N-cadherin is essential for retinoic acid-mediated cardiomyogenic differentiation in mouse embryonic stem cells

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Contraction forces developed by cardiomyocytes are transmitted across the plasma membrane through end-to-end connections between the myocytes, called intercalated disks, which enable the coordinated contraction of heart muscle. A component of the intercalated disk, the adherens junction, consists of the cell adhesion molecule, N-cadherin. Embryos lacking N-cadherin die at mid-gestation from cardiovascular abnormalities. We have evaluated the role of N-cadherin in cardiomyogenesis using *N-cadherin*-null mouse embryonic stem (ES) cells grown as embryoid bodies (EBs) *in vitro*. Myofibrillogenesis, the spatial orientation of myofibers, and intercellular contacts including desmosomes were normal in *N-cadherin*-null ES cell-derived cardiomyocytes. The effect of retinoic acid (RA), a stage and dose-dependent cardiogenic factor, was assessed in differentiating ES cells. all-trans (at) RA increased the number of ES cell-derived cardiomyocytes by ≈3-fold (at 3×10^{-9} M) in wt EBs. However, this effect was lost in *N-cadherin*-null EBs. In the presence of supplemented *at*-RA, the emergence of spontaneously beating cardiomyocytes appeared to be delayed and slightly less efficient in *N-cadherin*-null compared with wt and heterozygous EBs (frequencies of EBs with beating activity at 5 days: 54±18% vs. 96±0.5%, and 93±7%, respectively; peak frequencies of EBs with beating activity: 83±8% vs. 96±0.5% and 100%, respectively). In conclusion, cardiomyoyctes differentiating from *N-cadherin*-null ES cells *in vitro* show normal myofibrillogenesis and intercellular contacts, but impaired responses to early cardiogenic effects mediated by *at*-RA. These results suggest that N-cadherin may be essential for RA-induced cardiomyogenesis in mouse ES cells *in vitro*.

Key words: N-cadherin, heart, myocyte, retinoic acid, embryonic stem cell, differentiation, mouse.

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Paper accepted on July 2, 2007

European Journal of Histochemistry
2007; vol. 51 issue 3 (July-September):181-192

The coordinated interaction of neighboring cardiomyoyctes is required for normal cardiac development and function. End-to-end connections between cardiomyocytes, called intercalated disks, consist of multiple adhesive complexes including adherence junctions, desmosomes and gap junctions, which mediate proper cell-cell electromechanical coupling. Initial assembly of nascent myofibrils involves interaction with actin microfilaments at the plasma membrane (Lin *et al.*, 1989). Adherence junctions anchor actin microfilaments to the membrane at intercellular contact points. These junctional complexes contain cell adhesion molecules of the cadherin family. Classical cadherins are single-pass transmembrane glycoproteins that mediate calcium-dependent homophilic cell-cell adhesion (Takeichi, 1995). The adhesive activity is regulated by the cytoplasmic partners of cadherins, the catenins (Linask *et al.*, 1997; Lilien *et al.*, 2002), which link the cadherins to the actin cytoskeleton. Members of the cadherin family have distinct spatial and temporal patterns of expression during embryonic development as well as in adulthood (Takeichi, 1988). While most cell types express multiple members of the cadherin family, cardiomyocytes depend on one classical cadherin, N-cadherin (cadherin2). In cardiac muscle, N-cadherin is mainly located at the fascia adherens of intercalated disks and at close lateral contacts between neighboring cardiomyoyctes (Volk and Geiger, 1984). In addition, N-cadherin is mainly located in extrajunctional sites called costameres, where it co-localizes with α-actinin in the peripheral Z-disks of the sarcomeres (Goncharova *et al.*, 1992).

In the developing heart, N-cadherin is the only classical cadherin expressed in the myocardium, VE-cadherin being expressed in the endocardium. Previous studies have suggested possible roles for N-cadherin in several aspects of heart development including sorting out of the precardiac mesoderm (Lilien *et al.*, 2002), establishment of left-right

asymmetry (Garcia-Castro *et al.*, 2000), cardiac looping morphogenesis (Luo *et al.*, 2001), and myocardial trabeculation (Ong *et al.*, 1998). Several methodological approaches have been taken to assess the role of N-cadherin in heart development: antibodies that block N-cadherin function, truncated N-cadherin constructs acting as dominant-negative constructs, genetically modified mouse models and, most recently, zebrafisch mutants. N-cadherin-blocking antibodies caused abnormal myofibrillogenesis and decreased contractile activity in cultured cardiomyocytes isolated from chick embryos (Goncharova *et al.*, 1992). Essentially similar results were obtained using a dominant-negative N-cadherin construct microinjected into adult rat cardiomyocytes (Hertig *et al.*, 1996).

Germline deletion of the *N-cadherin* gene in mice results in embryonic lethality at approximately midgestation (Radice *et al.*, 1997). The lethal phenotype is characterized by multiple developmental abnormalities including defects in the yolk sac and blood vessel formation, neural tube malformation, irregularly shaped somites and a severe cardiovascular defect. In *N-cadherin*-null embryos, the primitive heart consists of loosely associated, round cardiomyocytes that surround the endocardium. Myocardial trabeculae are not properly formed. In chimeric mice derived from *N-cadherin*-null embryonic stem (ES) cells, *N-cadherin*-null cardiomyocytes do not participate in the formation of the myocardial wall in the developing heart (Kostetskii *et al.*, 2001), consisting with a functional defect of these cells. Cardiac-specific expression of either N-cadherin or E-cadherin partially rescues *N-cadherin*-null embryos indicating that the developmental cardiac defect is the cause of death in these embryos (Luo *et al.*, 2001). *N-cadherin*-null cardiomyocytes isolated at embryonic day 9 (E9) initially form small and loose cell aggregates in culture; however, they subsequently disassociate from one another and round up (Luo *et al.*, 2003). Cardiomyocytes isolated from *N-cadherin*-null embryos showed normal myofibrillogenesis but perturbed spatial orientation of the myofibrils (Luo *et al.*, 2003).

Retinoic acid (RA), the active form of vitamin A, is an important physiological modulator of cell differentiation. RA promotes differentiation of mouse ES cells and embryonic carcinoma (EC) cells into multiple cell lineages *in vitro*, in a concentration and developmental stage-dependent manner (Rohwedel *et al.*, 1999). In the first two days of embryoid body (EB) formation from ES cells (Strubing *et al.*, 1995) or EC cells (Edwards and McBurney, 1983), 10^{-8} M to 10^{-7} M RA promotes neurogenesis. After day 5, both 10^{-8} M and 10^{-9} M RA, both in the all-*trans* and in the 9-cis (9*c*-RA) configuration, resulted in a significant acceleration of cardiomyocyte formation in murine ES cells cultured in the presence of serum (Wobus *et al.*, 1997). Both *at*-RA and 9c-RA induced increased levels of α-cardiac myosin heavy chain (MyHC) and myosin light chain (MLC)-2v mRNA expression in EBs in early, but not in terminal developmental stages. They also increased the number of cardiomyocytes with Purkinje- and ventricle-like properties, while reducing that of pacemaker- and atrium-like cardiomyocytes. Exogenous 10^{-7} M RA preferentially induced atrial cardiomyocyte differentiation of murine EBs in another study (Hidaka *et al.*, 2003). In H9c2 myoblasts, RA promoted cardiac rather than skeletal muscle formation (Menard *et al.*, 1999).

Embryonic synthesis of RA is essential for cardiac morphogenesis in the mouse. Suppression of endogenous RA synthesis by disruption of the *raldh2* gene that encodes the first retinaldheyde dehydrogenase, which is expressed during early mouse post-implantation development, causes complex cardiac developmental abnormalities leading to death *in utero* at E10.5 (Niederreither *et al.*, 2001). In *raldh2* mutants, the developing ventricular myocardium consists of a thick layer of loosely attached cells, instead of trabeculae. In avian embryos, RA-deficiency causes a severe decrease in the expression of the GATA-4 transcription factor, a key cardiogenic factor (Watt *et al.*, 2004), in heart-forming regions (Kostetskii *et al.*, 1999). In RA-deficient avian embryos, heart-forming regions that show marked *GATA-4* gene downregulation display prominent morphological defects at later stages (Kostetskii *et al.*, 1999).

We have used *N-cadherin*-null mouse ES cells grown as EBs to study the role of N-cadherin in early cardiomyogenesis. This method allows to assess earlier stages of cardiac differentiation compared with approaches based on cultured cardiomyocytes isolated from *N-cadherin*-null embryos (Luo *et al.*, 2003) or treated with N-cadherin-blocking antibodies (Goncharova *et al.*, 1992). Using the latter approaches, cardiomyoyctes are formed and

cell-cell contacts between neighboring myoyctes are established *in vivo*. Isolation of myoyctes requires disruption of cell-cell contacts, which are subsequently re-established in culture. In addition, another advantage of our model is that early stages of cardiomyogenesis, including the initial formation of cell-cell contacts, can be studied under well-defined *in vitro* conditions, e.g., with respect to RA concentrations.

The aim of the present study was to compare cardiac myofibrillogenesis, as well as early cardiogenic effects of RA, in differentiating mouse ES cells in the presence or absence of N-cadherin.

Materials and Methods

Culture of ES cells

N-cadherin-null and heterozygous ES cell lines were generated by Dr. Glenn L. Radice (Philadelphia, USA), as described (Radice *et al.*, 1997). wt ES cells were obtained from the R1 ES cell line by Dr. A. Nagy (Toronto, Canada), as described (Nagy *et al.*, 1993). All ES cell lines were cultured in ES medium consisting of knock-out DMEM (82% V/V), 100x L-glutamine (1%), 100x non-essential amino acids (1%), 100x sodium pyruvate (1%), knock-out serum replacement (15%; all from Gibco), and 1 mmol/L β-mercaptoethanol (Sigma), essentially as described (Smith, 1992), with addition of 0.4% (V/V) medium conditioned by COS cells overexpressing leukaemia inhibiting factor (LIF).

In vitro ES cell differentiation

An ES cell suspension of 16,000 cells/mL in differentiation medium consisting of knock-out DMEM (82%), 100x L-glutamine (1%), 100x non-essential amino acids (1%), 100x sodium pyruvate (1%), fetal calf serum (15%), and 1 mmol/L β-mercaptoethanol was made, and drops of 30 μL were pipetted onto the inner side of 150-mm bacteriological Petri dish lids. Hanging drops were incubated at 37°C, 5% CO_2 for 2 days. During this time, cells collected at the bottom of the drops and aggregated, forming EBs. Hanging drops were then collected in 15 mL of differentiation medium and cultured in suspension in bacteriological Petri dishes for 2 days. They were then plated onto 100-mm tissue culture dishes coated with 0.1% gelatin (Fluka), at a density of 50 EBs per dish. In separate experiments, EBs were plated onto 35-mm tissue culture dishes at a density of 10 EBs per dish in differentiation medium supplemented with varying concentrations of *at*-RA (Sigma), or individually onto 48-well plates. Day 0 refers to the time point of plating of EBs onto gelatin-coated dishes.

Immunohistochemistry

Cells were washed with PBS, fixed with 4% paraformaldehyde (PFA) in PBS for 45 min, washed with PBS for 5 min three times, and permeabilized for 30 min in 0.2% Triton X-100 in PBS. Then, cells were blocked with 5% normal goat serum (NGS), 1% bovine serum albumin (BSA) in PBS for 30 min, and incubated with primary antibody mixtures diluted in buffer solution (155 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, 20 mM Tris-base, 1% BSA, pH 7.6) at 4°C overnight in a humid chamber. After washing with PBS containing 0.002% Triton X-100, cells were incubated with secondary antibody in the same buffer solution for 3 h at room temperature. After thorough washing in PBS containing 0.002% Triton X-100, the specimens were mounted in 70% glycerol/0.1 M Tris-HCl (pH 9.5) containing 50 mg/mL n-propyl gallate as an anti-fading agent, and sealed with nail polish. Primary antibodies were used at the following dilutions: mouse anti-myomesin mAb (mM anti-myomesin clone B4; Luo *et al.*, 2001), 1:100; mouse anti-sarcomeric α-actinin mAb (EA-53, Sigma), 1:500; rabbit anti-pan-cadherin Ab (Sigma), 1:200; rabbit anti-desmoplakin Ab (gift of Dr. Alison North, University of Manchester, Manchester, UK); 1:200; rabbit anti-mouse embryonic heart (EH) fragment of myomesin Ab (Agarkova *et al.*, 2000), 1:1000; mouse anti-N-cadherin mAb (clone 3B9, Zymed Laboratories), 1:100. Cy3-conjugated anti–mouse and anti–rabbit Igs (Jackson Laboratories) and FITC-conjugated anti–mouse and anti–rabbit Igs (Cappel) secondary antibodies were used at a 1:200-dilution. Controls for antibody specificity included omitting primary antibodies, replacing primary antibodies with isotype-matched control antibodies, and staining with each of the primary antibodies followed by the reciprocal secondary antibody. DAPI (Molecular Probes) was used at dilution 1:100 to visualize nuclei.

Microscopy

Specimens were analyzed by fluorescence microscopy. Images were acquired with a

Hamamatsu color chilled 3CCD camera, and processed with Adobe Photoshop 7.0 for Apple Macintosh.

Confocal microscopy

Specimen were analyzed by confocal microscopy on an inverted microscope "DM IRB/E" (Leica) equipped with a true confocal scanner TCS NT, a PL APO 63x/1.32 oil or a PL APO 40x/1.25 oil immersion objectives (Leica) and a He-Ne mixed gas laser. Images were processed by using a Silicon Graphics workstation with Imaris® software (Bitplane AG, Switzerland).

Temporal analysis of the appearance of spontaneously beating cardiomyocytes in EBs

To assess the time course of the emergence of spontaneously beating cardiomyocytes in *N-cadherin*-null, heterozygous and wt ES cells, EBs were plated individually in 48-well plates in the presence or absence of supplemented *at*-RA (96 wells per cell type and experimental condition), during the period from day 0-19 of differentiation. This experiment was performed twice. For each group, the number of wells with spontaneously beating cardiomyocytes was counted daily under an inverted light microscope and expressed as percentage of the total number of wells.

FACS analysis

FACS analysis was performed on EBs plated onto 35-mm tissue culture dishes at a density of 10 EBs per dish in differentiation medium supplemented with varying concentrations of *at*-RA. At 13 days of differentiation, cells were trypsinized, resuspended in PBS and fixed in 4% PFA in PBS for 15 min at 4 C°. Subsequent procedures were carried out on ice. Fixed cells were washed 2X (PBS/10%FCS), incubated with 400-fold diluted anti-mouse IgG sarcomeric α-actinin antibody (EA-53, Sigma) in PBS/10% FCS containing 0.2% Triton X-100 for 30 min. They were then washed 3X (PBS/10% FCS), incubated with FITC-conjugated goat anti-mouse IgG at a 800-fold dilution for 30 min, and washed 5x (PBS/10%FCS). Cells were analyzed on a FACSCalibur® system (Becton Dickinson) using the CellQuest™ software.

Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed on cells derived from wt, heterozygous, and *N-cadherin*-null ES cells at days 2 and 8 of differentiation. Total RNA was isolated with TRIzol Reagent (Invitrogen). For PCR, cDNA was denatured for 1 min at 95°C. PCR conditions were 95° for 30 sec, 66° for 40 sec, and 72° for 30 sec for the indicated numbers of cycles, which were within the range of linearity of the amplification. Equal cDNA inputs were used for each condition. PCR reaction samples (20 μL/tube) contained 0.1 μL each of sense and antisense primers (100 μM), 17.8 μL PCR mix solution (formulation: 40 μL dNTP, 40 μL Taq DNA polymerase, 5U/μL [Invitrogen], 200 μL 10x polymerase buffer, H_2O ad 2000 μL) and 2 μL cDNA. Primer pairs for marker amplification, designed with the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3), were as follows (GenBank accession numbers and numbers of amplification cycles are given in brackets): αMyHC: (NM_010856.2), 5′-CTG CTG GAG AGG TTA TTC CTC G-3′ and 5′-GGA AGA GTG AGC GGC GCA TCA AGG-3′ (24 cycles); MLC2v (NM_010861.2), 5′-TGT GGG TCA CCT GAG GCT GTG GTT CAG-3′ and 5′-GAA GGC TGA CTA TGT CCG GGA GAT GC-3′ (30 cycles); GATA-4 (NM_008092.2), 5′-CGA GAT GGG ACG GGA CAC-3′ and 5′-TTG ATG CCG TTC ATC TTG TGA-3′ (32 cycles); MyoD (NM_010866.1), 5′-GCT CTG ATG GCA TGA TGG ATT ACA GCG-3′ and 5-‘ATG CTG GAC AGG CAG TCG AGG C-3′ (32 cycles). The efficacy of reverse transcription across samples was verified by amplification of the β-tubulin sequence (NM_011655.2) using the following primers: 5′-TCA CTG TGC CTG AAC TTA CC-3′ and 5′-GGA ACA TAG CCG TAA ACT GC-3′ (24 cycles). One third of each PCR reaction was electrophoretically separated on 2% agarose gels containing 0.2 μg/mL of ethidium bromide, and gels were illuminated with UV light. Ethidium fluorescence signals were evaluated visually.

Results

Normal myofibrillogenesis in the absence of N-cadherin

N-cadherin-null, heterozygous, and wt mouse ES cells cultured in suspension as hanging drops formed EBs, which were then plated on gelatin-coated dishes. At 8 and 13 days of differentiation, EBs were immunostained for cell-cell contact proteins including cadherins, as well as for sarcomeric proteins, and analyzed by confocal microscopy. At 8

days, myofibrils, as evidenced by myomesin staining, were morphologically identical in wt and *N-cadherin*-null myocytes, including their spatial orientation (Figure 1A and D, respectively). In wt cells, staining with pan-cadherin antibodies recognizing multiple members of the cadherin family was comparable to staining with an N-cadherin-specific antibody (Figure 1B and 1H, respectively). Two morphologically distinct cell-cell contacts stained positive for N-cadherin: elongated structures running nearly parallel to the myofibrils, and shorter stretches perpendicular to them, corresponding to intercalated disc-like structures (Figure 1C/I; asterisks in inserts). Both pan-cadherin and N-cadherin stainings were negative in *N-cadherin*-null myocytes (Figure 1E and 1K, respectively), indicating lack of compensatory expression of other cadherin family members. At 13 days of differentiation, staining for desmoplakin, a component of desmosomes (a distinct cell-cell junction) was normal in *N-cadherin*-null myocytes (Figure 2E), suggesting preserved desmosome integrity and lack of compensatory desmoplakin upregulation in the absence of N-cadherin. Staining for sarcomeric α-actinin showed a normal density and a normal spatial organization of myofibrils in *N-cadherin*-null myocytes (Figure 3A/D). These stained positive for EH-myomesin (Figure 3C/F), indicating cardiac differentiation. EH-myomesin staining was antiperiodic with sarcomeric α-actinin, indicating correct assembly of Z-disks and M-bands in *N-cadherin*-null myocytes (Figure 3F).

Delayed appearance of beating N-cadherin-null cardiomyocytes during at-RA treatment

In separate experiments, EBs were plated individually onto 48-well plates in the presence or absence of supplemented *at*-RA during the period from day 1-19 of ES cell differentiation. Wells with spontaneously beating cardiomyocytes were counted daily for each cell group. In the absence of supplemented *at*-RA (Figure 4A), numbers of *N-cadherin*-null EBs with beating cardiomyocytes appeared to be slightly lower than those of wt and heterozygous EBs at corresponding time points (frequencies of EBs with beating cardiomyocytes at 5 days: 75± 20% *N-cadherin*-null EBs vs. 92±4% and 99± 1% wt and heterozygous EBs, respectively). Peak frequencies of wt and heterozygous EBs with spontaneously beating cardiomyocytes (97±1% and 99±1%, respectively) were reached by day 5-6, whereas peak frequencies of *N-cadherin*-null EBs with beating activity (90±2%) were reached around day 9. During *at*-RA treatment (Figure 4B), differences in the time course of emergence of spontaneous beating activity in *N-cadherin*-null and wt EBs tended to be more pronounced than in the absence of supplemented *at*-RA (frequencies of EBs with beating cardiomyocytes at 5 days: 54± 18% *N-cadherin*-null vs. 92±4% and 99±1% wt and heterozygous EBs, respectively). Peak frequencies of EBs with spontaneously beating cardiomyocytes in *N-cadherin*-null EBs (83±8%) were reached around day 9, whereas virtually all wt and heterozygous EBs (96±0.5% and 100%, respectively) contained beating cardiomyocytes at day 5-6.

at-RA increases numbers of wt but not N-cadherin-null ES cell-derived cardiomyocytes

The effect of *at*-RA on cardiac differentiation was assessed at the cellular level by FACS analysis of sarcomeric α-actinin expression in ES cells plated on gelatin-coated dishes at 13 days (Figure 5). At this time point, sarcomeric α-actinin expressing cells also expressed EH-myomesin (Figure 1L), indicating cardiac rather than skeletal differentiation. *at*-RA induced a dose-dependent increase in the number of sarcomeric α-actinin-positive cells by up to 3.1-fold, from 2.5% to 7.7% (at 3×10^{-9} M *at*-RA). This effect was lost in *N-cadherin*-null EBs. Higher *at*-RA concentrations inhibited cardiomyogenesis irrespective of presence or absence of N-cadherin. In the presence of supplemented *at*-RA, RT-PCR analysis suggested that α-MyHC mRNA expression was increased in wt cells at 8 days (but not at 2 days), but decreased in *N-cadherin*-null cells at 2 days (but not at 8 days). MLC2v mRNA expression was increased in wt (but not in *N-cadherin*-null) cells at 2 days (but not at 8 days). GATA-4 expression was increased in wt (but not in *N-cadherin*-null) cells at 2 days, and in both cell types at 8 days (Figure 6). β-tubulin mRNA expression appeared to be unchanged in all conditions.

Skeletal differentiation in the absence of N-cadherin

Microscopical analysis of EBs plated on gelatin-coated dishes suggested that, at 20 days of differentiation, the number of multinucleated cells with myotube-like morphology staining positive for sarcomeric α-actinin, but negative for EH-myomesin

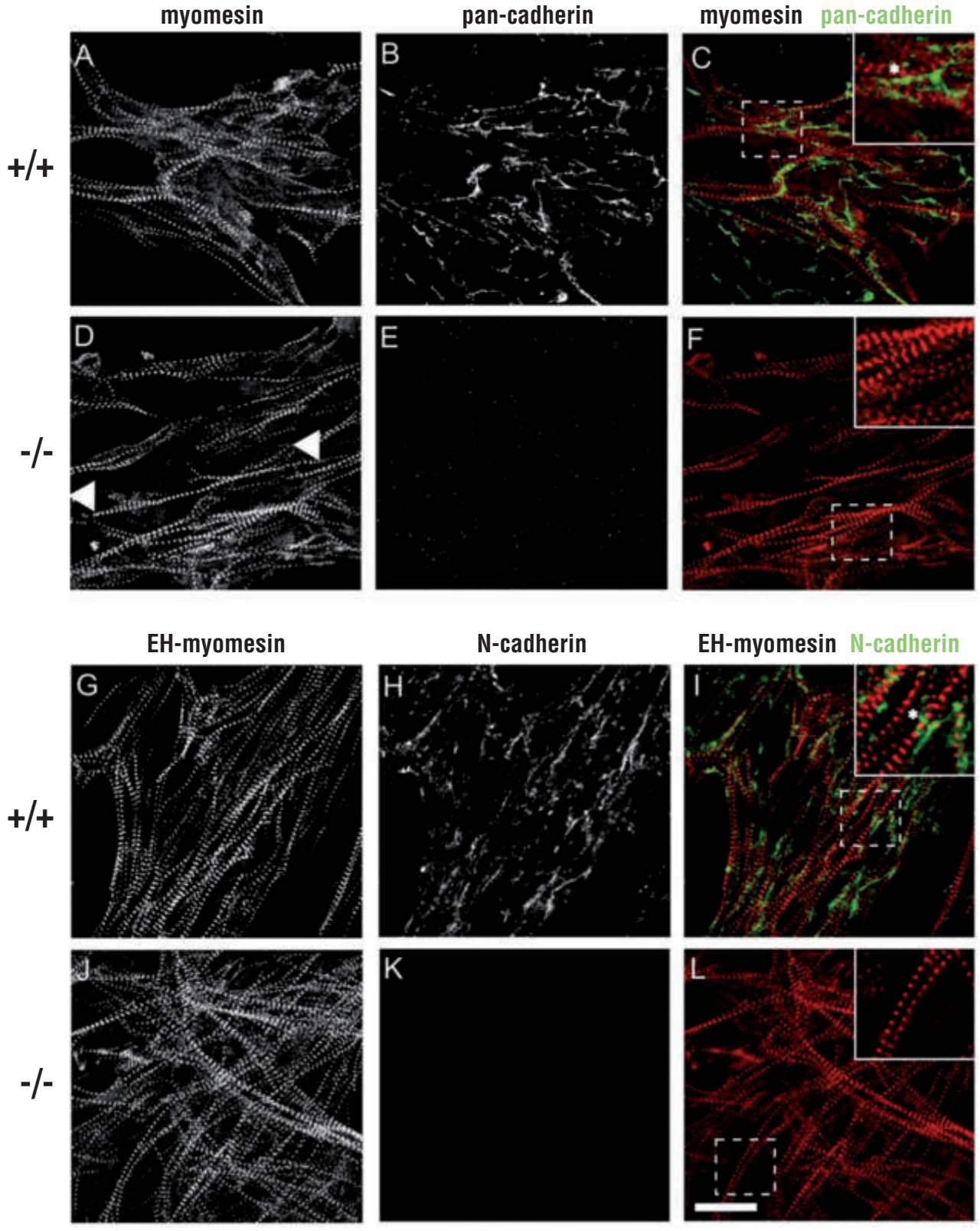
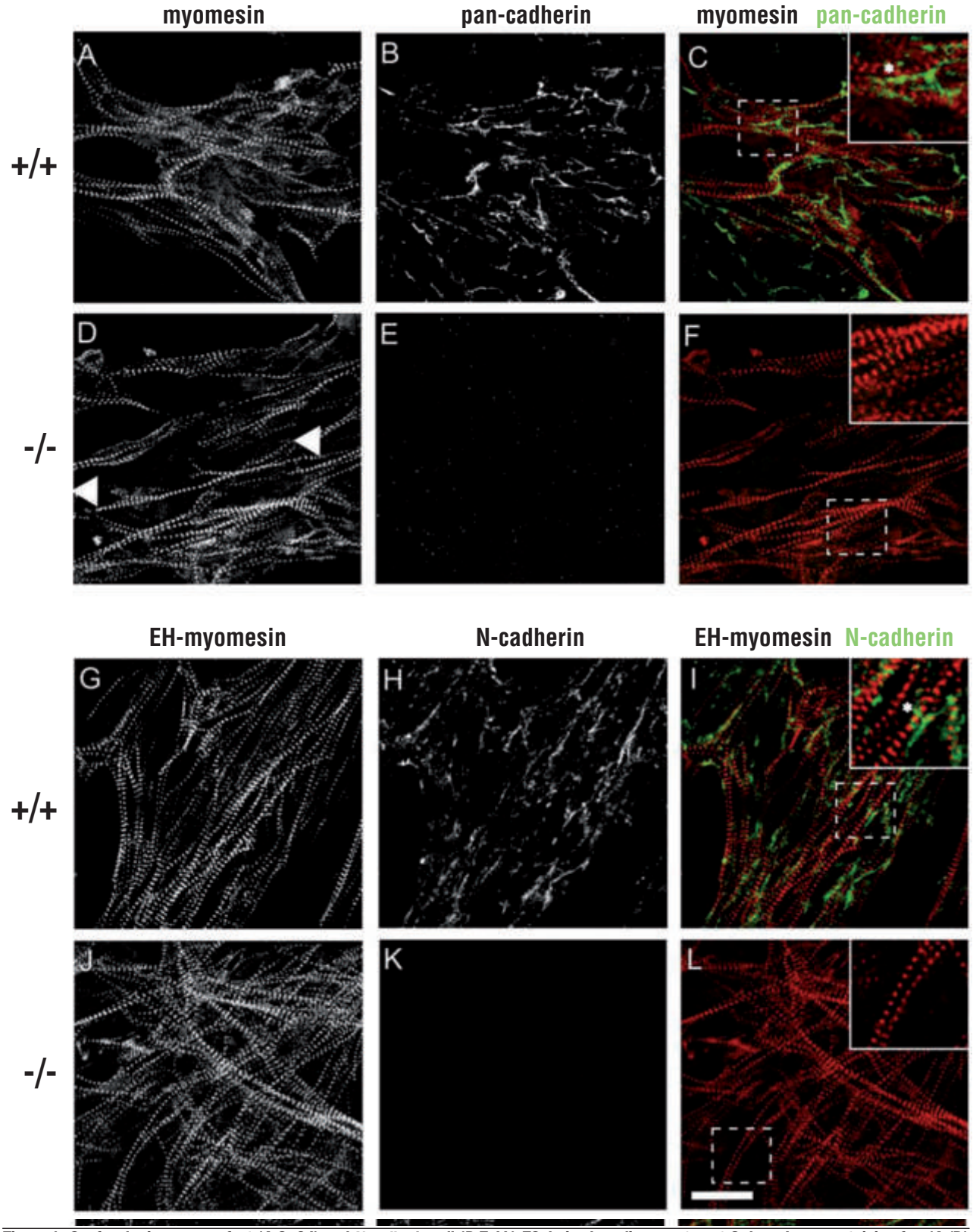


Figure 1. Confocal microscopy of wt (A-C, G-I) and *N-cadherin*-null (D-F, J-L) ES-derived cardiomyocytes at 8 days. Immunostaining for: (A/D) Myomesin. (B/E) Pan-cadherin. (C/F) Combined myomesin (red) and pan-cadherin (green). (G/J) EH-myomesin. (H/K) N-cadherin. (I/L) Combined EH-myomesin (red) and N-cadherin (green). In wt myocytes, unlike *N-cadherin*-null cells, both N-cadherin and pan-cadherin antibodies stain patches of cell membranes either parallel or perpendicular to the myofibrils (intercalated disk-like structures; asterisks in insets). Scale bar, 20 μm.



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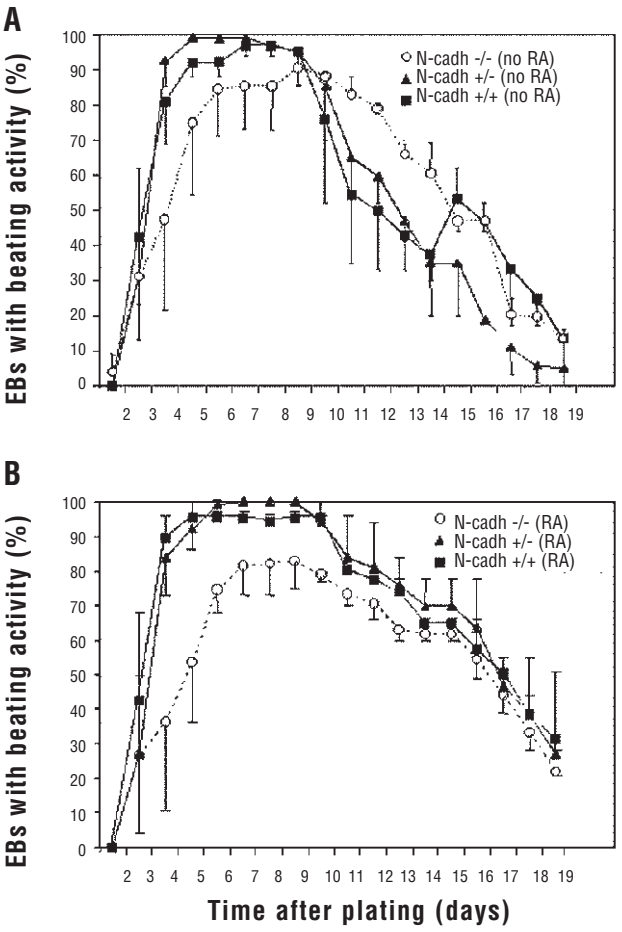

Figure 4. Temporal analysis of the appearance of spontaneously beating cardiomyocytes in EBs. (A) In the absence of supplemented at-RA. (B) In the presence of supplemented 10^{-8} M at-RA. Squares, wt; triangles, heterozygous; open circles, *N-cadherin*-null ES cells (mean values +SEM).

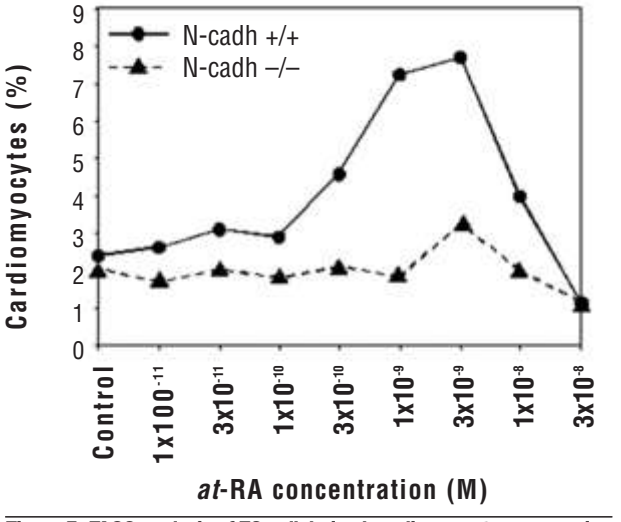

Figure 5. FACS analysis of ES cell-derived cardiomyocytes expressing sarcomeric α-actinin at 13 days in wt (circles; full line) and *N-cadherin*-null (triangles; dotted line) EBs in the presence of supplemented at-RA at the indicated concentrations. *at*-RA appears to increase the number of cardiomyocytes up to ≈3-fold in wt, but not in *N-cadherin*-null, EBs (one representative experiment out of two is shown).

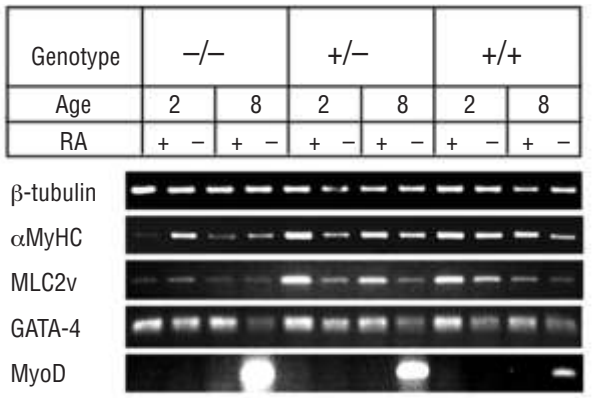

Figure 6. RT-PCR analysis of cardiac (αMyHC, MLC2v, GATA-4) and skeletal (MyoD) differentiation markers in EBs at 2 and 8 days. β-tubulin mRNA expression appears to be comparable in all conditions.

(indicating skeletal rather than cardiac muscle differentiation), was increased in *N-cadherin*-null EBs (Figure 7). In a separate experiment, EBs were plated individually on 48-well plates in the presence or absence of supplemented *at*-RA (46-47 wells per cell type and experimental condition). At 20 days, in the absence of *at*-RA treatment, skeletal myocytes were present in 40.4% *N-cadherin*-null compared with 10.6% wt EBs. RT-PCR analysis suggested that mRNA expression of the skeletal-specific myogenic determination factor, MyoD, was increased in *N-cadherin*-null cells at 8 days in the absence of supplemented *at*-RA (Figure 6). *at*-RA suppressed both MyoD expression and appearance of skeletal muscle cells in both *N-cadherin*-null and wt ES cells.

Discussion

In the absence of N-cadherin, the primitive heart consists of loosely associated, round cardiomyocytes that surround the endocardium but do not form myocardial trabeculae properly. These abnormalities may reflect defective cell-cell contacts resulting in impaired transmission of generated forces across neighboring myocytes. We have used *N-cadherin*-null ES cells grown as EBs *in vitro* to study the role of N-cadherin in early cardiomyogenesis. Our results indicate that myofibrillogenesis and intercellular contacts are normal in *N-cadherin*-null ES cell-derived cardiomyocytes. These findings are in general agreement with previous data in cardiomyocytes isolated from *N-cadherin*-null embryos (Luo *et al.*, 2003). Desmosomes were immunohistologically normal in *N-cadherin*-null

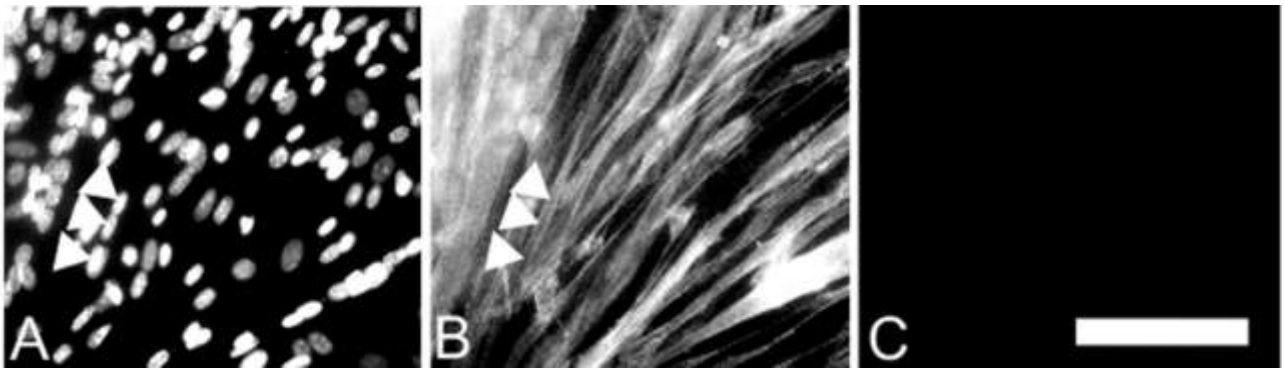

Figure 7. *N-cadherin*-null ES cells grown for 20 days in the absence of RA form skeletal myocytes. (A) DAPI staining. (B) Sarcomeric α-actinin staining. Polynucleated cells (arrowheads) staining positive for sarcomeric α-actinin are skeletal myofibers. (C) Negative EH-myomesin staining, indicating absence of (immature) cardiomyocytes. Scale bar, 100 μm.

ES cell-derived cardiomyocytes. Finally, the spatial orientation of myofibrils appeared to be normal in differentiating *N-cadherin*-null cardiomyocytes. This observation contrasts with previous data in cultured cardiomyocytes isolated from *N-cadherin*-null embryos (Luo *et al.*, 2003), as well as with data obtained with N-cadherin-blocking antibodies in cultured cardiomyocytes isolated from chick embryos (Goncharova *et al.*, 1992), or using a dominant-negative N-cadherin construct microinjected into adult rat cardiomyocytes (Hertig *et al.*, 1996). The reason for this difference is unclear, but it should be considered that cardiomyocytes differentiating in the embryonic heart *in vivo*, unlike those differentiating *in vitro*, are exposed to increased mechanical stress.

Retinoic acid induces cardiac differentiation in murine ES cells in a dose and stage-dependent manner. It has been shown (Wobus *et al.*, 1997) that formation of ES cell-derived cardiomyocytes at 10^{-8} and 10^{-9} M *at*-RA or 9c-RA is accelerated at early stages of differentiation (corresponding to days 4-10 in our model), but not at later stages (corresponding to day 16 in our model). Consistent with this report, the number of wt ES cell-derived cardiomyocytes in our study appeared to be increased in a dose-dependent manner during *at*-RA treatment (≈3-fold at 3×10^{-9} M). This effect of *at*-RA was essentially lost in *N-cadherin*-null ES cells. While virtually all wt and heterozygous EBs cultured in the presence of supplemented *at*-RA contained spontaneously beating cardiomoycytes by day 5-6, 17% of *N-cadherin*-null EBs did not, even at later time points. The time course of the emergence of spontaneous contractile activity in *N-cadherin*-null EBs appeared to be moderately delayed compared with wt and heterozygous EBs, as evidenced by the observation that same frequencies of EBs with spontaneously beating cardiomyocytes were reached 1-2 days later in *N-cadherin*-null than wt and heterozygous cells.

These results are in general agreement with most recent data in mouse ES cells grown in GMEM-based medium supplemented with the mesoderm-inducing factor, bone morphogenenetic protein 4 (BMP4; Honda *et al.*, 2006). In EBs, the cell population that expressed N-cadherin showed higher transcript levels of cardiogenic markers, *Nkx2.5*, *Tbx5*, and *Isl1* compared with the N-cadherin-negative cell fraction. After further differentiation on OP9 cells for 6 days, N-cadherin-positive cells differentiated to beating cardiomyocytes at higher rates (7.9-fold) than N-cadherin-negative cells. More cells in the N-cadherin-positive fraction stained positive for cardiac troponin-T than in the negative fraction. The authors suggested that the N-cadherin-positive cell population contains more cardiomyocyte progenitors differentiated from ES cells, and that N-cadherin may be used to select cardiomyocyte progenitors when ES cells are suppressed to differentiate into the neuronal lineage by a mesoderm-inducing factor (BMP4).

Recently, it has been reported that *glo* mutant zebrafish embryos with mutations in the *N-cadherin* gene show abnormal cardiomyocyte differentiation, with individual cardiomyocytes becoming round-shaped and loosely aggregated, leading to impaired increase of cardiac output over time (Bagatto *et al.*, 2006). Based on this observation, it is tempting to speculate that even a modest delay (by 1-2 days) in the appearance of spontaneous beating activity in differentiating *N-cadherin*-null

cardiomyocytes may have *in vivo* implications by affecting the time course of developed force by the primitive heart. Collectively, our observations in *N-cadherin*-null ES cells treated with *at*-RA, those in wt ES cells treated with BMP4 4 (Honda *et al.*, 2006), and data in *glo* mutant zebrafish embryos (Bagatto *et al.*, 2006) suggest an important role for N-cadherin in cardiac ES cell differentiation.

The molecular interactions of *at*-RA and N-cadherin during cardiomyogenesis remain poorly defined. *at*-RA upregulates *N-cadherin* gene expression in several cell types, including epithelial and breast cancer cells (Vermeulen *et al.*, 1995), P19 EC cells undergoing neural differentiation (Jonk *et al.*, 1994), and undifferentiated mesenchymal cells (Cho *et al.*, 2003). As mentioned above, RA-deficiency caused a severe decrease in the expression of GATA-4 in heart-forming regions in avian embryos (Kostetskii *et al.*, 1999). The area of the developing cardiac inflow tract that later displayed prominent morphological defects showed a complete lack of GATA-4 transcripts. Administration of retinol restored GATA-4 expression and completely rescued the RA-deficient phenotype. Another study showed that blocking the RA receptors RARα2 or RXRγ with specific antisense oligonucleotides inhibited GATA-4 expression in heart-forming regions (Romeih *et al.*, 2003). In these regions, GATA-4–specific siRNA selectively suppressed N-cadherin mRNA expression. Embryos null for the RA metabolizing enzyme RALDH2 showed a less developed endocardium with defect formation of trabeculae (Niederreither *et al.*, 2001). At the molecular level, RXRα interacts, through its DNA binding domain, with the zinc finger region (ZF2) of GATA-4 as well as additional site(s) within the COOH terminus of the protein (Clabby *et al.*, 2003). There is now good evidence to suggest that the induction of gene expression by RA can be mediated directly through activation of RA receptors or indirectly by its ability to induce GATA proteins (Murakami *et al.*, 1999). Consistent with this, it has been shown that GATA-4 function can partially rescue heart tube development in the absence of retinoids (Ghatpande *et al.*, 2006). Functional analysis of the *N-cadherin* gene promoter activity showed direct GATA-4 binding to the *N-cadherin* gene promoter region, which mediated transactivation of *N-cadherin* gene expression (Zhang *et al.*, 2003). Collectively, these results are consistent with a putative pathway whereby RA is upstream of GATA-4, and both factors are upstream of N-cadherin.

Finally, preliminary observations in the present study suggest that MyoD mRNA expression and skeletal muscle differentiation may be increased in *N-cadherin*-null EBs, but this will require confirmation in future studies.

In conclusion, our results suggest that, although myofibrillogenesis is normal in differentiating *N-cadherin*-null cardiomyocytes, N-cadherin may be essential for early cardiomyogenic effects of *at*-RA in mouse ES cells grown as EBs *in vitro*. This observation may contribute to explain the occurrence of cardiac defects and myocardial dysfunction in *N-cadherin*-null embryos.

Acknowledgements

We thank Dr. Glenn L. Radice (Philadelphia, USA) for providing us with both N-cadherin -/- and +/- ES cell lines, Dr. Andras Nagy (Toronto, Canada) for R1 ES cells, and Dr. Irina Agarkova (Zurich, Switzerland) for EH-myomesin antibodies. Drs. D. Auerbach, A. Hirschy, R. Eckner, I. Agarkova, and Ms. Sara Meier are gratefully acknowledged for scientific and technical support. R.B. was supported by a pre-doctoral training grant by the ETH Zürich, as well as by grants by the Swiss National Science Foundation (grant 31-63486 to JCP), by the Swiss University Conference, by the Gebert-Rüf Stiftung (grant P-038-01), by the Fondation Vaudoise de Cardiologie and the Teo Rossi di Montelera Foundation (to GV).

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