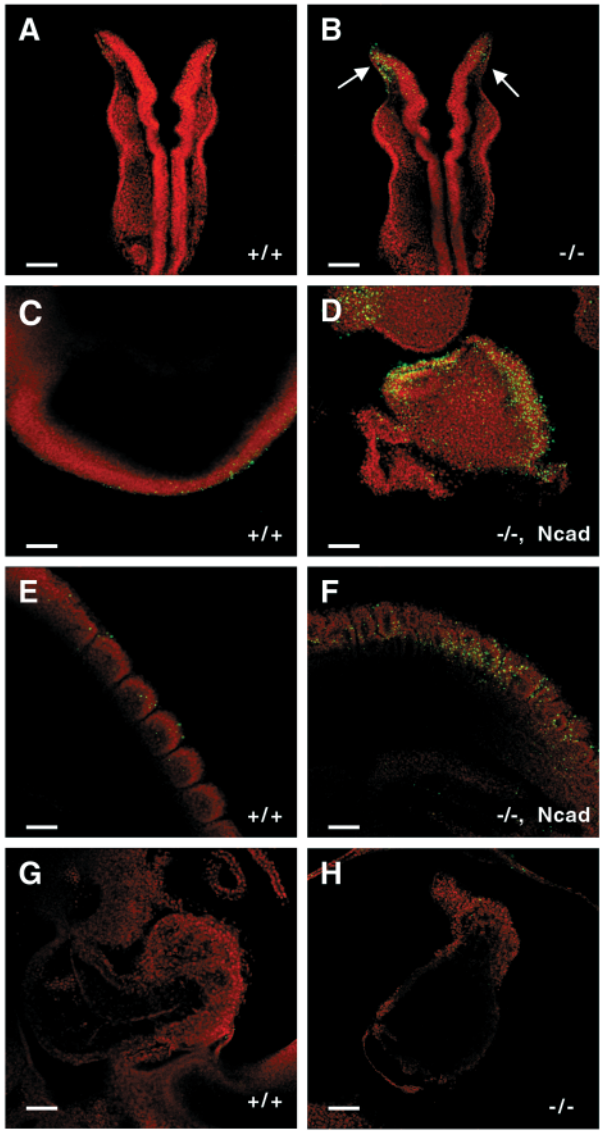


Fig. 8. Whole-mount TUNEL analysis of N-cadherin-null, cardiac-specific rescued and wild-type embryos. Embryos were examined by confocal microscopy at E8.5 (A,B) and E9.5 (C-F). N-cadherin-null embryos showed increased cell death in the anterior neural folds (arrows in B) compared with wild-type embryos at E8.5, before the cardiovascular phenotype is observed (A,B). A dramatic increase in cell death was also apparent in the neural folds (C,D) and somites (E,F) of the rescued embryo at E9.5. In contrast, no increase in apoptosis was observed in the mutant heart (G,H). Scale bars: 100 μm.

endothelial cell adhesion compared with N-cadherin (Carmeliet et al., 1999; Gory-Faure et al., 1999). In contrast, the dye never left the mutant heart and appeared to stop abruptly at the outflow tract, consistent with a narrow or collapsed outflow tract in the N-cadherin mutant embryos. In the constitutive N-cadherin-null embryos it was difficult to determine which region of the heart tube was responsible for the pericardial swelling, as the entire structure was missing N-cadherin. The analysis of a chimeric embryo with a unique contribution of N-cadherin-null cells to the outflow tract provided evidence that obstruction of the outflow tract was the primary cause of the pericardial swelling. To further refine the

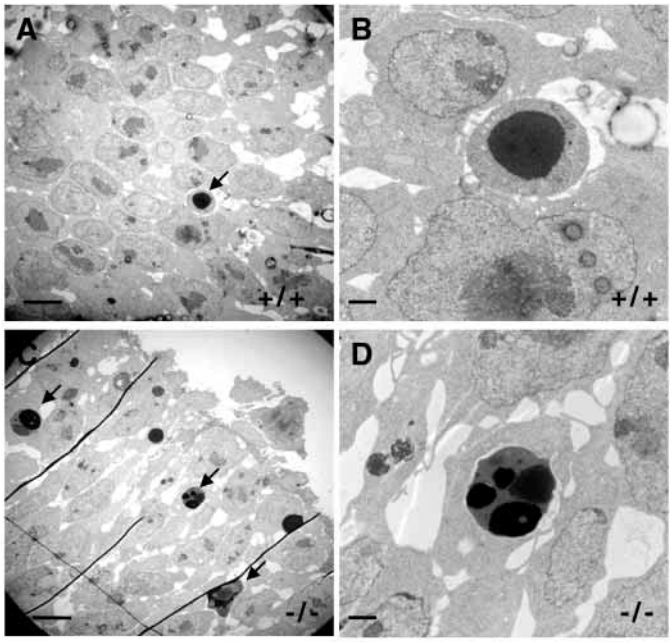


Fig. 9. Transmission electron microscopy of neuroepithelium of E8.5 embryos. Wild-type embryo (A,B) exhibited a low level of apoptotic cells (arrow) compared with the dramatic increase observed in the N-cadherin-null embryo (C,D). There appears to be more intercellular space between the neuroepithelial cells in the N-cadherin-null embryo compared with wild type. Scale bars: 10 μm in A,C; 2 μm in B,D.

role of N-cadherin in heart development a floxed N-cadherin allele in conjunction with a regional specific cardiac Cre transgene will be necessary.

Cadherin-mediated adhesion has been implicated in programmed cell death or apoptosis in various cell types. A dominant negative cadherin increased cell death in E-cadherin-positive enterocytes in the intestinal crypt of chimeric mice (Hermiston and Gordon, 1995). The mechanism by which cadherins mediate the cell survival effect is best described for VE-cadherin in endothelial cells. The growth factor VEGFA (vascular endothelial growth factor A) promotes endothelial cell survival via binding to VEGFR2 (KDR – Mouse Genome Informatics), thereby activating PI3-kinase and Akt (Nunez and del Peso, 1998). It was recently shown that VE-cadherin is required for VEGFA-mediated survival by forming a complex of VE-cadherin/β-catenin/PI3-kinase/VEGFR2 (Carmeliet et al., 1999). In contrast, the role of N-cadherin in cell survival is less well understood. Evidence from in vitro studies demonstrate that N-cadherin function is important for granulosa cell survival and this effect may be mediated through the FGF receptor (Makrigiannakis et al., 1999; Peluso et al., 1996). The growth factor bFGF promotes granulosa cell survival and decreased tyrosine-phosphorylation of the FGF receptor has been observed after N-cadherin-mediated adhesion was disrupted with anti-N-cadherin antibodies (Trolice et al., 1997). The increased apoptosis observed in the N-cadherin-null embryo is not caused indirectly by the cardiovascular defect (i.e. hypoxia induced), as the phenotype is already evident at E8.5, before the heart defect is apparent. Consistent with this finding, we observed a dramatic increase in apoptosis a day later in the neuroepithelium and somites of

rescued E9.5 embryos with normal cardiovascular function. Interestingly, no increase in apoptosis was observed in the mutant myocardium, suggesting an alternative pathway to N-cadherin-mediated cell survival in myocytes. Our results provide the first genetic evidence for N-cadherin-mediated cell survival in vivo.

In summary, the cardiac-specific rescued embryos allowed us to examine the role of N-cadherin in several developmental processes that are impossible to evaluate in the constitutive N-cadherin-null embryos, owing to the severe cardiovascular defect. The ability of E-cadherin to support early cardiac morphogenesis in the N-cadherin-null embryo provides the first example of functional replacement of a cadherin subtype during organogenesis. The rescue experiments allowed us to distinguish between primary and secondary effects caused by the mutation by restoring cardiovascular integrity with the transgene. However, the restricted expression pattern of the αMHC and βMHC promoters after cardiac chamber specification does not allow N-cadherin-mediated adhesion to continue throughout the myocardium; presumably the reason why the embryos eventually die around E11-E12. To extend the cardiac-specific rescue will require a cardiac promoter with more homogenous expression in the four-chamber heart. Alternatively, knowledge of the tissue-specific elements that regulate the endogenous N-cadherin gene may prove useful for such experiments. Examination of the chicken N-cadherin promoter has identified regulatory sequences necessary for neuronal-specific expression in transgenic mice; however, no expression was observed in the heart (Li et al., 1997). Further analysis of the N-cadherin promoter will be required to identify the regulatory elements required for cardiac expression. In future studies, the N-cadherin promoter may prove useful for further examining the ability of E-cadherin to functionally substitute for N-cadherin in other tissues, including brain and muscle. It would be interesting to determine if E-cadherin can support intercalated disc formation; however, the rescued embryos die before this stage of development. Our studies demonstrate that cardiac-specific rescue can serve as a complementary approach to conditional knockout technology by overcoming early cardiovascular defects, allowing gene function to be examined more carefully in noncardiac tissues.

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