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# N-cadherin-mediated cell adhesion determines the plasticity for cell alignment in response to mechanical stretch in cultured cardiomyocytes

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**Abstract**

Mechanical stretch has been implicated as the growth stimuli in the heart. Physiologically, mechanical stretch is reported to contribute to the orientation of cardiomyocytes, though the molecular mechanism remains to be elucidated. This study was designed to make clear functional significances of N-cadherin in plasticity of cell alignment in response to mechanical stretch. Neonatal rat cardiomyocytes, cultured on silicone dishes, were subjected to artificial uniaxial cyclic stretch. Mechanical stretch was started at certain times (3–75 h) after seeding and continued for 24 h. Stretch stimulation in 3 h after cultivation promoted cell orientation running parallel to tension direction. In contrast, cardiac myocytes fail to align when exposed to stretch 24–75 h after cultivation. To address the importance of N-cadherin in the responsiveness to stretch, the expression and distribution of N-cadherin were analyzed. Immediately after seeding, N-cadherin showed dispersed distributions. During cultivation, N-cadherin localized to cell–cell contacts accompanied by the upregulation of its protein. Next, to investigate influence of cell–cell adhesion, cardiomyocytes cultured for 72 h were replated by trypsin treatment and exposed to stretch 3 h after replating. The cardiomyocytes replated by trypsinization were oriented in parallel to tension direction by mechanical stretch. Finally, adenoviral transfection of dominant-negative N-cadherin recovered the ability to exhibit cell orientation in response to stretch. Our results suggested that N-cadherin was involved in the oriented responses of cardiomyocytes induced by mechanical stretch.


*Keywords:* Cardiomyocyte; N-cadherin; Mechanical stretch; Cell alignment; Cell adhesion

Cell–cell adhesion system is closely related with cytoskeletal structure. A number of studies have demonstrated that cadherin family proteins regulate cell–cell interaction and contribute to cytoskeletal organization through intracellular region by interacting with submembranous proteins [1–3]. The highly conserved cytoplasmic portion of cadherins interacts with catenins [4], which link the cadherin to the actin cytoskeleton [5]. Adherence junctions are also responsible for mechanical coupling between myocytes [6]. Cadherin family proteins are the key regulators in cell adhesion. In cardiac myocytes, N-cadherin is the major transmembrane components of the adherens junction. N-cadherin is abundantly expressed in the heart and localized to intercalated disks [7]. Then N-cadherin plays critical roles in myofibril organization [8].

Mechanical stretch is believed to be required for cardiac development and growth as a physiological stimulus and effective contraction of heart results from

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the arrangement of cardiomyocytes [9–11]. On the other hand, the disarrangement of cardiomyocytes is a characteristic of pathological hearts, especially hypertrophic cardiomyopathy (HCM) [12–15], though the mechanism of cardiac disarrangement remains to be unknown. Interestingly, recent studies revealed that stretch in early stage after cultivation induced cell orientation [16]. In this study, to make clear the regulatory mechanism for the oriented responses, we investigated the functional significances of N-cadherin on cell alignment in response to stretch.

## Materials and methods

*Antibodies.* Anti-N-cadherin antibody was purchased from BD Transduction Laboratories and anti-Cdk4 was from Santa Cruz Biotechnology. Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and anti-rabbit IgG were obtained from Santa Cruz Biotechnology. And Alexa 546-phalloidin and Alexa 488-conjugated goat anti-mouse IgG were from Molecular Probe.

*Cell culture.* Primary cultures of cardiac myocytes were prepared from 1-day-old Wistar rats as described previously [17]. All experimental procedures were approved by the Animal Care Committee of Osaka University. Cardiac myocytes were isolated by treatment with collagenase (Sigma) and trypsin (Difco). Cultures were enriched with cardiac myocytes by preplating for 1 h, to eliminate non-cardiac myocyte population. Non-adherent cells were plated at a density of $8.0 \times 10^5$ cells per silicone dish ($2 \times 2\ cm^2$). In the case of low cell density, cardiac myocytes were plated at a density of $3.0 \times 10^5$ cells per silicone dish. More than 90% cells were identified as cardiac myocytes, assessed by microscopic observation of cell beating (data not shown). The myocytes were cultured in Dulbecco's modified Eagle's medium/F-12 (ICN Biochemicals) supplemented with 5% newborn calf serum (ICN Biochemicals), 3 mM pyruvic acid, 100 μM ascorbic acid, 5 μg/mL insulin, 5 μg/mL transferrin, and 5 ng/mL sodium selenite (Roche) in the presence of 0.1 mM bromodeoxyuridine.

*Application of mechanical stretch.* The silicone chambers (Scholar Tec) were coated with 150 μg/mL collagen (Nitta Gelatin). To analyze the orientation response, cells were exposed to 20% cyclic stretch in uniaxial strain at 30 cycles/min for 24 h by a computer-controlled stepping motor (Scholar Tec).

*Immunofluorescent microscopy.* Cardiac myocytes were fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and incubated with PBS containing 0.5% Triton X-100 for 15 min. Washed with PBS, cells were incubated with primary antibodies in PBS for 30 min at room temperature. After washing, cells were incubated with secondary antibodies in PBS for 30 min at room temperature. The analysis was performed with the Olympus fluorescent microscopy systems.

*Western blot analysis.* Proteins were separated in 12.5% SDS–PAGE and transferred onto a polyvinylidene difluoride membrane. After blocking in T-PBS (0.05% Tween 20 in PBS) containing 5% skim milk, membranes were probed with anti-N-cadherin or anti-Cdk4 antibody. ECL system (Santa Cruz Biotechnology) was used for the detection.

*Adenoviral vectors.* Dominant-negative N-cadherin cDNA, which encodes truncated N-cadherin molecule consisting of a large deletion of the extracellular domain, was gifted by Dr. Jeffrey I. Gordon (Washington University) [18]. Adenovirus vector expressing dominant-negative N-cadherin was generated as described previously [19]. In brief, generation of recombinant adenoviral vector expressing dominant-negative N-cadherin mutant driven by the cytomegalovirus promoter was carried out through homologous recombination between pJM17 and the shuttle plasmids co-transfected in 293 cells. Viral vectors were purified by ultracentrifugation in the presence of CsCl.

Cardiac myocytes were transduced by the adenovirus vector at MOI 20 as described previously [20]. At this MOI, more than 90% of myocyte population was transduced (data not shown).

*Statistical analysis.* Depending on the design of the experiment, statistical significance was determined by Mann–Whitney *U* test. Differences were considered significant when the calculated *P* value was less than 0.05.

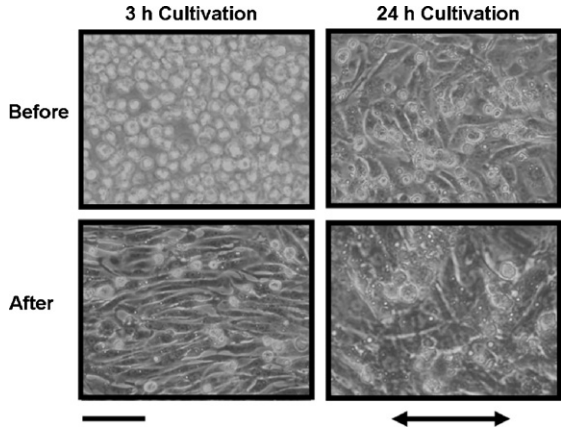


Fig. 1. Morphological responses are induced by stretch in cardiomyocytes. Representative microscopic images of cardiomyocytes before (upper panel) or after (lower panel) stretch stimulation were demonstrated. Stretch exposure started 3 (left panel) or 24 (right panel) hours after cell plating. Stretch direction is shown with arrow. The experiments were repeated four times with similar results. Bar, 50 μm.

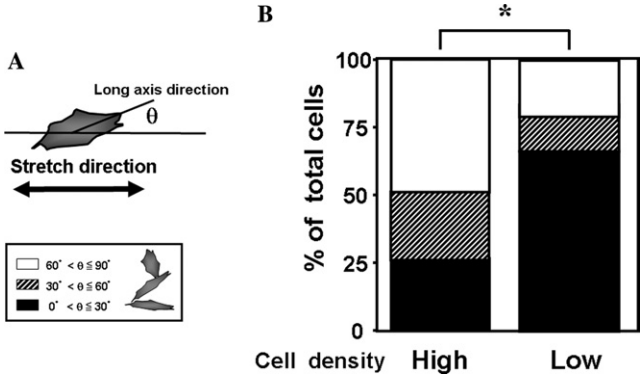


Fig. 2. Cardiac myocytes cultured at low cell density show the oriented response to stretch, but not those at high density. (A) The cell orientation was estimated as an angle ($\theta$) of the long axis with respect to the stretch direction. After stretch, the cells oriented at direction of 0° to 30° (black column), 30° to 60° (diagonal column), and 60° to 90° (white column) were counted. (B) The oriented cardiac myocytes was analyzed quantitatively. Cells were plated at high or low cell density. At low cell density, cells would develop few contacts with their neighbors. The percentages of cells in each direction were shown in culture at low (low) or high cell density (high). The experiments were repeated 3 times with similar results ($n = 210$ cells) (*$p < 0.01$; Mann–Whitney *U* test).

## Results and discussion

### *Mechanical stretch induces cell orientation in cardiomyocytes cultured for 3 h but not in those for 24 h*

Cardiac myocytes were plated on silicone chambers for 3 h and subjected to mechanical stretch. Three hours after seeding, cells attached to the chamber with round shape. After stretch stimulation, cell orientation was promoted in parallel to tension direction. When cells were subjected to stretch after 24 h cultivation, cell orientation was not induced. These data suggest that mechanical stretch transduces orientation signals in the cardiomyocytes that adhere loosely to substratum or to adjacent cells (see Fig. 1).

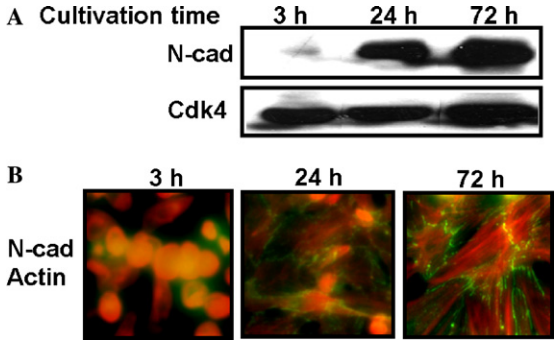


Fig. 3. Expression and distribution of N-cadherin in cultured cardiac myocytes. (A) Cell lysates were prepared from the cardiomyocytes 3, 24, or 72 h after seeding. The lysates were immunoblotted with anti-N-cadherin and anti-Cdk4 antibodies, an internal control. Cdk4 protein expression indicated that an equal amount of protein was loaded in each line. Note that N-cadherin was upregulated during cultivation. The experiments were repeated three times with similar results. (B) Cardiac myocytes were cultured for 3, 24 or 72 h and stained with phalloidin (red) and anti-N-cadherin antibody (green). Representative immunofluorescent micrographs are shown. The experiments were repeated three times with similar results.

### *Stretch-induced cell alignment is dependent on cell density*

To examine the effect of cell–cell contacts on cell orientation, we first analyzed whether the oriented response depends upon the confluency of the culture. As shown in Fig. 2, cells were oriented to the direction of stretch stimulation at low cell density, but not at high density. Importantly cells cultured at high cell density were almost confluent with cell–cell contacts, whereas cells at low cell density developed few contacts with their adjacent cells. Collectively, these findings suggest that cell orientation in response to stretch is inhibited by cell–cell adhesion.

### *The plasticity of N-cadherin-mediated cell–cell adhesion is required for the cell alignment*

In cardiac myocytes, N-cadherin is the major molecule of cell–cell adhesion system. To evaluate the importance of N-cadherin in cell orientation, we examined the expression and distribution of N-cadherin in cultured cardiac myocytes before stretch stimulation. N-cadherin was upregulated during cultivation after seeding with the peak at 72 h (Fig. 3A). Immunofluorescent microscopy demonstrated that N-cadherin was diffusely distributed around cytoplasmic membrane 3 h after seeding, and that N-cadherin was localized at cell–cell contacts when cultured for 72 h (Fig. 3B). These findings suggest that cardiomyocytes could respond to stretch, resulting in cell alignment, when cells adhere to cell substratum without formation of cadherin-mediated cell adhesion.

Thus, to examine whether or not immaturity of N-cadherin-mediated cell adhesion is prerequisite for orientation response against mechanical stretch, we analyzed the effects of the modulation of cell adhesion by reseeding the cardiomyocytes with trypsinization. The expression of N-cadherin is lower in cells cultured for 3 h after trypsinization, compared with that in cells

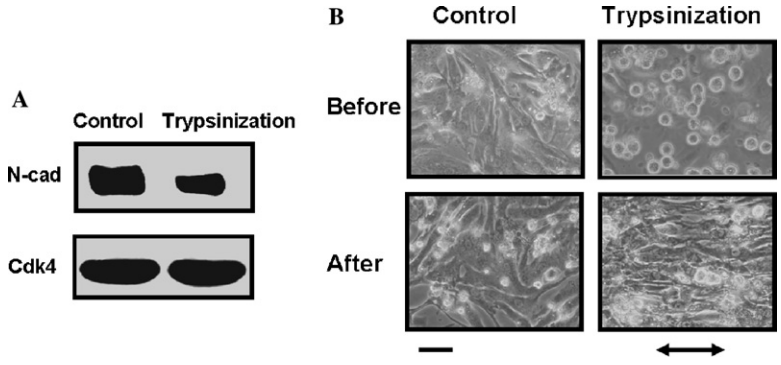


Fig. 4. Modulation of cell contact alters the oriented responses to stretch. (A) After 72 h cultivation, cardiac myocytes were collected by 0.2% trypsin–0.02% EDTA in PBS, reseeded on chamber, and incubated for 3 h (Trypsinization). Cells cultured for 75 h without trypsinization were used as control (Control). Cell lysates were immunoblotted with anti-N-cadherin and anti-Cdk4 antibodies, an internal control. The experiments were repeated three times with similar results. (B) The cells were exposed to mechanical stretch with (right panel) or without (left panel) trypsinization. Representative microscopic images of cardiomyocytes before (upper panel) or after (lower panel) stretch stimulation were demonstrated. Stretch direction is shown with arrow. The experiments were repeated three times with similar results. Bar, 50 μm.

cultured without reseeding (Fig. 4A). In parallel, the modulation of cell–cell adhesion by trypsinization enabled cardiomyocytes to rearrange their orientation following stretch stimulation (Fig. 4B).

To address the functional significance of the down-regulation of N-cadherin protein, we examined the effect of inhibition of N-cadherin function on orientation response in cardiomyocytes with cell–cell adhesion. In this experiment, we used the adenovirus vector expressing dominant-negative N-cadherin [20], which lacks the extracellular domain of N-cadherin. After cultivation for 24 h, cells were transfected with dominant-negative N-cadherin or β-galactosidase and were exposed to mechanical stretch. Cardiac myocytes transfected with β-galactosidase did not align in response to stretch, while adenoviral gene transfer of dominant-negative N-cadherin restored the ability (Fig. 5). These data demonstrated that the modulation of N-cadherin function confers the susceptibility of cardiomyocytes to cell rearrangement in response to stretch.

It remains to be revealed how cell alignment is determined in vivo. Our findings presented here could propose that the disturbance of N-cadherin function results in the impaired arrangement of cardiomyocytes.

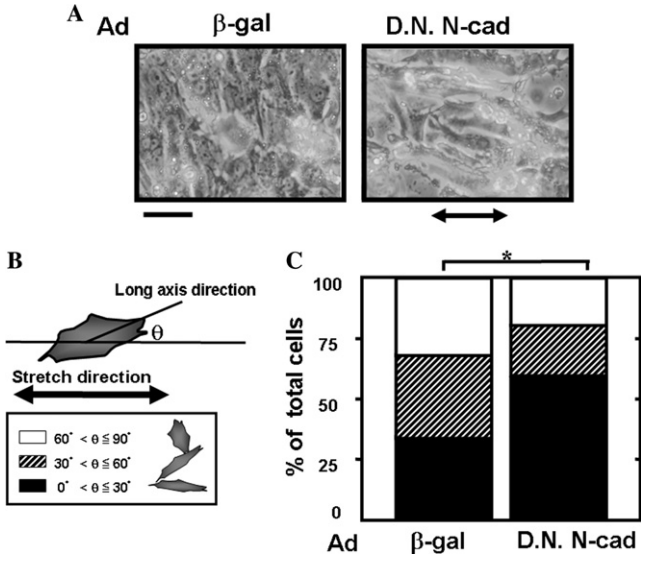


Fig. 5. Adenoviral gene transfer of dominant-negative N-cadherin restores the ability to align in response to stretch. After 24 h cultivation, cells were infected with adenoviral vector expressing dominant-negative N-cadherin (D.N.N-cad) or β-galactosidase (β-gal) at MOI 20 and exposed to mechanical stretch. (A) Representative microscopic images of cardiomyocytes after stretch stimulation were demonstrated. Stretch direction is shown with arrow. The experiments were repeated four times with similar results. Bar, 50 μm. (B,C) The oriented cardiac myocytes were analyzed quantitatively. The cell orientation was estimated as an angle ($\theta$) of the long axis with respect to the stretch direction. The cells oriented at direction of 0° to 30° (black column), 30° to 60° (diagonal column), and 60° to 90° (white column) were counted. The percentages of cells in each direction are shown (C). The experiments were repeated three times with similar results. ($n = 330$ cells) (*$p < 0.01$; Mann–Whitney $U$ test).

Interestingly, this proposal is supported by clinical findings that N-cadherin expression is altered in cardiomyopathic hearts, which show the disarrangement of cardiac myocytes, known as "disarray" [15,21].

In summary, we demonstrated that N-cadherin regulates the oriented responses of cardiomyocytes caused by mechanical stretch. The plasticity for cell alignment in response to stretch is determined by N-cadherin-mediated cell adhesion in cardiac myocytes.

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