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N-Cadherin is a prospective cell surface marker of human mesenchymal stem cells that have high ability for cardiomyocyte differentiation

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ABSTRACT

Mesenchymal stem cells (MSCs) are among the most promising sources of stem cells for regenerative medicine. However, the range of their differentiation ability is very limited. In this study, we explored prospective cell surface markers of human MSCs that readily differentiate into cardiomyocytes. When the cardiomyogenic differentiation potential and the expression of cell surface markers involved in heart development were analyzed using various immortalized human MSC lines, the MSCs with high expression of N-cadherin showed a higher probability of differentiation into beating cardiomyocytes. The differentiated cardiomyocytes expressed terminally differentiated cardiomyocyte-specific markers such as α-actinin, cardiac troponin T, and connexin-43. A similar correlation was observed with primary human MSCs derived from bone marrow and adipose tissue. Moreover, N-cadherin-positive MSCs isolated with N-cadherin antibody-conjugated magnetic beads showed an apparently higher ability to differentiate into cardiomyocytes than the N-cadherin-negative population. Quantitative polymerase chain reaction analyses demonstrated that the N-cadherin-positive population expressed significantly elevated levels of cardiomyogenic progenitor-specific transcription factors, including *Nkx2.5*, *Hand1*, and *GATA4* mRNAs. Our results suggest that N-cadherin is a novel prospective cell surface marker of human MSCs that show a better ability for cardiomyocyte differentiation.

1. Introduction

Stem cell therapy is expected to be an alternative regenerative medicine. In addition to embryonic stem (ES) cells and induced pluripotent stem (iPS) cells, mesenchymal stem cells (MSCs) have been shown to differentiate into various cell types including osteoblasts, chondrocytes, adipocytes, neurons, skeletal muscle fibers, and cardiomyocytes *in vitro*. However, the differentiation ability of MSCs toward cardiomyocytes is still limited [1–3]. To overcome this problem, cell surface markers specific for cardiomyogenic progenitor cells could be used to enrich better population for regenerative medicine of heart failure.

Flk-1, a vascular endothelial growth factor receptor (VEGFR2), has been reported to be a prospective cell surface marker of cardiomyocyte progenitor cells during heart development [4,5]. Flk-1 is expressed in the progenitors of multiple mesodermal lineages, including cardiac, endothelial, and vascular smooth muscle cells [5]. c-Kit (CD117) is a transmembrane tyrosine kinase receptor for Stem cell factor, and used as a cell surface marker for hematopoietic progenitors, melanocytes, mast cells, and spermatogonial stem cells. Recent research has suggested that c-Kit could be a putative cell surface marker for cardiomyogenic progenitor cells in the neonatal heart [6].

A Ca^{2+}-dependent cell–cell adhesion molecule, N-cadherin, is also expressed on cardiomyocyte progenitor cells during mouse development. N-Cadherin expression is observed in the precardiac

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mesoderm at E8.5 in mice and continues to be expressed in the whole heart during development. N-Cadherin-knockout mice died by E10 because of defects in the primitive heart. Although myocardial tissue was initially formed in the knockout mouse embryos, the myocytes were subsequently dissociated, and the heart tube failed to develop [7].

In this study, we explored cell surface markers of human MSCs that have a high ability to differentiate into cardiomyocytes. We show that N-cadherin is a prospective cell surface marker of MSCs with high cardiomyogenic potential.

2. Materials and methods

2.1. Cell culture

Human MSC cell lines, UE7T-13, UE6E7T-11, UBE6T-15, UE6E7T-12, UE7T-9, and UE6E7T-2, were obtained from the JCRB Cell Bank (Osaka, Japan). They were immortalized by retrovirus gene transfer of a combination of *bmi-1*, *E6*, *E7*, and/or *hTERT* genes to human bone marrow stromal cells harvested from a 91-year-old woman [8,10]. The EPC-214 cell line was similarly immortalized at the National Research Institute for Child Health and Development (NRICHD), Japan [9]. These cell lines were maintained in DMEM high glucose (Wako) supplemented with 10% fetal calf serum (FCS; Roche). As for the primary MSCs, ANP0425 and 0607NC, were obtained from Dr. Ohgushi (National Institute of Advanced Industrial Science and Technology, Japan). MSC-R36_2 cells, MSC-R36_3 cells, and Yub623 cells were obtained from the RIKEN BRC Cell Bank (Ibaraki, Japan). Primary MSCs derived from adipose tissue (ASCs), including 09-036 (36) cells, 10-008 (8) cells, 05-055 (55) cells, and 05-076 (76) cells, were prepared at the University of Tokyo, School of Medicine Tokyo, Japan. KN-SC (KN) cells, MY-SC (MY) cells, and NN-SC (NN) cells were prepared at the Research Institute National Center for Global Health and Medicine (NCGM), Japan. Other ASCs were purchased from Invitrogen. For the ASCs, all samples except KN-SC were obtained from women aged 22–45 years (KN-SC was derived from a 41-year-old man). All of these primary cells were maintained in MesenPRO RS Basal Medium supplemented with MesenPRO RS Growth Supplement (GIBCO). Cells were maintained in a humidified incubator at 37 °C with an atmosphere of 5% CO_2. All the experiments using human materials were approved by the Human Ethics Committee at AIST, NRICHD, NCGM, and the University of Tokyo. Human umbilical vein endothelial cells (HUVEC) were cultured in RPMI-1640 supplemented with EGM-2 SingleQuots (LONZA) and penicillin/streptomycin (Wako). TF-1 cells were cultured in RPMI-1640 supplemented with 10% FBS, 2 ng/mL rhGM-CSF, and penicillin/streptomycin (Wako).

2.2. Preparation of mouse fetal cardiomyocytes

The fetal hearts of E16.5 ICR mice were cut into small pieces and washed with phosphate-buffered saline (PBS). They were incubated with 0.15% trypsin and 0.012% EDTA in PBS at 37 °C for 10 min under gentle stirring. The supernatant containing the dissociated cardiomyocytes was mixed with DMEM supplemented with 10% FCS, and centrifuged at 1000 rpm for 5 min. The pellet was then re-suspended in 10 mL of DMEM with 10% FCS and incubated on a glass dish for 1 h to remove fibroblasts. The floating cardiomyocytes were collected and re-plated at $5 \times 10^5/cm^2$ on gelatin-coated glass bottom dishes (Asahi Techno Glass). All the experiments using animals were approved by the Animal Experiment Committee at AIST.

2.3. Immunoblotting analysis

Human MSC cell lines were homogenized in a lysis buffer containing 20 mM Tris–HCl (pH 7.4), 300 mM NaCl, 0.5 mM EDTA, 1% NP-40, and a complete protease inhibitor cocktail (Roche). After centrifugation at 13,000 rpm for 10 min at 4 °C, equal protein amounts were separated by SDS–PAGE (5–20%). The blots were incubated with antibodies against N-cadherin (1:200; C3865, Sigma), Flk-1 (1:100, 10347; IBL), c-Kit (1:200, AF332; R&D Systems), Integrin-α4 (1:200; sc-14008, Santa Cruz), VCAM-1 (1:200; sc-8304, Santa Cruz), PDGFRα (1:200; 323503, BioLegend), Nkx2.5 (1:200; sc-14033, Santa Cruz), GATA4 (1:200; sc-9053, Santa Cruz), or β-tubulin (1:1000, RB-9249; NeoMarkers). Proteins were detected with an enhanced chemiluminescence (ECL) reagent (Super-Signal West Femto Maximum Sensitivity Substrate, Pierce) using an LAS-3000 Image Analyzer (Fuji Film).

2.4. Flow cytometry analysis

All MSCs were harvested with cell dissociation buffer (GIBCO) and blocked with normal sheep IgG on ice for 1 h. Cells were incubated with biotinylated anti-N-cadherin antibody (1:100, BAF1388; R&D System), anti-Flk-1 antibody (1:100, 10347; IBL), and APC-conjugated anti-c-Kit antibody (1:100, 550412; Becton Dickinson) on ice for 1 h. The N-cadherin antibody was fluorescently labeled using Allophycocyanin-Alexa Fluor 750 streptavidin (Molecular Probes). The Flk-1 antibody was fluorescently labeled with the Alexa Fluor 488-conjugated secondary antibody (Molecular Probes). Cells were resuspended in buffer with propidium iodide (Sigma). Analysis was performed with a FACS Aria (Becton Dickinson) and FlowJo software (TOMY Digital Biology) with propidium iodide-negative population. The data were obtained from at least two independent experiments.

3. Results

MSCs are a mixture of primary adherent cells derived from the stroma of adult tissues. The multipotency of MSCs rapidly decreases as the passage number increases. Therefore, it is not easy to obtain reproducible data from these heterogeneous primary cells. To overcome these problems, we took advantage of immortalized human MSC clones with *bmi-1*, *TERT*, *E6*, and/or *E7*, which retain their multipotent differentiation ability over a long time when cultured *in vitro* [8].

Cardiac differentiation of human MSCs was performed by co-culturing with mouse fetal cardiomyocytes, which is a well-established method to differentiate MSCs into electro-physiologically validated cardiomyocytes [9,10]. Human MSC cell lines were labeled with a GFP-expressing lentivirus and then cultured on a cardiomyocyte feeder cells prepared from mouse embryonic heart tissue (Fig. S1A). On day 7, human MSC lines such as EPC-214 and UE7T-13 differentiated into cardiomyocytes. Around 5% of these GFP-labeled MSCs differentiated into beating cardiomyocytes (Fig. 1A). GFP-positive, differentiated cardiomyocytes showed autonomously periodical contractions (Fig. S1B). Significant number of GFP-positive human MSCs expressed cardiomyocyte-specific terminal differentiation markers (Fig. S1C, left and middle, Fig. S1D, left, and a confocal image Fig. S1E). On the other hand, some cell lines did not differentiate into beating cardiomyocytes under identical conditions (UE7T-9, UE6E7T-2) (Fig. S1C right and Fig. S1D right).

Next, the efficiency of human MSCs differentiation into spontaneously beating cardiomyocytes was quantified by counting the number of GFP-positive and spontaneously beating cardiomyocytes with a fluorescence microscope on day 7 (Fig. 1A). The

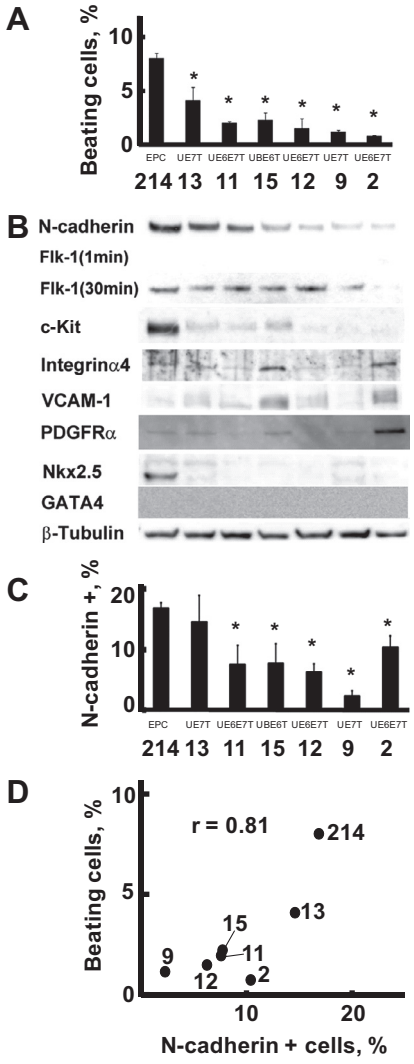

Fig. 1. Correlation between cardiomyogenic differentiation efficiency and cell surface protein marker expression in human MSC cell lines. (A) Autonomously beating cardiomyocytes differentiated from GFP-labeled human MSC lines, EPC-214, UE7T-13, UE6E7T-11, UBE6T-15, UE6E7T-12, UE7T-9, and UE6E7T-2, were counted under a fluorescence microscope. (B) The expression of cell surface proteins and transcription factors related to cardiovascular development were analyzed by immunoblotting of whole cell lysates. (C) Flow cytometric analysis of cell surface expression of N-cadherin in human MSC lines. (D) Correlation between the differentiation efficiency into cardiomyocytes and cell surface N-cadherin expression in human MSC lines. The vertical axis represents the differentiation efficiency. The horizontal axis represents cell surface expression of N-cadherin. The correlation coefficient (r) is shown on the graph. $^{*}P < 0.05$.

expression of various cell surface proteins, which are essential for the development of the heart *in vivo* or are specifically expressed in cardiovascular progenitor cells, was examined by immunoblotting (Fig. 1B). Among these markers, the expression of N-cadherin showed a good correlation with the differentiation efficiency toward beating cardiomyocytes. The MSC lines highly expressing N-cadherin showed higher differentiation ability toward cardiomyocytes. Flk-1 also showed an expression pattern similar to that of N-cadherin. However, the expression levels in human MSCs were very low. Only an extremely long exposure (30 min) enabled us to detect the Flk-1 protein bands (Fig. 1B). The expression of c-Kit showed some correlation with the cardiomyogenic differentiation abilities of these cells, although the expression levels of c-Kit in some human MSCs that readily differentiated into cardiomyocytes were very low (Fig. 1B, UE7T-13, UE6E7T-11, and UBE6T-15).

Other cell surface proteins have been reported as essential for heart development. Integrin α4 is essential for the development of the heart and placenta [11]; a homozygous null mutant of integrin α4 caused embryonic lethality due to defects in the epicardium and coronary vessel development, leading to cardiac hemorrhage, in addition to failure of fusion between the allantois and chorion during placentation. Knockout mice of vascular cell adhesion molecule 1 (VCAM-1) displayed a reduction in the compact layer of the ventricular myocardium and intraventricular septum [12]. Platelet-derived growth factor receptor α (Pdgfrα) is expressed in cardiac progenitor cells in the posterior part of the secondary heart field. Pdgfrα is also expressed in the valves and pericardia of the heart at E12.5–16.5 [13]. However, the expression of these cell surface proteins did not show a strong correlation with the differentiation ability of human MSCs into cardiomyocytes (Fig. 1A and B).

Next, we verified the cell surface-specific expression of N-cadherin, Flk-1, and c-Kit in living MSCs by flow cytometry. Flk-1 was barely detectable on the cell surface of human MSCs (Fig. 2A), although a positive control, HUVEC, showed strong cell surface expression of Flk-1 (Fig. 2A, right), indicating that human MSCs do not express detectable amounts of Flk-1 on the plasma membrane. The cell surface expression of c-Kit was also relatively low (Fig. 2B), and the MSC lines with higher differentiation ability toward cardiomyocytes did not show a significant amount of cell surface expression of c-Kit (Fig. 2B, UE7T-13).

By contrast, N-cadherin was readily detectable in the human MSC lines with high differentiation ability toward beating cardiomyocytes (Fig. 2C). When the cell surface expression of N-cadherin (Fig. 1C) and the differentiation ability into beating cardiomyocytes (Fig. 1A) were compared with human MSC cell lines, a strong correlation was observed between these 2 events ($r = 0.81$; Fig. 1D). Immunofluorescence analysis of the human MSC line EPC-214, which readily differentiated into cardiomyocytes, showed characteristic localization of N-cadherin in cell-to-cell contacts in addition to the uniform expression on the plasma membrane (Fig. 2D). However, UE7T-9 cells, which expressed N-cadherin at low levels and did not differentiate into cardiomyocytes, did not show significant expression of N-cadherin (Fig. 2D, right). These results suggest that N-cadherin could be a good prospective cell surface marker of cardiomyogenic human MSCs.

Next, we validated the expression of N-cadherin with various primary human MSCs. Human bone marrow-derives MSCs (BMSCs) cultured for a limited number of passages gave a good correlation between the cell surface expression of N-cadherin and the differentiation ability into beating cardiomyocytes (Fig. 3A and B). Human MSCs derived from adipose tissue (ASCs) also showed similar results (Fig. 3D and E). The Pearson's correlation coefficients of cell surface expression of N-cadherin and differentiation efficiency toward beating cardiomyocytes in BMSCs and ASCs were good in both cases (0.55 and 0.77, respectively; Fig. 3C and F). As for c-Kit, we failed to detect significant expression of c-Kit in primary MSCs that showed distinct cardiomyogenic differentiation abilities (Fig. S2, 36_2). c-Kit protein could be detected on the cell surface of some primary ASCs (Fig. S2B, 1212). However, the isolation of c-Kit-positive cells from these primary ASCs was not successful by flow cytometry.

To determine whether the N-cadherin-positive population of human MSCs has higher differentiation ability into cardiomyocytes than N-cadherin-negative MSCs, we established the separation conditions of N-cadherin-positive cells using MACS (Fig. S3). Then, the N-cadherin-positive fraction was concentrated from a primary culture of human ASCs (1212 used in Fig. 3) using the same method (Fig. 4A); these cells were further cultured on mouse embryonic heart feeder cells for 7 days. The enriched ASC fraction expressing cell surface N-cadherin showed a 4-fold higher cardiomyogenic differentiation ability than the N-cadherin-negative fraction (Fig. 4B).

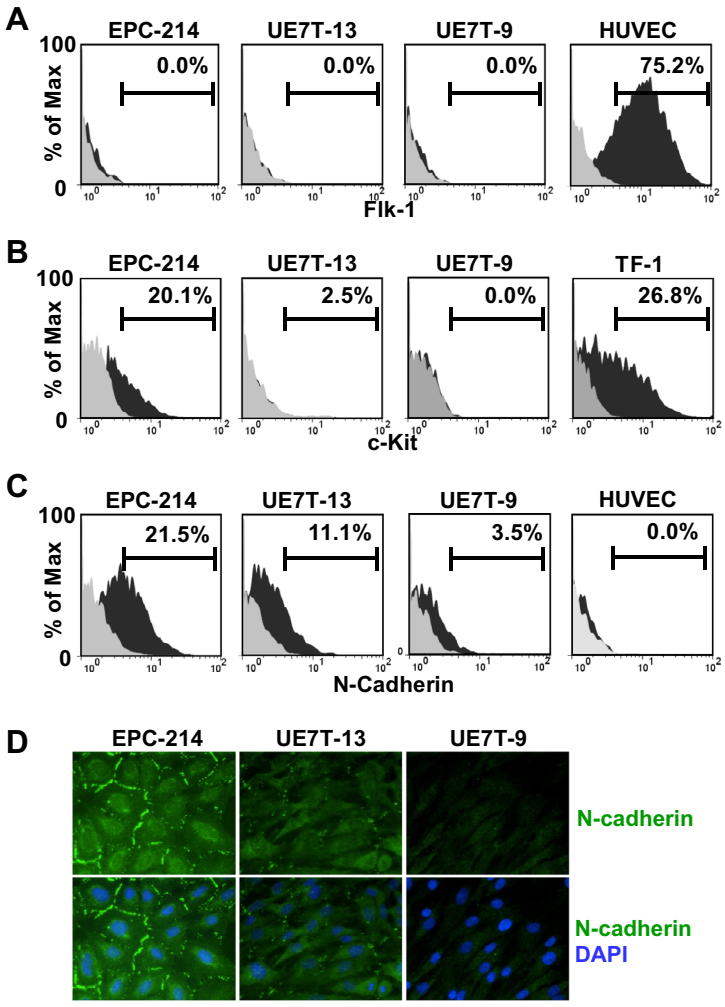

Fig. 2. Representative FACS plots of cell surface protein expression in human MSC cell lines. The live cells were immunostained with (A) Flk-1, (B) c-Kit, or (C) N-cadherin antibodies. Propidium iodide-positive cells were excluded from the analysis. (D) Localization of N-cadherin in human MSCs. The indicated human MSC lines were immunostained with N-cadherin antibody (green) and DAPI (blue). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

To characterize the cardiomyogenic N-cadherin-positive population, we analyzed the gene expression profiles of the MACS-sorted fractions with microarray. Gene ontology analysis of 3056 genes (among 44,000 genes) that exhibited more than a 1.5-fold difference identified molecular functions associated with zinc ion binding, transition metal ion binding, and nucleic acid binding (Fig. S4A) and biological functions involved in DNA binding, gene expression, transcription, and metabolic processes of nucleic acid (Fig. S4B). These results suggested that N-cadherin-positive cells showed higher expression of specific DNA-binding proteins and elevated metabolic activity. On the other hand, the N-cadherin-negative population showed higher expression of genes involved in the MHC class I protein complex, vacuole organization, and GTPase activity (Fig. S4C and S4D).

When the expression of various lineage marker genes was compared, the N-cadherin-positive fraction showed up-regulated expression of genes involved in the differentiation of cardiomyocytes and skeletal myocytes, such as *Nkx2.5*, *Hand1*, *Tnni3* (*cTnI*), and *Myog* (Fig. 4C). In contrast, ectodermal and endodermal lineage markers were the same among the MACS-sorted fractions, with the exception of *Pax4*, a transcription factor involved in pancreatic development. Although MSCs efficiently differentiate into osteoblasts, chondrocytes, and adipocytes, these specific markers did not show a large difference. The expression of MSC-specific cell surface markers was not increased in the N-cadherin-positive fraction.

Quantitative PCR analysis revealed significant up-regulation of a cardiomyogenic precursor-specific gene, *Nkx2.5*, in the N-cadherin-positive fraction, for more than 200-times higher than that in the N-cadherin-negative fraction. Two other transcription factors, *Hand1* and *Gata4*, but not Tbx5, also showed significantly elevated expression in the N-cadherin-positive fraction (Fig. 4D). Interestingly, the expression of *Myog*, a transcription factor involved in skeletal muscle development, was also elevated in the N-cadherin-positive fraction. Although terminal markers for cardiomyocytes, such as *Anp* and *cTnI*, showed higher expression in the N-cadherin-positive fraction (Fig. 4E), the expression levels of these terminal markers were very low, suggesting that N-cadherin-positive cells may be ready for differentiation, but not terminally differentiated into cardiomyocytes.

Interestingly, the expression of some pluripotency-specific genes such as *Oct4* (*Pou5f1*), *Sall4*, and *Nanog* was significantly up-regulated in the N-cadherin-positive population (Fig. 4F and G). However, these expression in human MSCs was not as high as that in human ES cells (Fig. 4F), suggesting that these genes up-regulated in N-cadherin-positive MSCs may not exhibit pluripotency as observed in ES/iPS cells.

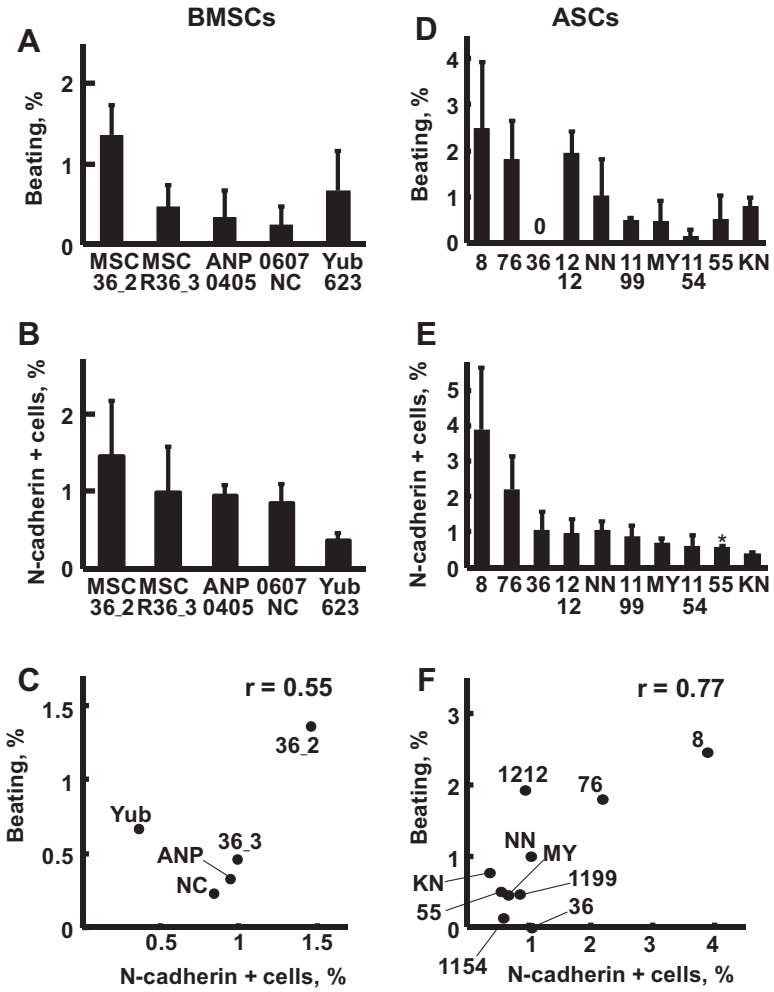

Fig. 3. Differentiation efficiency of primary human MSCs into cardiomyocytes and cell surface expression of N-cadherin. In this experiment, primary human MSCs derived from (A–C) the bone marrow (BMSCs) or (D–F) adipose-derived tissue (ASCs) were used. (A, D) Differentiation efficiency of primary human MSCs into cardiomyocytes. Autonomously beating cardiomyocytes differentiated from GFP-labeled human (A) BMSCs or (D) ASCs were counted using a microscope. (B, E) Flow cytometric analysis of cell surface expression of N-cadherin in primary human (B) BMSCs or (E) ASCs. $^{*}P < 0.05$. (C, F) Correlation between the differentiation efficiency into beating cardiomyocytes and the cell surface N-cadherin expression of primary human (C) BMSCs or (F) ASCs. The correlation coefficient (r) is shown on the graph.

4. Discussion

In this study, we have identified N-cadherin as a reliable cell surface marker for human MSCs with higher differentiation ability toward cardiomyocytes. N-cadherin is continuously expressed from cardiomyogenic progenitor cells to mature cardiomyocytes in the adult heart. N-cadherin maintains the functional gap junction complex at the plasma membrane in the adult heart, and conditional knockout of N-cadherin in mice resulted in arrhythmia in adult hearts with significant decreases in Cn43 and Cn40 [17].

We have previously shown that the cardiomyogenic progenitor cells differentiated from mouse ES cells expressed high levels of N-cadherin on the cell surface membrane, and an antibody against N-cadherin could be used to concentrate the progenitor cells from a heterogeneous cell population differentiated from mouse ES cells [18]. Although the possible differentiation pathway of cardiomyocytes from pluripotent ES cells and multipotent MSCs may not be the same, N-cadherin could be a common progenitor marker of the cardiomyogenic cells derived from these stem cells.

In addition to cardiomyogenic genes, we observed increased expression of pluripotency-specific transcription factors of ES cells, such as *Oct4*, *Sall4*, and *Nanog*, in the N-cadherin-positive fraction. Recently, *Oct4* has been suggested to be the gatekeeper into and out of the reprogramming expressway [19]. Therefore, the elevated expression of *Oct4* and related transcription factors could positively modulate the differentiation ability of MSCs. For example, overexpression of the *Oct4* gene enhanced the differentiation ability of MSCs [14], and knockdown of *Oct4* caused loss of multiple differentiation potential [15]. Nanog was also shown to possess similar activity in BMSCs [16]. Therefore, N-cadherin-positive cells with up-regulated expression of *Oct4* and other transcription factors responsible for cardiomyogenesis may increase the differentiation ability of MSCs into cardiomyocytes.

N-Cadherin is localized in the cell–cell contacts of cardiomyocytes and plays essential roles for formation of the cardiac intercalated disk structure that electromechanically couples adjacent cardiac myocytes. Addition of antibodies against N-cadherin to the cultured cardiomyocytes [20], mesodermal explants [21] or injected into embryos [22] caused a reduction in the number of myofibrils and destroy stress fibers [23]. In primary cardiomyocytes dissociated from adult rat heart, N-cadherin diffusely distributed around the cell periphery begins to co-localize with desmocollin, plakoglobin, and plakophilin-2 at the cell contact sites. The newly generated adhesive contacts sequentially recruit desmoplakin, intermediate filaments, connexin-43, and ankyrin-G. Subsequently, the voltage-gated sodium channel is incorporated into mature intercalated disks. This assembly process requires the clustering of transmembrane adhesive contacts with N-cadherin

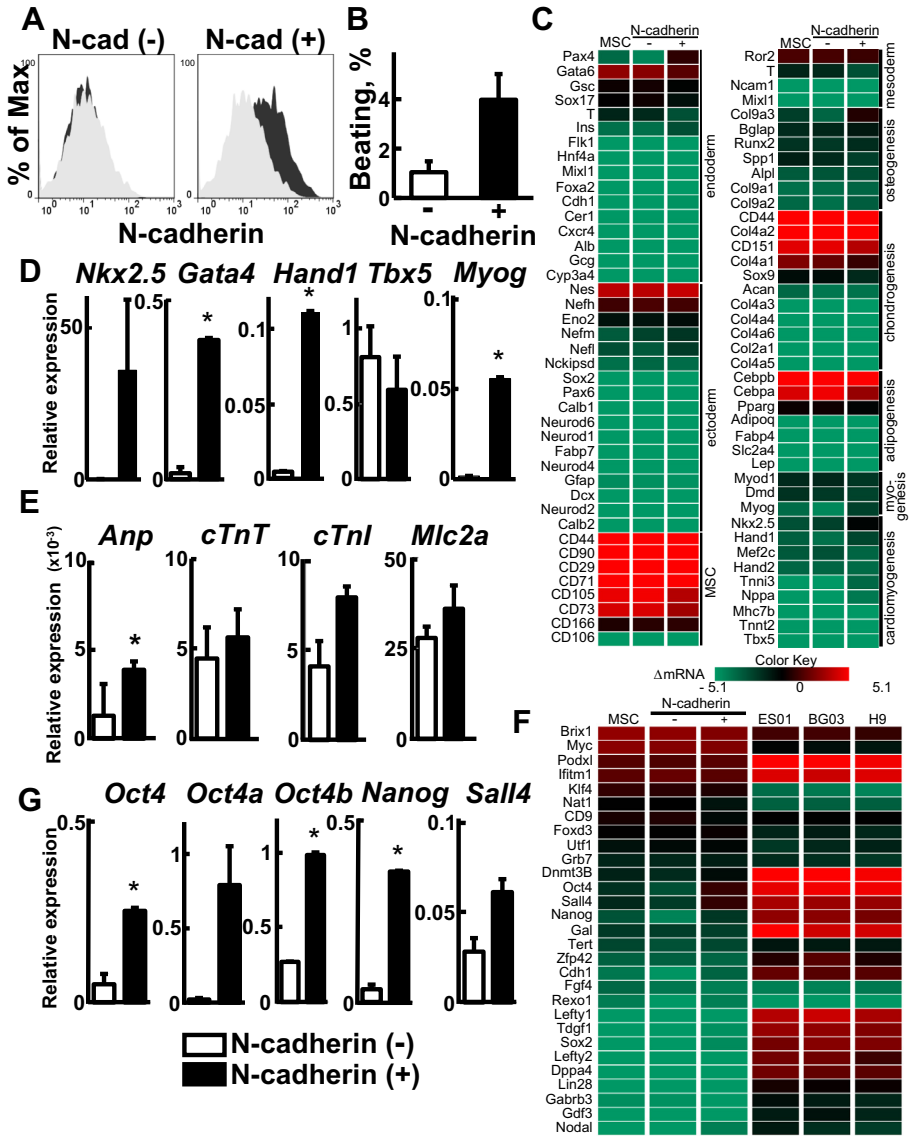

Fig. 4. Separation of N-cadherin-positive cells from the primary MSCs derived from adipose tissue (1212) with anti-N-cadherin antibody-conjugated magnetic beads. (A) The FACS histograms represent cell surface N-cadherin expression in the N-cadherin-negative fraction (left) and N-cadherin-positive fraction (right). (B) Differentiation efficiency of the purified primary ASCs into beating cardiomyocytes. Bar graphs represent the mean value of differentiation efficiency obtained from two independent experiments. (C) Heat map profile of lineage-specific differentiation marker expression in ASCs. The N-cadherin-positive fraction showed elevated expression of specific genes involved in cardiomyogenesis. (D, E) qPCR analysis of cardiomyogenic progenitor-specific transcription factors (D) and terminal differentiation markers for heart development (E). (F) Heat map profile of pluripotency-specific marker expression in human ASCs and human ES cells (ES01, BG03, and H9). (G) qPCR analysis of the expression of pluripotency-specific transcription factors in MACS-sorted fractions. $^{*}P < 0.05$. (For interpretation of the references to colour in (C) and (F), the reader is referred to the web version of this article.)

[24]. Therefore, MSCs with higher expression of N-cadherin may have preferable potential for the differentiation into cardiomyocytes. N-cadherin is expressed in pericytes and is involved in the interaction between pericytes and endothelial cells during vessel formation *in vivo* [25,26]. Pericytes in MSCs could be one of the possible cell sources that show higher differentiation ability toward cardiomyocytes.

Disclosure statement

No competing financial interests exist.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.081.

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