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Directed differentiation of human mesenchymal stem cells toward a cardiomyogenic fate commitment through formation of cell aggregates☆

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ABSTRACT

Cell morphology is known to modulate the multipotential lineage commitment of stem cells. We provide a new strategy to induce the early lineage commitment of human mesenchymal stem cells (hMSCs) toward a cardiomyogenic fate through the formation of cell aggregates. A surface-immobilized polyamidoamine dendrimer with fifth generation of dendron structure was used during the culturing of hMSCs. These hMSCs cultured on the G5 surface formed aggregates through active migration and division. More than 22% of cardiac troponin-T (cTnT)-positive (cTnT$^+$) cells in aggregates formed on the dendrimer surface; the population formed on the dendrimer surface was higher than that in conventional culture vessel. When cell aggregate was reseeded onto a fresh G5 surface, single cells migrated out of the aggregates, proliferated, and formed new aggregates. This passage method, accompanied with repetitive aggregate dispersion and formation, was applied to cultures over 40 days. The proportion of cTnT$^+$ cells increased to 62% by the end of third passage. Our results suggest that culturing hMSCs on G5 surface results in directed commitment of the hMSCs toward a cardiomyocyte-like fate.

1. Introduction

Mesenchymal stem cells (MSCs) are attracting increased attention because they possess multi-lineage developmental potential and the capacity for self-renewal *in vivo* [1,2]. MSCs are multipotent cells, initially isolated from the bone marrow, and are noted for their ability to differentiate into osteoblasts, chondrocytes, and adipocytes [2]. As an autologous source of stem cells, MSCs have been considered a possible source of cells that can be applied to regenerative therapies [1]. To facilitate *in vitro* development, many researchers have attempted to design microenvironments that mimic the stem cell niche, thereby driving cells down their preferred differentiation pathway [3–5]. The niche paradigm outlines three interactions involving cells, culture medium components, adjacent cells and substrates; these are cell-soluble factor, cell–cell, and cell–substrate interactions [3]. *In vitro* strategies for regulating stem cell behavior attempt to mimic developmental mechanisms and establish artificial environments that promote specific cell fates [2,5]. The majority of previous research studies have focused on dynamic process that are tightly orchestrated by the sequential expression of multiple signal transduction proteins and transcription factors working in a combination [4,6]. A number of signaling pathways (Wnts/Nodal) and growth factors (bone morphogenic proteins and fibroblast growth factor) have been implicated in the development of specialized cardiac subtypes [4–6]. The times and concentrations at which these factors affect differentiation toward the desired fate have been optimized. Although human MSCs (hMSCs) possess a self-renewing capacity, they display heterogeneous responses upon the induction of differentiation, resulting in a mixture of differentiated cells [5,6]. Zhang et al. [7] reported that culturing MSCs for 21 days with 5-azacytidine in culture medium induced 45% cardiomyocyte-like cells that were cardiac troponin-T positive (cTnT$^+$); however, other differentiation pathways were also followed leading to a heterogeneous population of differentiated cells. Fully functional cardiomyocytes with striated cytoskeleton and proper electrical coupling for hMSCs have not been observed. Overall, it appears that the best differentiation strategies produce homogenous populations of cardiomyocytes; these strategies tend to be reproducible, result in cells of the appropriate quality, and produce large quantities of cells.

In previous studies, the use of a dendrimer surface has been proposed as a method of regulating the morphology and function of cells [8–11]. Polyamidoamine dendrimers have been immobilized to culture surfaces, with the variations in generation number of the dendrimer surface shown to cause morphological changes in

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

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hMSCs. The observed morphological changes were accompanied by dynamic cytoskeletal formation in cells on the culture surface. Subsequent formation of cell aggregates led to cardiomyogenic differentiation of hMSCs *in vitro* [10]. It is believed that endogenous Rho family GTPase signaling pathways, accompanied by morphological changes, are an intrinsic cue for modulating transcription factors.

In the present study, we used a fifth generation of dendrimer surface in an attempt to induce initiation of lineage commitment of hMSCs toward a cardiomyogenic fate. Novel approaches to cell culture including aggregate dispersion and formation during passage were trialed, and prolonged culturing was conducted to enhance the homogeneity of the cardiomyocyte-like cell population.

2. Materials and methods

2.1. Cells and culture conditions

Human bone marrow-derived MSCs were obtained from Lonza (Lot no. 8F3543; Walkersville, MD, USA). Routine subcultures of hMSCs were conducted in a 75-cm^2 flask (Corning Costar, Cambridge, MA, USA) using hMSC growth medium (Lonza) at 37 °C in a humidified atmosphere containing 5% CO_2. Upon reaching 70% confluence, cells were detached by enzymatic treatment with a 0.1% trypsin/0.02% EDTA solution (Sigma–Aldrich, St. Louis, MO, USA). Cells that had undergone less than five passages were used in subsequent experiments.

For all experiments, hMSCs were expanded for the specified number of days in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) supplemented with 10% fetal bovine serum (Invitrogen, Grand Island, NY, USA) and antibiotics (100 U/cm^3 penicillin G, 0.1 mg/cm^3 streptomycin and 0.25 mg/cm^3 amphotericin B; Invitrogen). Cultures were grown in square 8-well plate with and without dendrimer immobilized to the surface. The seeding density was fixed at a viable cell concentration of 5.0×10^3 cells/cm^2 (X_0). Culture medium was replenished every 3 days. If necessary, round-bottomed 96-well plates were used to generate floating cell aggregates, which were used to prepare aggregates of hMSCs as controls.

2.2. Surface preparation

The dendrimer surface was prepared using the conventional tissue culture polystyrene (PS) surface of a square 8-well plate (surface area; 10.5 cm^2, Nunc, Roskilde, Denmark), as described previous report [8]. Briefly, a G5 surface was created, under sterile conditions, by changing the generation number of synthesized dendrimers over four reactions. Hydroxyl groups were displayed on the plain surface by pouring potassium *tert*-butoxide into the wells. Then, aqueous glutaraldehyde was introduced into the wells, with the wells were then treated with a tris(2-aminoethyl) amine solution to produce a dendron structure. The wells were rinsed with sterile water and the previous step repeated until the fifth generation of dendrimers was synthesized. To display glucose as a terminal ligand, D-glucose was applied.

2.3. Determination of cell growth and differentiation of aggregates

To determine the cell number (X_T), cells were enzymatically detached using a 0.1% trypsin/0.02% EDTA solution, followed by direct counting of suspended cells using trypan blue exclusion on a hemocytometer.

To analyze hMSC differentiation into cardiomyocytes, cells cultured for various periods were stained with a nuclear stain and an antibody against cTnT (Materials and Methods in the Supporting Information). For quantitative assessment of cardiomyogenic differentiation, suspended cells were re-seeded after harvesting from the cultures as described above. The numbers of $cTnT^+$ and the $DAPI^+$ nuclei were evaluated to determine the ratio of $cTnT^+$ (X_P) to $DAPI^+$ (X_T) cells.

2.4. Statistical analysis

All experiments were performed at least three times and data expressed as means with standard deviations. Student's *t*-test was used to determine statistical significances among the data sets; *p*-values less than 0.01 and 0.05 were considered significant.

3. Results

3.1. Dynamic behavior and spontaneous cardiomyogenic differentiation of hMSCs

Cultures of hMSCs were grown on G5 and PS surfaces in 8-well plates, and in round-bottom 96-well plates, with non-adhesive culture surfaces, for 10 days. Time-lapse observations (Fig. 1A and Movie S1) revealed that the morphology of almost all cells on the G5 surface was round with temporal stretching. After 3 days, these cells formed three-dimensional cell aggregates through coalescence between cells and aggregates. A remarkable change in shape with repeated extension and contraction of aggregates was occasionally observed, although the aggregates on G5 surface were loosely attached; these were relatively easy to collect after tapping of the culture vessels. However, cells on the PS surface were flat and demonstrated continuous stretching (Fig. 1B and Movie S2). Cells in round-bottom 96-well plates formed aggregates that did not adhere to the bottom of wells. These aggregates maintained a uniform size and shape over time (Fig. 1C).

To evaluate the degree of cardiomyocyte differentiation, single cells suspension of hMSCs were re-seeded on the PS surface after harvesting, and identified by immunofluorescent staining for cTnT. Fluorescent microscopy (Fig. 2) revealed two main types of cells cultured on G5 surface: small spindle-like and large polygonal cells. Staining for cTnT and F-actin revealed thin filaments corresponding to cTnT in the cytoplasm of the large polygonal cells. This pattern was not observed in cells with spindle-like cells.

To examine cell aggregates formed in response to environmental cues, time profiles of cell growth and differentiation were

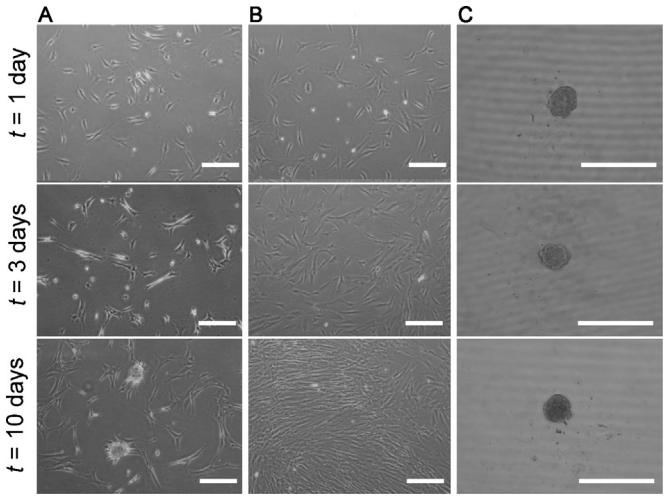

Fig. 1. Morphological characterization of hMSCs cultured on the G5 surface (A), PS surface (B), and round-bottom 96-well plate (C). Scale bars indicate 100 μm.