**Migration-driven aggregate behaviors of human mesenchymal stem cells
on a dendrimer-immobilized surface direct differentiation toward a
cardiomyogenic fate commitment**

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Footnote: Much of this work forms the basis of the Ph.D. dissertation of Yuuki Ogawa

ABSTRACT

Dynamic behaviors of cell aggregates on a dendrimer surface were investigated to drive the directed differentiation of human mesenchymal stem cells (hMSCs) toward a cardiomyogenic lineage. Cell aggregates on the polyamidoamine dendrimer surface with fifth-generation (G5) of dendron structure showed dynamic changes in morphology associated with repetitive stretching and contracting during migration. Spatial-temporal observations revealed cellular movement in single aggregates by their morphological change through stretching and contracting on the G5 surface, suggesting that the dynamic behavior of aggregate causes mixing of cells. However, aggregates without cell–substrate adhesions on the low-binding culture surface sustained their spherical morphology without cellular movement within a single aggregate. Furthermore, β -catenin was observed at nuclei in aggregates on the G5 surface, and expression of the cardiomyocyte marker cardiac Troponin T (cTnT) was detected. However, β -catenin localized to the nuclei only in the outer region of the aggregate on the low-binding culture surface, and cTnT expression was restricted at the exterior surface of the aggregates. These observations indicate that cell mixing within aggregates on the G5 surface induced the directed differentiation of hMSCs toward a cardiomyogenic lineage by nuclear translocation of β -catenin through dissociation of cell–cell adhesions. These results suggest that migration-driven aggregate behaviors on the dendrimer surface caused repeated morphological changes of aggregate through stretching and contracting, leading to the directed differentiation of hMSCs toward a cardiomyogenic fate commitment.

[Key words: migration-driven aggregate behaviors, dendrimer surface, cell–cell adhesion, cardiomyogenic lineage, human mesenchymal stem cells]

INTRODUCTION

Human mesenchymal stem cells (hMSCs) can differentiate into several cell types, such as chondrocytes, adipocytes, osteoblasts, neurons, and cardiomyocytes (1-3). Uncommitted hMSCs have extensive proliferative ability in culture while retaining their multilineage differentiation potential, which makes them attractive candidates for cell-based therapeutic approaches (4). However, the low differentiation efficiency and poor reproducibility of hMSCs is a major limitation to their clinical use.

To establish cellular microenvironments that promote differentiation into objective cell types, many researchers have focused on exogenous stimulants such as growth factors and extracellular matrices to mimic the native microenvironment (2-5). Transforming growth factor- β (TGF- β) superfamily members direct differentiation of hMSCs toward a specific lineage: Mohanty et al. (5) reported that TGF- β 1 contributes to hMSC differentiation into a cardiomyogenic lineage, showing 21% cardiomyocyte-like cells. An alternative approach to hMSC differentiation involves endogenous stimulation by regulation of cell behaviors (6-8). The endogenous Rho GTPase family includes RhoA, Rac1, and Cdc42 and regulates the assembly and disassembly of actin cytoskeleton, thereby leading to changes in cellular behaviors such as cell migration (9-12). Rac1 activation is required to promote actin polymerization for cell extension, whereas RhoA activity regulates the formation of actin stress fibers and is antagonistic toward Rac1 activity (10,11). Activation of Rac1 induces differentiation of hMSCs into the myogenic lineage, and its inactivation induces differentiation into the chondrogenic lineage (6). Thus, endogenous Rho family GTPase signaling pathways are considered to play a central role in regulation of cell fate decisions, and the fate choice relies on modulation of the network of transcription factors. Furthermore, stimulation of cell-cell adhesion by aggregate culture has been shown to facilitate hMSC differentiation (13), suggesting that aggregate culture and intercellular communication play significant roles in regulating cell differentiation.

In our previous studies (14-16), we demonstrated that formation of hMSC aggregates on the

dendrimer surface through active migration with morphological changes led to directed differentiation of hMSCs toward a cardiomyogenic fate commitment. Repetitive collapse and re-formation of the aggregates increased the population of cardiomyocyte-like cells by passage culture of aggregates on a new dendrimer surface (15). In this study, we investigated the migratory behaviors of hMSC aggregates on the dendrimer surface to enhance directed differentiation toward a cardiomyogenic lineage. Based on examination of aggregate migratory behaviors on the dendrimer surface, we discuss the fundamental mechanisms of cell–cell and cell–substrate adhesion in relation to guidance of cell fate.

MATERIALS AND METHODS

Cells and culture conditions

Bone marrow-derived hMSCs (Lot no. 0000183402; Lonza, Walkersville, MD, USA) were used in the following experiments. Routine subcultures were conducted as described previously (16). For all experiments, hMSCs were harvested in Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and antibiotics (Life Technologies, Carlsbad, CA, USA). The seeding density was 5.0×10^3 cells/cm², and medium changes were performed every 3 days.

hMSCs were exposed to medium with 50 µg/mL Rac1 activator HMG-1 (Sigma-Aldrich) or 5 µg/mL Rac1 inhibitor NSC23766 (Calbiochem, Merck, Darmstadt, Germany) from day 3 to day 10 to activate or inhibit migratory behaviors of cell aggregates, respectively.

Preparation of dendrimer-immobilized surface

The fifth-generation dendrimer surface (G5 surface) was prepared on the tissue-cultured polystyrene (PS) surface of a square 8-well plate (Nunc, Roskilde, Denmark) and 35 mm culture dish (Ibidi GmbH, Martinsried, Germany) as described previously (16). Briefly, hydroxyl groups on the starting materials were displayed by addition of potassium *tert*-butoxide. Then, dendron structures

1 were constructed by alternately adding glutaraldehyde and Tris(2-aminoethyl)amine solution (pH 9.0,
2 adjusted with 0.1 mol/L NaOH), respectively, five times. D-glucose was displayed as a terminal
3 ligand.

4 **Time-lapse observations**

5 Time-lapse observations were carried out by obtaining images every 10 min at several positions
6 using an observation tool (BioStudio-T; Nikon Engineering, Kanagawa, Japan).

7 To track individual nuclei within the aggregates, the cells were transfected with nucleus-green
8 fluorescent protein (GFP) by baculovirus (CellLight nucleus-GFP; Life Technologies) according to
9 the commercially available protocol. There was no significant difference in migratory behavior and
10 differentiation between cultures of transfected cells and untransfected cells; therefore, transfected
11 cells were not isolated from untransfected cells. Cells were then stained with a cytoplasmic dye
12 (CellTracker™ Orange CMTMR; Life Technologies) used to distinguish hMSC aggregate behavior
13 according to the commercially available protocol. Images were obtained every 20 min using a
14 time-lapse confocal laser scanning microscope (FV-10i; Olympus, Tokyo, Japan) through a 60×
15 objective lens. Tracking imaging analysis of the migration of individual cells within the aggregate
16 was performed using image processing software (Imaris; Bitplane AG, Zurich, Switzerland).

17 **Immunofluorescence staining**

18 Immunostaining was carried out as described previously (14). Briefly, cells were fixed with 4%
19 paraformaldehyde. Then, the cells were permeabilized by incubation in PBS with 0.5%
20 polyoxyethylene (10) octylphenyl ether. After masking non-specific proteins (Block Ace; Dainippon
21 Sumitomo Pharma Co., Ltd., Osaka, Japan), the cells were incubated with anti-β-catenin primary
22 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-cardiac Troponin T (cTnT)
23 antibody (Abcam, Cambridge, UK). The cells were reacted with secondary antibody (Alexa Flour
24 488-conjugated anti-mouse IgG; Life Technologies). Nuclei and F-actin were stained with
25 4',6-diamidino-2-phenylindole (DAPI) and rhodamine phalloidin (Life Technologies) respectively.

Images were obtained using a confocal laser scanning microscope (FV-1000; Olympus) through a 60× objective lens.

Determination of the cardiomyogenic potential of aggregates

Quantitative assessment of cardiomyogenic differentiation was conducted as described previously (15). Briefly, cells incubated for a specified number of days were collected by enzymatic treatment and re-seeded on the PS surface. The cells were stained using cTnT antibody as above. The ratio of cTnT-positive cell number to DAPI-positive cell number (X_P/X_T) was calculated.

Statistical analysis

All experiments were performed at least three times, and data are expressed as means with standard deviations. Student's *t*-test and the Tukey-Kramer method were used to determine statistical significances among the data sets; *p*-values less than 0.01 were considered significant.

RESULTS

Dynamic behaviors of hMSC aggregates

To understand the behavioral changes in hMSC aggregates, we performed time-lapse imaging of representative aggregates cultured on the G5 surface and low-binding culture surface. The cells on the G5 surface showed repetitive morphological changes through stretching and contracting during migration, and their migratory behaviors were repeated for cultivation from day 7 to day 10 (Movie S1). However, cells on the low-binding culture surface formed ball-like cell aggregates and maintained this shape over the entire observation period (Movie S2).

To observe the movement of nuclei within a single aggregate during migration, the nucleus-GFP-transfected hMSC aggregates were labeled with cell-tracker dye, and time-lapse series were acquired using a confocal fluorescence microscope at day 8. On the G5 surface, active cell migration was observed in cells located at the periphery of the aggregates, and cell migration leading to the stretching and contracting of aggregates was observed (Fig. 1, Movie S3, and Movie S4). The

Fig. 1

nuclear position of cells attached to the surface (marked by a cross in each image) moved toward the upper side of the aggregate during contraction, indicating nuclear mixing within the aggregate. However, on the low-binding culture surface, aggregates maintained their spherical morphology without any drastic change in nuclear position (Fig. 1 and Movie S5). These results considered that the dramatic change in aggregate morphology through active migration on the G5 surface caused mixing of cells in aggregate.

β -catenin translocation into nucleus and cTnT-based cardiomyogenic potential

Translocation of β -catenin into the nucleus and cardiomyogenic potential were observed by immunostaining the hMSC aggregates for β -catenin and cTnT on the G5 surface and low-binding culture surface at day 8. Punctate β -catenin staining was observed at contact sites between adjacent cells irrespective of the surface (Fig. 2). Comparison β -catenin nuclear translocation within aggregates revealed its distribution in the nucleus throughout the entire aggregate from central to peripheral regions on the G5 surface, whereas it was detected in the nucleus only at the peripheral region on the low-binding culture surface (Fig. 2A and Fig. 2B).

Similarly, cTnT was strongly detected throughout the entire aggregate on the G5 surface at day 8 (Fig. 3A) but only in the peripheral region of the aggregate on the low-binding culture surface. The quantitative estimation of the cardiomyogenic potential was calculated by the ratio of cTnT positive cells (X_P/X_T) in culture of hMSCs on both surfaces. As shown in Fig. S1, the X_P/X_T value in hMSCs cultured on the G5 surface was 2.2-fold higher than that on the low-binding culture surface.

Effect of migratory behaviors on cardiomyogenic potential of hMSC aggregates

The effect of migratory behaviors on the cardiomyogenic potential of hMSCs was studied by exposure to medium with 50 μ g/mL Rac1 activator or 5 μ g/mL Rac1 inhibitor from day 3 to day 10, and mRNA expression of Rac1 was examined at days 8 and 10 in hMSCs cultured under these conditions. The expression levels of Rac1 in hMSCs cultured on the G5 surface were significantly increased or decreased with exposure to Rac1 activator and Rac1 inhibitor both at days 8 and 10,

1 compared to those without exposure (Fig. S2).

2 In cultures exposed to Rac1 activator, the aggregates on the G5 surface maintained dynamic
3 morphological changes through stretching and contracting from day 7 to day 10 (Movie S6),
4 although the morphological change of aggregate on the G5 surface without exposure decreased from
5 day 7 to day 10. In addition, Rac1 activator-exposed cells exhibited mixing of cells in the aggregate
6 at days 8 and 10 through active migration of cultured cells attached to a surface (Movie S7 and
7 Movie S8), although the movement was suppressed at day 10 in culture without exposure (Movie S9).
8 This migratory pattern of nuclei within the aggregate mimicked that in unexposed cells at day 8. In
9 contrast, incubation with the Rac1 inhibitor led to a flattened and well-spread aggregate morphology
10 without any spontaneous periodic change (Movie S10). The coordinated movement was suppressed
11 at days 8 and 10, which are correlated with suppression of the active migration of leader cells to
12 surface (Movie S11 and Movie S12).

13 The cardiomyogenic potential, the X_P/X_T value was calculated in culture of hMSCs on the G5
14 surface with Rac1 activator or Rac1 inhibitor from day 3 to day 10. As shown in Fig. 4, the X_P/X_T Fig. 4
15 value with Rac1 activator was higher than that without Rac1 activator at both days 8 and 10. In
16 addition, the X_P/X_T value of Rac1 activator-exposed cells at day 10 was higher than that at day 8.
17 However, the X_P/X_T values of Rac1 inhibitor-exposed cells both at days 8 and 10 were lower than
18 that of unexposed cells. In addition, the X_P/X_T values for aggregates cultured on the low-binding
19 culture surface with Rac1 activator or Rac1 inhibitor were evaluated. In contrast to hMSC aggregates
20 on the G5 surface, the X_P/X_T values were the same as those derived from unexposed cells at day 10,
21 regardless of culture conditions (Fig. S3).

22 DISCUSSION

23 **Surface-driven migratory behavior of aggregates leads to coordinated movement within**
24 **the aggregate**

1 The formation of aggregates is an important event influencing the fate of cultured stem cells,
2 and the mechanisms of aggregate migratory behaviors need to be elucidated to understand cellular
3 differentiation. In this study, we investigated their effects on the directed differentiation of hMSCs
4 toward a cardiomyogenic fate commitment accompanied by dynamic migration behaviors within
5 the aggregates on the G5 surface (Fig. S4). The main feature of aggregate migration behavior is the
6 emergence of leader cells and their subsequent migration that is accompanied by nearby cells called
7 follower cells, thereby causing collective migration. Time-lapse observation (Movie S1) revealed
8 that aggregates grown on the G5 surface actively migrated and showed morphological fluctuations
9 with repetitive stretching and contracting. In addition, time-lapse fluorescent observation of nuclei
10 (Movie S2) revealed that dynamic change of aggregate morphology on the G5 surface caused the
11 cellular mixing in aggregate. However, aggregates without cell–substrate adhesion on the
12 low-binding culture surface maintained their morphology without any drastic change of nuclear
13 location within a single aggregate (Movie S5). In the analysis of lineage commitment of hMSCs
14 toward a cardiomyogenic fate, the expression of cTnT was prominently seen throughout aggregates
15 on the G5 surface but was exclusively located around the aggregates on the low-binding culture
16 surface (Fig. 2 and Fig. 3). For further enhancement of directed differentiation of hMSCs toward a
17 cardiomyogenic lineage, we applied the Rac1 activator and Rac1 inhibitor to regulate the migratory
18 behaviors of the aggregates on the G5 surface. In culture, exposure to the Rac1 activator caused
19 repetitive morphological changes through stretching and contracting during active migration of
20 cells on the G5 surface and enhanced directed differentiation of hMSCs toward a cardiomyogenic
21 lineage (Movie S6 and Fig. 4). The difference in migratory behavior of cell aggregates resulted in
22 varied properties between attached aggregates and unattached, floating aggregates. The dramatic
23 morphological change of aggregates through active migration on the G5 surface can lead to
24 coordinated movement (passive) of individual cells within the aggregate. Migration determines the
25 balance between cell contractile forces and cell–substrate adhesion through the rearrangement of

1 integrin-mediated cell adhesion; thus, cell contractions can cause a secondary wave of
2 mechano-regulated outside-in and internal cell signal changes that control cell fate decisions (17).
3 Taken together, these findings revealed that the coordinated movement of cells driven by cell
4 adhesion to the G5 surface led to directed differentiation of hMSCs toward a cardiomyogenic fate
5 commitment.

6 **Migration-driven aggregate behaviors direct differentiation toward a cardiomyogenic**
7 **fate commitment through cell–cell dissociation**

8 The spontaneous formation and migratory behaviors of aggregates affect cell–substrate and
9 cell–cell adhesions within aggregates and thereby regulate cell differentiation signaling (14,15).
10 Within migrating aggregates, cell–cell and cell–substrate adhesions are integrated so that cells
11 maintain interactions with neighboring cells and the underlying substratum. The coordinated
12 organization of cadherin-based and integrin-based adhesion complexes is required for coordinated
13 movement of large groups of cells, and spatiotemporal regulation of Rho family GTPases and actin
14 dynamics is required for collective cell migration (17-20). These mechanisms are dependent on the
15 synthesis and degradation of the extracellular matrix (7). Our previous reports indicated that the
16 dynamic cellular behaviors on the G5 surface were changed due to the adsorption and assembly of
17 fibronectin (16). On the G5 surface, adsorption of fibronectin induces the extension of cells, and
18 that fibronectin assembly affected by matrix metalloproteinases plays important roles in the
19 stabilization of focal adhesions associated with cytoskeletal formations. Because paxillin
20 phosphorylation enhances the turnover of focal adhesion with lamellipodium protrusion, focal
21 adhesion turnover is thought to be promoted by active cell migration through paxillin
22 phosphorylation (16). In addition, cadherins at cell–cell adhesion sites may show responses to
23 mechanical signals similar to paxillin at focal adhesions, which is supported by the similarity of
24 interactions in cell–cell and cell–substrate adhesion structures with the actin cytoskeleton (16,18).
25 These results suggest that extracellular signals can be locally integrated by the endogenous Rho

family GTPases to coordinate the dynamic cytoskeletal rearrangements and cell-cell communication necessary for directing stem cell fates. The β -catenin plays important roles in stem cell differentiation as a transcription factor and cell-cell adhesion by connecting to N-cadherin. Dynamic changes in β -catenin expression and transcriptional activity were regulated in response to altered cell adhesion during migration. Consistent with previous results (15), cell aggregates on the G5 surface induced cTnT expression due to the morphological changes, and the results from this study suggest that β -catenin signaling in response to changes in migration behaviors of hMSC aggregates may be implicated in the guidance of cardiomyogenic fate. β -catenin was expressed at the interface among cells in the aggregates on the G5 surface and the low-binding culture surface. In addition, on the G5 surface, β -catenin at the nuclei and cTnT were observed throughout the aggregates, whereas they were only detected at the outer layer of the aggregates on the low-binding culture surface (Fig. 2 and Fig. 3). The changes in β -catenin transcriptional activity with relocalization of the position of each cell within the aggregate, which made dynamic morphological changes on the G5 surface combined with changes in N-cadherin expression indicate that cell association during aggregate migration can affect subsequent pathway activation and differentiation. It is likely that similar interrelated signaling between cadherins and β -catenin may enable the pool of cadherin-bound β -catenin to become available for nuclear translocation and signaling upon remodeling of cadherins during active migration of aggregates. The cytoplasmic stabilization of dephosphorylated β -catenin permits the translocation of β -catenin to the nucleus, where it acts as a transcriptional co-activator with T-cell factor/lymphoid enhancer-binding factor to regulate the transcription of target genes during stem cell differentiation (21-25). Therefore, the morphological changes in aggregates due to adsorption and assembly of fibronectin on the G5 surface led to the dissociation of cell-cell adhesion, introducing to the directed differentiation toward a cardiomyogenic lineage through transcriptional activity of β -catenin.

In conclusion, the present study indicates that the migratory behaviors of aggregates on the G5

1 surface associated with morphological changes in the aggregates through stretching and contracting
2 induce directed differentiation of hMSCs toward a cardiomyogenic fate commitment. The active
3 migration of cells on the G5 surface leads to coordinated movement of cells within the aggregates
4 during stretching and contracting. We found that the nuclear translocation of β -catenin due to the
5 cell movement within the aggregate plays important roles in the directed differentiation of hMSCs
6 toward a cardiomyogenic fate commitment. These results suggest that the G5 surface offers a
7 model for substrate design based on aggregate migratory behaviors as a tool for driving a
8 cardiomyogenic lineage specification in an ex vivo stem cell culture system.

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FIGURE LEGENDS

FIG. 1. Still images of time-lapse fluorescence observations of cytoplasm (red) and GFP-transfected nuclei (green) in hMSC aggregates cultured on the G5 surface (A–D) and low-binding culture surface (E and F) at day 8. The images show 3D-constructed images of aggregates. Panels A–D show the morphological fluctuations with repetitive stretching (A, B) and contracting (C, D) of aggregates grown on the G5 surface. Scale bars: 50 μm .

FIG. 2. Immunostaining of β -catenin (green) in hMSCs cultured on the G5 surface (A) and low-binding culture surface (B) at day 8. Cell nuclei were stained with DAPI (blue). Panels A and B show confocal images of β -catenin and nuclei in the middle sections of x - z stack images. Panels A1–B2 show enlarged images of the white boxed areas of panels A and B. Red arrows show the translocation of β -catenin into the nucleus. Scale bars: 50 μm (A, B) and 10 μm (A1, B2).

FIG. 3. Immunostaining of cTnT (green) in hMSCs cultured on the G5 surface (A) and low-binding culture surface (B) at day 8. Cell nuclei were stained with DAPI (blue). The confocal images of cTnT are from the middle section of the x - z stack images. Scale bars: 50 μm .

FIG. 4. Ratio of cTnT positive cells, X_P/X_T , in hMSCs on the G5 surface in culture conditions when exposed to medium without (A) and with Rac1 activator (B) and Rac1 inhibitor (C) from day 3 to day 10 by co-immunostaining with cTnT and DAPI after re-seeding to the PS surface by collecting all cells with enzymatic treatment at days 8 and 10. Open and closed bars show X_P/X_T at days 8 and 10, respectively. Vertical bars indicate the standard deviations ($n = 3$). Statistical significance was according to one-way analysis of variance (ANOVA) and Tukey-Kramer post-hoc test ($*p < 0.01$).

Fig 1

Fig 2

Fig 3

Fig 4