Journal of Bioscience and Bioengineering
VOL. xx No. xx, 1–6, 2016

www.elsevier.com/locate/jbiosc

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

Yuuki Ogawa, Mee-Hae Kim, and Masahiro Kino-oka*

Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan

Received 22 December 2015; accepted 19 April 2016
Available online xxx

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]

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-β1 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

* Corresponding author. Tel.: +81 6 6879 7444; fax: +81 6 6879 4246.
E-mail address: kino-oka@bio.eng.osaka-u.ac.jp (M. Kino-oka).
Much of this work forms the basis of the Ph.D. dissertation of Yuuki Ogawa.

1389-1723/$ – see front matter
http://dx.doi.org/10.1016/j.jbiosc.2016.04.005

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

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

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

Immunofluorescence staining Immunostaining was carried out as described previously (14). Briefly, cells were fixed with 4% paraformaldehyde. Then, the cells were permeabilized by incubation in PBS with 0.5% polyoxyethylene (10) octylphenyl ether. After masking non-specific proteins (Block Ace; Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan), the cells were incubated with anti-β-catenin primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-cardiac Troponin T (cTnT) antibody (Abcam, Cambridge, UK). The cells were reacted with secondary antibody (Alexa Flour 488-conjugated anti-mouse IgG; Life Technologies). Nuclei and F-actin were stained with 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).

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.jbiosc.2016.04.005.

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, Movies S3 and S4). The 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

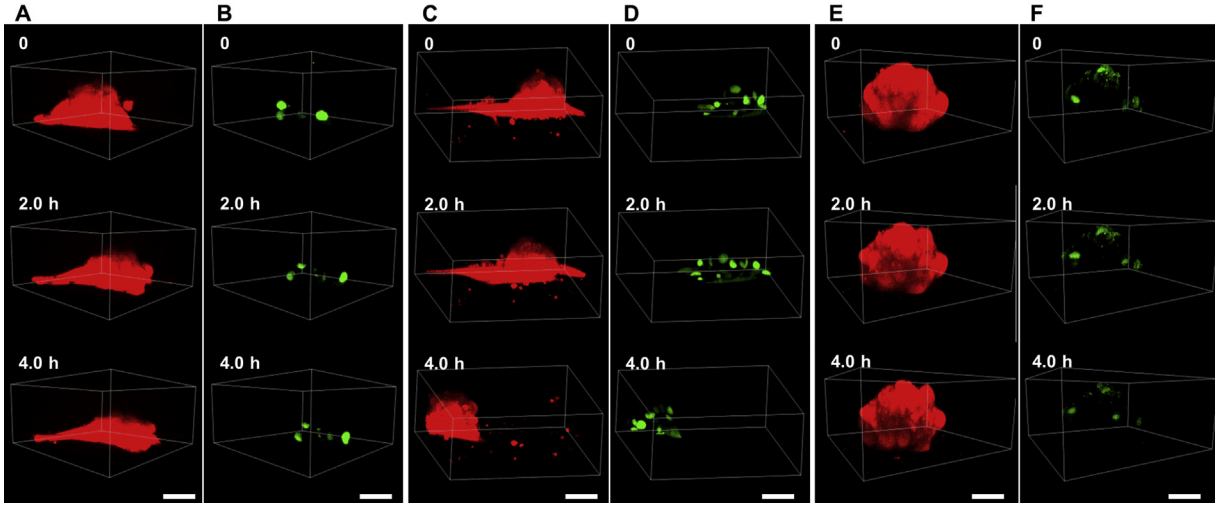

FIG. 1. Still images of time-lapse fluorescence observations of cytoplasm (red) and GFP-transfected nuclei (green) in hMSC aggregates cultured on the G5 surface (A–D) and low-binding culture surface (E, F) at day 8. The images show 3D-constructed images of aggregates. Panels A–D show the morphological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

Please cite this article in press as: Ogawa, Y., et al., Migration-driven aggregate behaviors of human mesenchymal stem cells on a dendrimer-immobilized surface direct differentiation toward a..., J. Biosci. Bioeng., (2016), http://dx.doi.org/10.1016/j.jbiosc.2016.04.005