Constitutive overexpression of N-cadherin in mouse embryonic stem cells led to marked changes in the phenotype and adhesion properties of these cells. The changes included the formation of smaller embryonic bodies, elevated mRNA and total protein levels of N-cadherin, and increased amounts of p120 catenin and connexin-43. N-cadherin cells exhibited decreased attachment to non-cell surfaces, while their adhesiveness to each other and to rat neonatal cardiomyocytes was significantly elevated. The findings suggest that N-cadherin overexpression can facilitate electromechanical integration of stem cells into excitable tissues with endogenously high levels of N-cadherin, such as the heart and brain.

Introduction

Replacement of diseased tissue by pluripotent stem cells or their differentiated derivatives holds enormous therapeutic potential.1,2 Yet, the single major problem is short term survival and limited engraftment of the donated cell population.3 With respect to cardiac grafts, incomplete cell engraftment is particularly dangerous, as islands of uncoupled donor cells can be arrhythmogenic.4,5 To date, a limited number of strategies can be used to improve the electromechanical coupling between the donor cell population and host cardiomyocytes.

Cadherins are homophilic adhesion molecules that hold cells together in a “zipper-like” fashion. N-cadherin is the predominate isoform found in cardiac muscle.6 The tendency of stem cell-derived cardiac grafts to segregate into clusters may be explained by the dissimilar isoforms and/or expression level of cadherins between the grafted cells and host cardiomyocytes.7,8 Therefore, one can suggest that upregulation of N-cadherin in donor cells can be an approach to alleviate cell clusterization and to enhance the adhesiveness of donor cells to the myocardium.

This report describes a new line of mouse embryonic stem cells (ESC) that constitutively overexpress N-cadherin (NCadR1 cells) and, as a result, are more adhesive to cardiomyocytes. In addition, these cells have significantly higher levels of connexin-43, suggesting a higher degree of electrical engraftment.

Results

Morphology of N-cadherin overexpressing ESC and EBs. The proliferation rate, shape and size of undifferentiated NCadR1 cells were not different from control R1 cells (Fig. 1A and B). NCadR1 cells maintained their pluripotency, and were able to differentiate into multiple cell types upon removal of LIF and/or formation of EBs. Pluripotency of ESC was confirmed by PCR using Oct-4 as a marker (Fig. 1C). The expression of N-cadherin and connexin-43 in ESC colonies was tested by semi-quantitative PCR and found to be comparable between R1 and NCadR1 cells (Fig. 1C).

When NCadR1 cells were allowed to differentiate via either LIF withdrawal or EB formation the amount of N-cadherin protein started to increase due to the activation of the cytomegalovirus promoter.11 A higher copy number of the N-cadherin gene was confirmed in differentiated NCadR1 cells by comparing total genomic DNA to that of control R1 cells, using semi-quantitative PCR (n = 6). N-cadherin mRNA overexpression was verified by RT-PCR (n = 6), and protein overexpression was verified by western blot analysis as shown below.

Spontaneous cardiac differentiation of NCadR1 cells during EB development was confirmed by the presence of two cardiac specific markers, myosin cardiac heavy chain and troponin-T (Fig. 1D), which were expressed similarly in NCadR1 and R1 EBs. This confirmed our visual observation that there were no significant differences in the number of beating EBs in suspension or when plated. Interestingly, spontaneously beating areas of NcadR1 EBs often appeared to be “buried” inside the dense EB, resulting in a jittering motion rather than the more conventional “contracting” edge of EB.

NCadR1 EBs were 30% smaller in size, compared to control R1 EBs (Fig. 2A). This difference in EB size was attributed to tighter cell packing, since upon dissociation of EBs, neither the size nor the number of individual cells was different between control
and NCadR1. With long-term cultivation (40–50 days), 40% of R1 EBs developed macroscopic cyst-like structures, whereas NCadR1 EBs remained dense and compact (Fig. 2B). The observed morphological differences appeared to be specific to the NCadR1 line, as an alternative genetic manipulation of R1 cells (transfection with GFP-gene under cardiac specific α-myosin heavy chain gene promoter) did not modify the EB size or cell adhesive properties (data not shown).

The different adhesive properties of control R1 and NCadR1 cells were underlined by experiments in which a 1:1 mixture of these two cell types failed to form EBs (Fig. 2C). This cell sorting was observed in the majority of hanging drops. In the few cases where chimeric R1-NCadR1 EBs developed, these EBs were malformed (non-spherical), and there was a clear tendency for NCadR1 cells to cluster together (Fig. 2C, bottom). These observations are in agreement with previous studies, which have shown that cells expressing high levels of cadherin sort from cells expressing low levels of cadherin.

Cell attachment. EBs (20–25 days) were dissociated, and the resulting cell suspension was cultured under standard cell conditions. NCadR1 EB-derived cells spread poorly on a variety of non-cell surfaces, such as culture-treated dishes, untreated glass, or laminin or gelatin-treated glass coverslips (Fig. 3A, top). NCadR1 cells prefer to adhere to one another, forming areas of tightly associated cells and often clumps (Fig. 3A bottom). Most importantly, NCadR1 exhibited elevated adhesion to cells with high levels of endogenous N-cadherin expression. This observation is illustrated in Figure 3B, in which EB-derived cells were pre-loaded with a long-term live cell tracer and then allowed to attach to a cardiomyocyte layer. The amount of adherent cells which attached to the underlying non-fluorescent myocyte layer was significantly higher in NCadR1 samples (Fig. 3B).

Changes in p120 catenin. Cadherin-cadherin interactions between neighboring cells are regulated by a number of proteins, including the catenins, which links the cadherin protein to the cytoskeleton. In particular, p120 catenin is essential to N-cadherin trafficking and stabilization within the plasma membrane. Since p120 catenin can be sequestered by excess cytosolic N-cadherin, we had to confirm the abundance of p120 catenin in NCadR1 cells. Western blot analysis revealed an increase in both total and membranous p120 catenin in NCadR1 EB-derived cells (Fig. 4 and data not shown). Immunostaining confirmed the marked elevation of p120 catenin in NCadR1-derived cells as compared to the controls (Fig. 4).

Levels of connexin-43 in NCadR1 cells. Previous studies suggest that cardiac gap junctions form only after mechanical junctions are in place, as cadherin expression and the formation of adherens junctions serve as a driving force for connexin expression and gap junction formation. While the expression levels of connexin-43 in undifferentiated ESC was comparable between the two cell lines (Fig. 1C), differentiated EB-derived NCadR1 cells exhibited a 7-fold increase in connexin-43 expression (Fig. 4). Immunostaining and RT-PCR confirmed the increased levels of connexin-43 expression in NCadR1 cells. By using a gap junction specific connexin-43 antibody (IF1, Fred Hutchinson Cancer Research Center) we have confirmed that the increase in connexin-43 can be traced to the increase in gap junctional protein (data not shown).

Discussion

Although the increased amount of N-cadherin is the most straightforward explanation for the described phenotypic changes, additional factors can also be at play. Other cadherin isoforms may be expressed in a greater amount, or these cadherins may form more stable complexes at cell-to-cell junctions. Our preliminary studies have not revealed major changes in the total amount of either VE-cadherin or E-cadherin in NCadR1 cells, but this possibility needs to be further explored. Alternatively, an increase in cytosolic N-cadherin levels can trigger pathways that increase the expression level of junction-stabilizing proteins. One such protein, p120 catenin, showed enhanced expression in the membranes.
N-cadherin overexpression and adhesion properties of embryonic stem cells

Wnt family has numerous roles in embryonic development and in stem cell biology. In our studies, we did not detect a difference in β-catenin protein expression between NCadR1 and R1 control (data not shown). Nevertheless, the possible involvement of the Wnt signaling pathway in relation to the phenotypical differences in NCadR1 cells should be explored further.

The data have confirmed our initial hypothesis that overexpression of N-cadherin in differentiating cells might lead to an increase amount of membranous connexin-43. In cardiac cells, formation of mechanical junctions serves as a prerequisite for gap junctional assembly, acting as a driving force for connexin expression. Another line of evidence came from the knockout mice, as induced deletion of the N-cadherin gene led to the disruption of not only mechanical, but also electrical coupling. These and other studies provide a mechanistical explanation between the overexpression of N-cadherin and the observed increase in the amount of connexin-43 protein in NCadR1 cells.

Overexpression of a few key genes is a known tool to trigger multiple pathways and cause phenotypic changes. This was underscored by the ground-breaking studies which used targeted gene reprogramming to confer pluripotency to somatic cells. We used a similar strategy to test a hypothesis that overexpression of N-cadherin in differentiating ESC can act as a trigger to enhance both mechanical and electrical connectivity of their progeny. Our data suggest a new means to improve stem cell engraftment into excitable tissues with endogenously high levels of N-cadherin (such as the heart and brain), although additional studies are necessary to fully implement this strategy. Importantly, we have shown that N-cadherin overexpression did not preclude cardiac differentiation. This suggests that indirectly increasing the expression level of connexin-43 is preferential to constitutive connexin-43 overexpression, which has been shown to impede cardiac differentiation.

Similarly, if N-cadherin overexpression is used to enhance neuronal engraftment, it would be essential to confirm the presence of neuronal markers in the differentiated progeny. In the future, functional assessment will be conducted on N-cadherin overexpressing cells to determine whether or not these cells graft more efficiently.

Materials and Methods

Reagents and antibodies. Mouse anti-connexin-43 antibody, horse radish peroxidase (HRP) conjugated goat-anti-mouse or goat-anti-rabbit IgG and all other chemicals were purchased from Sigma Chemical (St. Louis, MO) unless otherwise specified. Rabbit anti-N-cadherin antibody, goat-anti-troponin T, alkaline phosphatase (AP) conjugated goat-anti-mouse, goat-anti-rabbit or

![Figure 2. Appearance of NCadR1 and R1 embryoid bodies.](image_url)

- **A** Appearance of 2-day old embryoid bodies (EB) derived from R1 and NCadR1 ESC, visualized in phase contrast. The graph shows the statistically smaller size of NCadR1 EBs, compared to R1 control (n = 5, p < 0.001).
- **B** Macrophscopic cystic structures seen in many R1, but not NCadR1, upon long-term cultivation of EBs (n = 6). Images of 60 mm culture dishes with visible cystic EBs are shown.
- **C** Round EBs were formed in 100% of the hanging drops which contained either R1 or NCadR1 cells loaded with a different color of CellTracker. In comparison, 1:1 mixture of R1 and NCadR1 cells failed to form EBs (bottom left) in >70% of the drops. In the remaining cases, EB were asymmetrical, malformed and dissociated into smaller cell clusters with time (bottom right). Scale bar 50 micrometers.

...of NCadR1 cells. The p120 catenin protein has multiple roles, depending on its phosphorylation status. It will be interesting to address the phosphorylation status of p120 catenin in NCadR1 cells, and to determine whether this property has any additional impact on the adhesiveness of these cells.

N-cadherin overexpression can modify the expression and localization of β-catenin, which is a key player in the Wnt pathway. The...
donkey-anti-goat IgG were purchased from Santa Cruz Biotechnology. Mouse anti-heavy-chain cardiac myosin antibody was purchased from Abcam and mouse anti-p120 catenin antibody was purchased from BD Transduction Laboratories. Other secondary antibodies included goat-anti-mouse-Alexa 488 (Invitrogen/Molecular Probes) and goat-anti-rabbit-Cy5 (Jackson ImmunoResearch). Nuclear staining and long-term staining of live cells were performed using the SelectFX Nuclear staining kit and CellTracker, respectively (Invitrogen).

**N-cadherin overexpression.** The N-cadherin gene is highly conserved between Mus musculus and Rattus norvegicus, showing 99% gene identity in the translated region, as confirmed by a BLAST search. For cloning purposes, the Rat norvegicus gene can be considered identical to the Mus musculus gene. A 2,721 bp N-cadherin gene was amplified from a cDNA library obtained from rat testis. EcoR1 and Xba1 restriction digest sites were inserted at the 3’ and 5’ ends to facilitate ligation into a pTracer-CMV vector, which also contains a zeocin antibiotic resistance gene. The resulting plasmid was designated as pT-NCad and used for stable transfection. Mouse R1 ESC were cultured according to the manufacturer’s protocol (ATCC). R1 ESC were transfected with the pT-NCad construct using the FuGene6 transfection system (Roche Molecular Biochemicals), and transfected cells were selected via antibiotic pressure. Undifferentiated R1 and NCadR1 ESC were split every 48 h, and maintained in stem cell medium supplemented with LIF (Chemicon).

**Cell differentiation and attachment studies.** Cells were differentiated by generating embryoid bodies (EB), via the hanging drop method. Briefly, 500 cells were suspended in 20 μl of 20% FBS stem cell media, in the absence of LIF. EBs were allowed to develop in suspension for 48 hr before harvesting. Aggregated EBs were cultured for the indicated time periods. To assess the proliferative capacity of NCadR1 versus R1 cells, 20 EBs from each cell type were dissociated and the total number of cells were counted at day 1, 2, 4 and 6. Alternatively, NCadR1 and R1 cells were seeded onto 12-well plates (1 x 10^5/well) and proliferation was assessed by counting the total number of cells per well on the designated day. To analyze cell attachment properties and to perform immunostaining, EBs were dissociated into a single cell suspension using Dispase (Gibco) and trypsin-EDTA (ATCC). For attachment studies, dissociated cells were loaded with CellTracker dye according to the manufacturer’s instructions (Molecular Probes). Cells were then seeded onto laminin or gelatin-treated glass coverslips. Alternatively, they were plated onto neonatal cardiomyocyte layers. The latter were prepared from 1–2 day-old Sprague-Dawley rats, as described previously. After 18 hours of co-culture, the medium was changed twice to remove unattached cells. Fluorescence was acquired from five random areas of each coverslip using Zeiss LSM512 confocal imaging system.

**PCR analysis.** Total RNA was isolated from either control R1 or stably transfected NCadR1 cells, or from EBs (20–25 days differentiation) using Trizol reagent (Invitrogen). RNA was treated with DNase I (Invitrogen) to remove DNA contamination, and then converted to cDNA via Affinityscript QPCR cDNA synthesis.

![Figure 3](image-url)
kit (Stratagene). An ABI Prism 7300 light cycler was used to perform real-time PCR using SYBR green Q-PCR mastermix (Applied Biosystems, Foster City CA). Alternatively, semi-quantitative PCR was performed by quantitating cDNA and adding equal amounts to Choice Taq Blue mastermix (Denville). Samples were resolved on a 2% agarose gel. The following primers were used in PCR experiments: N-cadherin forward AGG GTG GAC GTG ATT GTA GC, N-cadherin reverse CTG TTG GGG TCT GTC AGG AT; β-Acin reverse TGT TAC CAA CTG GGA TGT TAC, β-Acin forward GGG GTG TTT TCC AGA AAC CTG GGA CAA, GGA CGA CA, β-Acin reverse GGG GTG TTT TCC AGA AAC CTG GGA CAA, GGA CGA CA, β-Acin forward GGT GGT TTT TCC ACT CTA ACA ATG, GGT GGT TTT TCC ACT CTA ACA ATG; N-cadherin forward AGG GTG GAC GTG ATT GTA GC, N-cadherin reverse CTG TTG GGG TCT GTC AGG AT; β-Acin reverse TGT TAC CAA CTG GGA TGT TAC, β-Acin forward GGG GTG TTT TCC AGA AAC CTG GGA CAA, GGA CGA CA, β-Acin reverse GGG GTG TTT TCC AGA AAC CTG GGA CAA, GGA CGA CA, β-Acin forward GGT GGT TTT TCC ACT CTA ACA ATG, GGT GGT TTT TCC ACT CTA ACA ATG.

Western blots and immunocytochemistry. Samples were fixed using a standard 4% paraformaldehyde protocol, followed by staining with rabbit N-cadherin (1:50), mouse connexin-43 (1:500), and mouse p120 catenin (1:500) antibodies and detected by goat-anti-rabbit Cy5 (1:1,000) or goat-anti-mouse Alexa 488 probes, respectively. Images were acquired using a Zeiss LSM510 confocal imaging system using identical setting to compare R1 and NCadR1 samples. Conclusions are based on the analysis of at least two different sets of experiments, each containing a duplicate number of coverslips per staining. Total protein was isolated in homogenization buffer containing 250 mM sucrose, 10 mM KCl, 10 mM imidazole, 5 mM EDTA and 2 mM dithiothreitol with 10% glycerol, pH 7.8. Samples were sonicated 3 x 10 sec, and cell debris was removed. Equal amounts of protein were loaded onto precast 4–20% gradient gels (Pierce). Representative blots and images are shown.

Acknowledgements
We thank Dr. Ara Arutunyan for helpful discussions. Dr. C. Yan Cheng (Population Council, Center for Biomedical Research) is acknowledged for providing the N-cadherin-expressing plasmid. Support by the NIH and the American Heart Association is gratefully acknowledged.

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