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Establishment of High Throughput Screening System Using Human Umbilical Cord-derived Mesenchymal Stem Cells

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The use of high throughput screening (HTS) in drug development is principally for the selection new drug candidates or screening of chemical toxicants. This system minimizes the experimental environment and allows for the screening of candidates at the same time. Umbilical cord-derived stem cells have some of the characteristics of fetal stem cell and have several advantages such as the ease with which they can be obtained and lack of ethical issues. To establish a HTS system, optimized conditions that mimic typical cell culture conditions in a minimal space such as 96 well plates are needed for stem cell growth. We have thus established a novel HTS system using human umbilical cord derived-mesenchymal stem cells (hUC-MSCs). To determine the optimal cell number, hUC-MSCs were serially diluted and seeded at 750, 500, 200 and 100 cells per well on 96 well plates. The maintenance efficiencies of these dilutions were compared for 3, 7, 9, and 14 days. The fetal bovine serum (FBS) concentration (20, 10, 5 and 1%) and the cell numbers (750, 500 and 200 cells/well) were compared for 3, 5 and 7 days. In addition, we evaluated the optimal conditions for cell cycle block. These four independent optimization experiments were conducted using an MTT assay. In the results, the optimal conditions for a HTS system using hUC-MSCs were determined to be 300 cell/well cultured for 8 days with 1 or 5% FBS. In addition, we demonstrated that the optimal conditions for a cell cycle block in this culture system are 48 hours in the absence of FBS. In addition, we selected four types of novel small molecule candidates using our HTS system which demonstrates the feasibility if using hUC-MSCs for this type of screen. Moreover, the four candidate compounds can be tested for stem cell research application.

Key words: human umbilical cord derived mesenchymal stem cells, high throughput screening, small molecule

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Introduction

Mesenchymal stem cells (MSCs) are generally defined as multi-potent cells with self-renewal ability; they are undifferentiated [1] and exist in the many mesenchymal tissues, including fat [2], umbilical cord blood [3], amniotic fluid [4], placenta [5], dental pulp [6], the salivary glands [7], the synovial membrane [8], the vocal fold [9], and the umbilical cord [10]. Despite the great interest in MSCs, there is no standard methodology for cell preparation at present [11]. MSCs are characterized by their ability to adhere to plastic cell culture dishes [12] and their specific surface markers as well as their multi-lineage differentiation capacity under adequate conditions [11]. Because of this ability, MSCs are considered an attractive material for tissue engineering and cell-based therapies [13,14].

Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) are isolated from discarded human umbilical cords after birth. The human umbilical cord is composed of 2 arteries and 1 vein surrounded by Wharton's jelly [15]. hUC-MSCs are purified from Wharton's jelly and umbilical cord vein by chopping up the whole cord and digesting it in

enzyme or catheterizing and filling it with enzyme [16,17]. These cells are characterized by the expressions of cell surface markers, including CD14, CD29, CD44, CD73, CD90, and CD105, and the absence of the expressions of CD14, CD34, and CD45. In addition, they have fibroblastoid morphology [16,17]. Currently, bone marrow is the most common source of MSCs for clinical studies [18]. However, the number of human bone marrow MSCs (hBM-MSCs) decreases significantly with donor age [19]. Therefore, hUC-MSCs isolated from newborn tissue are considered an alternative source of MSCs to hBM-MSCs.

High-throughput screening (HTS) is an in vitro assay for screening the efficiency and toxicity of new drug candidates. HTS is widely used in pharmacology and related fields to discover novel molecule candidates because it enables rapid and quantitative research [20]. It is specifically used to discover drugs for treating rare diseases [21,22].

HTS is generally based on testing certain target molecules using easily cultured cell lines [23]. However, the use of hUC-MSCs, which have multi-potent ability, seems to be applicable for various tissues. Moreover, a recent study used hBM-MSCs in chondrogenic research in 96-well plates [20,24,25] and osteogenesis studies [23] using HTS.

However, there is no hUC-MSC-based HTS standard at present. Therefore, we established a standardized optimal HTS system using hUC-MSCs in 96-well plates. We determined the optimal conditions for hUC-MSCs, including cell number, culture duration, FBS conditions, and duration of FBS starvation. Moreover, we selected four candidate small molecules using an established HTS system.

Materials and Methods

Isolation and purification of human umbilical cord-derived mesenchymal stem cells

Human umbilical cord was obtained and processed within 24 h after delivery. All experiment was approved by institutional review board (IRB No. S-D20090001) from School of Dentistry, Seoul National University. Human umbilical cord was carried in HBSS (Welgene, Daegu, South Korea) containing 3× antibiotic antimycotic (Gibco, Grand Island, NY, USA) and washed DPBS (Welgene, Daegu, South Korea) several times. Cord was sectioned along with a vein 2~3 cm long. Then put down with a sectioned-side in a culture dish which contained with 0.1% collagenase type I (Gibco, Grand Island, NY, USA) and cultured for 30 min at 37°C, 5% CO_2. After incubation, the umbilical cord vein was scrapped with a scrapper to isolate endotherial stem cells. That umbilical cord vein put down again with sectioned-side in a culture dish that contained with 0.1% collagenase type I (Gibco, Grand Island, NY, USA) and 0.24% dispase (Gibco, Grand Island, NY, USA) and cultured for 2 h at 37°C, 5% CO_2. After 2 h, the umbilical cord vein was scrapped with a scrapper. Enzyme solution was gathered and centrifuged for 4 min at 400 g. Then cell pellet was suspended in cell culture medium which DMEM (Welgene, Daegu, South Korea) containing 20% FBS, 1% antibiotic antimycotic (Gibco, Grand Island, NY, USA) and plated in 35 cm^2 cell culture dish (Nunc, Naperville, IL, USA). Cell culture was maintained at 37°C, 5% CO_2. After 3 days, cell culture medium was replaced and cell culture medium was changed every 2 days.

Fluorescence-activated cell sorting (FACS) analysis

MSCs markers such as CD10, CD29, CD44, CD73, CD90, CD105 and hematopoietic markers such as CD14, CD34, CD45, CD117 were attached and analyzed. Each antibodies were conjugated follow as: FITC-conjugated mouse anti-human CD14, CD34, CD45, CD90 and PE-conjugated mouse anti-human CD73, CD117 and PE.Cy5-conjugated mouse anti-human CD10 and PE.Cy7-conjugated mouse anti-human CD44 and APC-conjugated mouse anti-human CD29, CD105 (All from eBioscience, San Diego, CA, USA). hUC-MSCs passage 5 was used. hUC-MSCs 2.5×10^5 cells/mL were washed in FACS buffer (PBS, 2% FBS, 0.02% Sodium azide) and incubated with primary antibodies for 30 min in ice. After incubation, cells were washed and fixed with 4% paraformaldehyde at 4°C and analyzed through FACS calibur (Becton Dickinson, San Joes, CA, USA).

Experimental design

(1) Determine the optimal cell seeding numbers (750, 500, 200 and 100 cells) and (2) cell culture period (3, 5 and 7 days) and FBS concentrations (1, 5, 10, and 20%). To synthesize the same cell cycle, FBS-starvation experiment was performed. First, (3) 24 h experiment were performed to find the proper FBS-starvation concentration (0 and 1%), then 48 h FBS-starvation experiment were performed for finding optimal period. Cells were seeded in 96 well plates and incubated in 200 μL of 20% FBS containing cell culture media and all experiments were performed using MTT assay kit.

Optimization of the cell seeding numbers for HTS using hUC-MSCs

To determine the optimal cell seeding numbers, hUC-MSCs were serially diluted in 96 well plates that cell seeding number 750, 500, 200 and 100 cells. The four different cell number conditions compared for 3, 7, 9 and 14 days.

Optimization of cell culture period and FBS condition for HTS using hUC-MSCs

In order to determine the optimal FBS concentration, experimental conditions was fixed that cell seeding number 200, 500 and 750 cells and culture period 3, 5 and 7 days as well as FBS concentrations 1, 5, 10 and 20%.

Optimization of FBS-starvation condition for cell synchronization

To determine the optimal conditions of FBS-starvation, cell viability assay for FBS condition and period condition was performed. First, FBS condition was determined among FBS 0 or 1% with different cell numbers (200, 300 and 500 cells), culture period (4 and 6 days) at 24 h FBS-starvation. Cells were seeded in 96 well plates with 20% FBS containing cell culture media and incubated for 1 day. After incubation, FBS-starvation was performed with 0 or 1% FBS containing cell culture media for 24 h. Second, we performed 48 h FBS-starvation with different condition that cell number (300 and 500 cells), culture period (5 and 7 days) and FBS condition (0.1, 0.3, 0.5, 1, 3 and 5%).

Screening of small molecule library of hUC-MSCs using established HTS system

After establishment of the optimal HTS system for hUC-MSCs in 96 well plates, we conducted to screen the small molecule library using established HTS system. hUC-MSCs were seeded in 96 well plates with 20% FBS containing cell culture media and attached for 24 h. After 24 h, FBS-starvation for 48 h with 0% FBS was performed, then 10 nM small molecule candidates were treated to each well with 1 or 5% FBS media. Cells were incubated for 5 days and media change was performed 2 times. Through previous screening experiments, we selected the four small molecules which have cell viability and cell proliferation capacity by MTT and BrdU assay. To investigate cell viability, MTT assay was performed for the short-term (3 days and without FBS-starvation) and long-term (6 days and 48h FBS-starvation), also, cell proliferation assay that BrdU was performed for short-term (3 days and without FBS-starvation).

Cell viability assay (MTT assay)

MTT assay was measured according to manufacturer's instructions (EZ CyToX; Daeil lab service, Seoul, South Korea). MTT reagent was treated directly to the samples and incubated for 2 h at 37°C, 5% CO_2. The absorbance was measured at 480 nm for ELISA (S500, BIO-RAD, Camarillo, CA, USA).

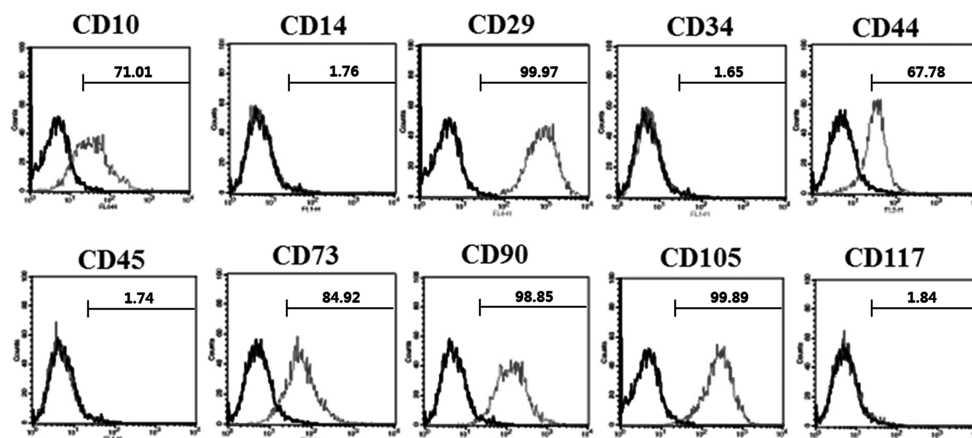
Cell proliferation assay (BrdU assay)

BrdU assay was assessed by manufacturer's instructions (BrdU Cell Proliferation Assay, Millipore, Billerica, MA, USA). Briefly, the medium was removed and BrdU reagent was added and incubated at 37°C for 24 h. After incubation, fixing solution was added at the plates and incubated at room temperature for 30 min and washing with washing buffer. Then detection antibody was added and incubated at room temperature for 1 h and washed. Goat anti-mouse IgG was added and incubated at 37°C for 30 min. After washing, substrate was added and incubated at room temperature for 30 min (in the dark). After 30 min, stop solution was added and the absorbance measured at 450 nm by ELISA (S500, BIO-RAD, Camarillo, CA, USA).

Results

In the FACS analysis (Supplementary Fig. 1), hUC-MSC were positive for MSC markers, including CD10 (71.01%), CD29 (99.97%), CD44 (67.78%), CD73 (84.92%), CD90 (98.85%), and CD105 (99.89%), and negative for hematopoietic markers, including CD14 (1.76%), CD34 (1.65%), CD45 (1.74%), and CD117 (1.84%).

We determined the optimal cell seeding numbers to obtain the characteristics of hUC-MSCs in 96-well plates (Fig. 1). The four different cell number conditions exhibited similar OD levels at 3 days; however, at 7 days, the growth rates of 500 and 750 cells increased remarkably compared with that at 3 days (zero growth and 3.84-, 6.05-, and 4.23-fold increases with 100, 200, 500, and 750 cells, respectively). At 9 days, the growth rates of 100, 200, 500, and 750 cells increased by 2.35-, 4.29-, 2.49-, and 2.69-fold compared to those at 7 days, respectively. However, at 14 days, the 200-,



Supplementary Fig. 1. FACS analysis of surface-marker expression on hUC-MSCs. CD 10, 14, 29, 34, 44, 45, 73, 90, 105 and 117 markers were used and cell type was UC-MSCs passage 5. Each antibody was stained at 4°C for 30min and then detected.

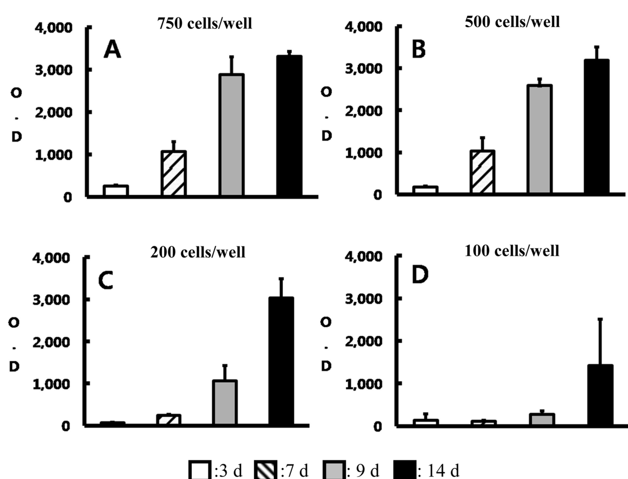


Fig. 1. Optimization of the cell seeding numbers for HTS using hUC-MSCs. In order to decide the optimal cell seeding number, hUC-MSCs were serially diluted at 750, 500, 200 and 100 cells/well on 96-well plates and were cultured for 3, 7, 9, and 14 days (□: 3 d, ▧: 7 d, ■: 9 d, ■: 14 d). FBS concentration was 20%. Cell viability was measured by MTT assay. (A) 750 cells/well were reached over-confluent at day 9, (B) 500 cells/well were reached over-confluent at day 9, (C) 200 cells/well were reached over-confluent at day 14, (D) 100 cells/well were reached over-confluent at day 14. Error bars represent standard deviation of the mean (n = 3).

500-, and 750-cell groups reached over-confluence. Therefore, we determined the optimal cell seeding number to be between 200 and 500 cells, and the optimal cell culture period to be between 7 and 9 days.

Next, we determined the optimal FBS concentration under the modified conditions (Fig. 2). At 3 days, 200 cells exhibited viability on 20% FBS but not on 1%, 5%, or 10% FBS (OD value: 44). At 5 days, 200 cells exhibited viability on 10% and 20% FBS (OD: 23 and 59.3, respectively) but not on 1% or 5% FBS. At 7 days, all FBS conditions exhibited cell viability as follows: 1%, 5%, 10%, and 20% FBS exhibited OD values of 394.3, 666.3, 657.3, and 634.6, respectively. In cells cultured in 10% FBS, the growth rate at

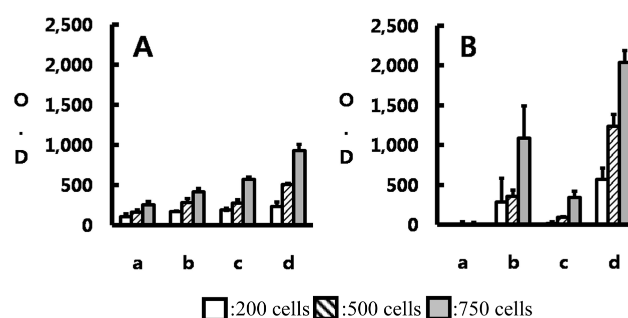


Fig. 3. Optimization of FBS starvation conditions for cell synchronization. To find the optimal FBS concentration for cell cycle synchronization, FBS starvation was performed for 24 h. Cells were seeded in 96 well plates with 20% FBS containing media and attached for 1 day. (□: 200 ▧: 500 cells, ■: 750 cells). After 1 day, the cell media was changed with FBS 0% or 1% containing media; then, the cells were incubated for 24 h. Next, cell media was changed with 0% and 20% or 1% and 20% (FBS concentration; (a): starvation 0% → changed 0%, (b): starvation 0% → changed 20%, (c): starvation 1% → changed 1%, (d): starvation 1% → changed 20%). (A) The cells were cultured for 4 days after FBS starvation, (B) the cells were cultured for 6 days after FBS starvation; group (a) not shown cell viability.

7 days was 28-fold higher than that at 5 days (OD: 23 vs. 657.3); however, cells cultured in 5%, 10%, and 20% FBS showed similar cell viability. At 3 days, 500 cells grown in 1%, 5%, 10%, and 20% FBS had OD values of 85, 89.8, 128.3, and 132, respectively. At 5 days, cells exhibited increased cell viability compared with that at 3 days: cells grown in 1%, 5%, 10%, and 20% FBS had OD values 4.72-, 7.16-, 5.28-, and 5.27-fold greater than those at 3 days, respectively; those at 7 days were 2.94-, 3-, 3.06-, and 3-fold greater, respectively. At 3 days, 750 cells grown in 1%, 5%, 10%, and 20% FBS had OD values of 148.6, 219.3, 230.3, and 261.3, respectively. Compared with the growth rate in 1% FBS, the cells grew 1.47-, 1.54-, and 1.75-fold faster in 5%, 10%, and 20% FBS, respectively. Compared with cell viability at 3 days, cell viability at 5 days in 1%, 5%, 10%, and 20% FBS increased by 4.45-, 3.01-, 5.16-, and 4.62-

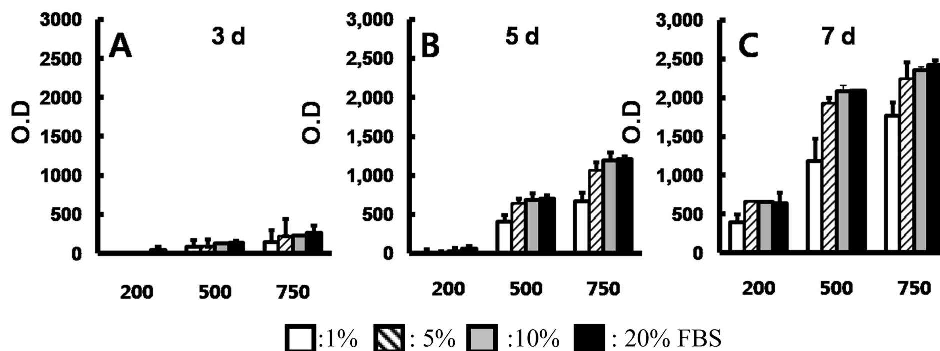


Fig. 2. Optimization of cell culture period and FBS conditions for HTS using hUC-MSCs. To determine the optimal culture period and FBS conditions, hUC-MSCs were serially diluted at 750, 500, and 200 cells/well on 96-well plates and were cultured for 3, 5, and 7 days. FBS concentration were 20%, 10%, 5%, and 1% (□: 1%, ▧: 5%, ■: 10%, ■: 20%). Cell viability was measured by MTT assay. (A) Culture period for 3 days, (B) culture period for 5 days, (C) culture period for 7 days; 500 cells/well and 750 cells/well were reached over-confluent at FBS 10% and 5%, respectively. Error bars represent standard deviation of the mean (n = 3).

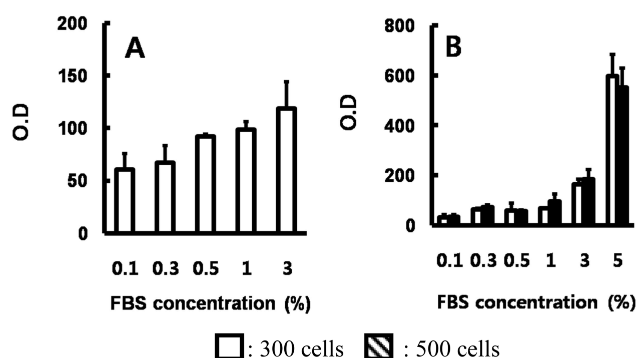


Fig. 4. Optimization of FBS starvation conditions for cell synchronization. To determine the optimum duration for cell cycle synchronization, FBS starvation was performed for 48 h. (A) 300 cells/well (□: 300 cells) were cultured for 5 days after FBS starvation; FBS concentrations were 0.1, 0.3, 0.5, 1 and 3%. (B) 300 cells/well and 500 cells/well (□: 300 cells, ▧: 500 cells) were cultured for 6 days after FBS starvation; FBS concentration 0.1, 0.3, 0.5, 1, 3 and 5%.

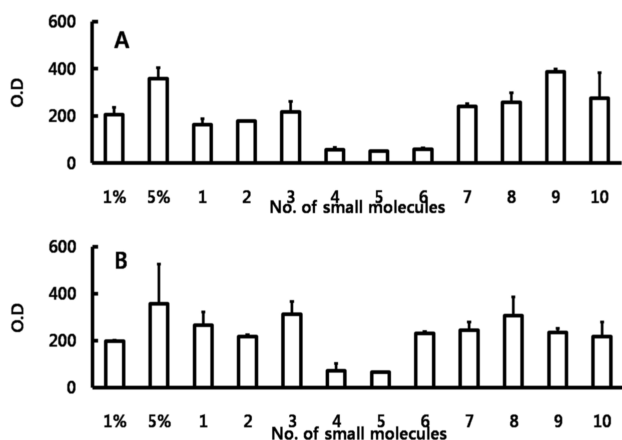


Fig. 5. Cell viability assay of ten small molecules for 3 days. (A) Five of the small molecules showed similar or greater viability ability compared with 1% control; cell culture conditions were as follows: cell number 300 cells/well and 1% FBS containing media. FBS starvation was not performed. (B) Eight of the small molecules showed similar or greater viability ability compared with 1% control; cell culture conditions were as follows: cell number 300 cells/well and 5% FBS containing media. FBS starvation was not performed. All experiments identified through MTT assay and small molecule concentration was 10 nM.

fold, respectively; compared with growth at 5 days, growth at 7 days increased by 2.66-, 2.11-, 1.98-, and 2-fold, respectively.

In order to synchronize the states of the cells, we performed FBS starvation experiments to synchronize the cell cycle. An MTT assay was carried out after 24-h FBS starvation to determine the optimal FBS starvation concentration (Fig. 3). The following conditions were used: (a) 0% FBS starvation → 0% FBS, (b) 0% FBS starvation → 20% FBS, (c) 1% FBS starvation → 1% FBS, and (d) 1% FBS starvation → 20% FBS. Group (a) exhibited cell viability at 4 days but not at 6 days. However, in group (b), the cell viability of 200,

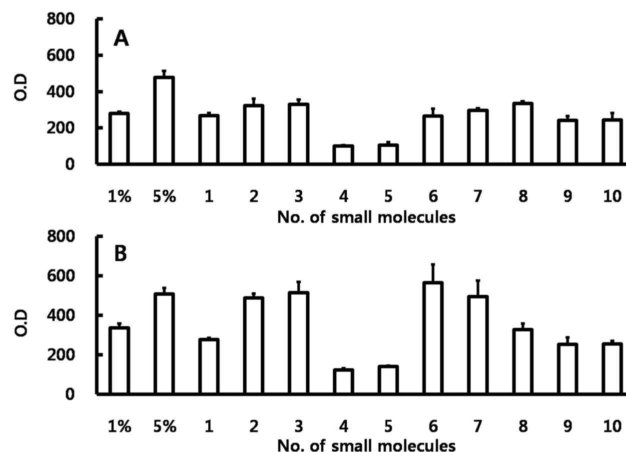


Fig. 6. Cell viability assay of ten of small molecules for 6 days. hUC-MSCs were cultured with 1% (A) or 5% (B) FBS containing media for 6 days. FBS starvation was performed for 48 h. After FBS starvation, media was changed to 1% or 5% FBS containing media. (A) Five of the small molecules showed similar or greater viability ability compared with 1% control, (B) Four of the small molecules showed similar or greater viability ability compared with 1% control. All experiments identified through MTT assay and small molecule concentration was 10 nM. (□: 300 cells).

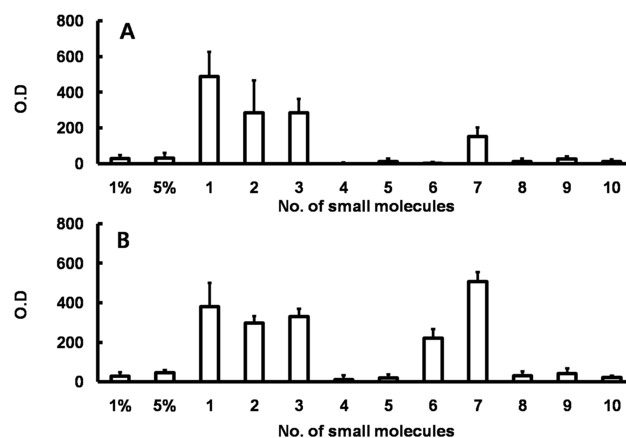


Fig. 7. Cell proliferation assay of ten small molecules for 3days. hUC-MSCs were cultured with 1% (A) or 5% (B) FBS containing media for 3 days. FBS starvation was not performed. After FBS starvation, media was changed to 1% or 5% FBS containing media. (A) Four of the small molecules showed similar or greater proliferation ability compared with 1% control, (B) Five of the small molecules showed similar or greater proliferation ability compared with 1% control. All experiments identified through BrdU assay and small molecule concentration was 10 nM. (□: 300 cells).

500, and 750 cells increased by 1.67-, 1.24-, and 2.6-fold from 4 to 6 days, respectively. At 4 days in group (c), 200, 500, and 750 cells had 187, 276, and 570 viable cells, respectively; cell viability decreased to 14, 92, and 334 cells at 6 days, respectively. Lastly, the cell viability of 200, 500, and 750 cells in group (d) increased by 2.42-, 2.42-, and 2.18-fold from 4 to 6 days, respectively. After 24-h FBS starvation, 48-h FBS starvation was performed with the previously modified conditions of 24-h FBS starvation (Fig.

7). At 5 days, cell viability according to OD increased in a dose-dependent manner (60.8%, 67.4%, 92.3%, 99.8%, and 119%); a similar trend was observed at 7 days (300 cells: 32.3%, 63%, 58.6%, 69.3%, 164.6%, and 598%; 500 cells: 34.6%, 74%, 56.6%, 95.6%, 185.6%, and 551.6). Moreover, seeding 300 and 500 cells produced similar viability.

We screened 619 small molecules using a previously established HTS system (data not shown). Ten small molecules were selected on the basis of the results of the cell viability experiments. To determine which small molecules have the greatest positive effect on viability ability among the 10, cell viability assays were performed for 3 days without FBS starvation (Fig. 4). Five of the small molecules increased cell viability compared to the 1% control as follows: nos. 3, 7, 8, 9, and 10 by 1.05-, 1.17-, 1.25-, 1.89-, and 1.34-fold, respectively (Fig. 4A). The 5% FBS results indicated that 8 small molecules showed similar or greater viability ability compared with the 1% control (Fig. 4B). In addition, this experiment was performed for 6 days with 48-h FBS starvation (Fig. 5). The 1% FBS results indicate that four small molecules, namely, nos. 2, 3, 7, and 8, increased cell viability by 1.15-, 1.17-, 1.06-, and 1.19-fold compared with the 1% control, respectively (Fig. 5A). The 5% FBS results showed that nos. 2, 3, 6, and 7 increased cell viability by 1.44-, 1.52-, 1.68-, and 1.46-fold compared with the 1% control, respectively (Fig. 5B). The assay was performed to determine which small molecules have the highest proliferation ability among the ten small molecules for 3 days with no FBS starvation (Fig. 6). The 1% FBS results showed that nos. 1, 2, 3, and 7 increased proliferation compared with the 1% control by 18-, 10-, 10-, and 5.62-fold, respectively (Fig. 6A). In addition, the 5% FBS results indicated that nos. 1, 2, 3, 6, and 7 increased cell proliferation by 13-, 10-, 11-, 7.85-, and 18-fold compared with the 1% control, respectively (Fig. 6B).

Discussion

The results of our study demonstrate the feasibility of performing HTS assays with hUC-MSCs. This system may be useful for drug discovery and toxicity tests. In order to perform HTS assays, it is important that to adapt the culture conditions for limited spaces such as 96-well plates (0.32 cm^2. However, until now, there was no optimal HTS system for hUC-MSCs. Therefore, we designed an optimal HTS system using 96-well plates. Sabrina *et al*. used human embryonic stem cells for HTS assays with the following conditions: 6,000 cells/well in 384-well plates (0.06 cm^2) and 6 days culture duration [26]; hBM-MSCs were used for HTS and seeded at 12,000 cells/cm^2 in 384-well plates and cultured for 8 days [27]. Ding *et al.* performed HTS assays for mouse P19 cells; P19 cells were seeded at 2,000 cells/well in 384-well plates and cultured for 4 days [28]. Alves *et al*. conducted HTS assays with hBM-MSCs seeded at 2,000 cells/well in 96-well plates and cultured for 4 days [23]. However, their cell numbers are equivocal: they used 2,000 cells/well in their method and reported 2,000 cells/cm^2 in their data. In our laboratory, hUC-MSCs were seeded at 100,000 cells per 60 mm^2 (21.5 cm^2) and subcultured after 3 or 4 days. Therefore, in 96-well plates, the hUC-MSCs were seeded at 1490.3 cells/well. However, at this density, the cells soon reached over-confluence. Therefore, in the present study, hUC-MSCs were serially diluted and seeded at 750, 500, 200, or 100 cells/well on 96-well plates to determine optimal cell numbers (Fig. 1). To assay cell viability, we used tetrazolium dyes such as MTT [29]. The results of the first experiment indicate that the optimal conditions for HTS are between 200 and 500 cells and 7 and 9 days since the cells did not reach over-confluence (less than 2,000/OD).

Next, we carried out a second experiment to determine the optimal FBS conditions (Fig. 2). The results of the second experiment indicate that the groups of 500 and 750 cells reach over-confluence with 5%, 10%, and 20% FBS at 7 days. Then, we confirmed the optimal HTS conditions to be 1% to 5% FBS and 7 days for culture duration. The results indicated that 200 cells exhibited a slower growth rate from 3 to 5 days compared to 500 and 750 cells. However, from 5 to 7 days, 200 cells grew faster than 500 and 750 cells.

When FBS-containing media were added to FBS-starved cells, the cell cycle was synchronized and subsequently promoted [30]. FBS starvation was not performed in the abovementioned HTS experiment. However, we carried out FBS starvation to prevent FBS from interfering with the action of small molecules. First, the experiment was performed to examine 0% or 1% FBS conditions (Fig. 3). As seen in Fig. 3A, the results show a dose-dependent increase. However, at 6 days, 0% FBS starvation showed no cell viability, while 1% FBS starvation did show cell viability. As shown in Fig. 3A and B, 0% and 1% FBS starvation followed by addition of 20% FBS-containing media showed dose-dependent or similar results. These results indicate that 0% FBS is the optimal condition for cell-cycle starvation since 1% FBS still resulted in cell viability despite FBS starvation. Therefore, we performed 48-h FBS starvation and culturing for 5 and 7 days. With 48 h of FBS starvation at 5 days, all FBS groups showed dose-dependent results. Furthermore, at 7 days, the OD values of all FBS concentrations of 300 and 500 cells showed similar results. Thus, we determined that the 48-h FBS starvation period is better than 24 h; moreover, for cell seeding number, 300 cells were better than 500 cells (Fig. 4).

Based on established HTS systems, we determined that the optimal conditions for the HTS system for hUC-MSCs are 300 cells, 1% FBS, 8 days culture duration, and 0% FBS starvation for 48 h.

To induce chondrogenesis in MSCs, growth factors such as TGFβ or BMPs are essential materials. However, the molecular mechanisms of these growth factors involved in

the phenotypic conversion are only partially known [20]. Therefore, determining which small molecules enhance the proliferative ability of cells is important for inducing chondrogenesis. Due to the development of HTS techniques, millions of small molecules can be screened simultaneously. Therefore, in the present study, we established an optimal system for avoiding over-confluence of hUC-MSCs in limited 96-well environments. Under this optimal system, we determined four small molecules that enhance the proliferative ability of hUC-MSC using cell viability and proliferative assays.

The results of the present study demonstrate the successful creation of an HTS assay using hUC-MSCs in an academic laboratory without automatic settings. Moreover, this system will be useful for developing models for drug discovery, in studies of cell biology, and toxicity tests using hUC-MSCs. Further studies should reveal the action mechanisms of these four small molecules. In addition, the four novel small molecules are expected to be studied for their chondrogenic effects on hUC-MSCs.

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References

1. Caplan AI. Mesenchymal stem cells. J Orthop Res. 1991; 9:641-650.
2. Xu Y, Malladi P, Wagner DR, Longaker MT. Adipose-derived mesenchymal cells as a potential cell source for skeletal regeneration. Curr Opin Mol Ther. 2005;7:300-305.
3. Bieback K, Kluter H. Mesenchymal stromal cells from umbilical cord blood. Curr Stem Cell Res Ther. 2007;2: 310-323.
4. Tsai MS, Lee JL, Chang YJ, Hwang SM. Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. Hum Reprod. 2004;19:1450-1456.
5. Igura K, Zhang X, Takahashi K, Mitsuru A, Yamaguchi S, *et al*. Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta. Cytotherapy. 2004;6:543-553.
6. Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res. 2003;18:696-704.
7. Rotter N, Oder J, Schlenke P, Lindner U, Bohrnsen F, *et al*. Isolation and characterization of adult stem cells from human salivary glands. Stem Cells Dev. 2008;17:509-518.
8. De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. Arthritis Rheum. 2001;44:1928-1942.
9. Hanson SE, Kim J, Johnson BH, Bradley B, Breunig MJ, *et al*. Characterization of mesenchymal stem cells from human vocal fold fibroblasts. Laryngoscope. 2010;120:546-551.
10. Girdlestone J, Limbani VA, Cutler AJ, Navarrete CV. Efficient expansion of mesenchymal stromal cells from umbilical cord under low serum conditions. Cytotherapy. 2009;11:738-748.
11. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, *et al*. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8: 315-317.
12. Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet. 1970;3:393-403.
13. Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol. 2007;213:341-347.
14. Satija NK, Singh VK, Verma YK, Gupta P, Sharma S, *et al*. Mesenchymal stem cell-based therapy: a new paradigm in regenerative medicine. J Cell Mol Med. 2009;13:4385-4402.
15. Sobolewski K, Bankowski E, Chyczewski L, Jaworski S. Collagen and glycosaminoglycans of Wharton's jelly. Biol Neonate. 1997;71:11-21.
16. Covas DT, Siufi JL, Silva AR, Orellana MD. Isolation and culture of umbilical vein mesenchymal stem cells. Braz J Med Biol Res. 2003;36:1179-1183.
17. Weiss ML, Troyer DL. Stem cells in the umbilical cord. Stem Cell Rev. 2006;2:155-162.
18. Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. Exp Hematol. 2000;28:875-884.
19. Rao MS, Mattson MP. Stem cells and aging: expanding the possibilities. Mech Ageing Dev. 2001;122:713-734.
20. Huang AH, Motlekar NA, Stein A, Diamond SL, Shore EM, *et al*. High-throughput screening for modulators of mesenchymal stem cell chondrogenesis. Ann Biomed Eng. 2008;36:1909-1921.
21. Cotticelli MG, Rasmussen L, Kushner NL, McKellip S, Sosa MI, *et al*. Primary and Secondary Drug Screening Assays for Friedreich Ataxia. J Biomol Screen. 2011
22. Varma H, Lo DC, Stockwell BR. High-Throughput and High-Content Screening for Huntington's Disease Therapeutics. In: Lo DC, Hughes RE, editors. Neurobiology of Huntington's Disease: Applications to Drug Discovery. Boca Raton (FL). 2011
23. Alves H, Dechering K, Van Blitterswijk C, De Boer J. High-throughput assay for the identification of compounds regulating osteogenic differentiation of human mesenchymal stromal cells. PLoS One. 2011;6:e26678.
24. Penick KJ, Solchaga LA, Welter JF. High-throughput aggregate culture system to assess the chondrogenic potential of mesenchymal stem cells. Biotechniques. 2005;39: 687-691.
25. Welter JF, Solchaga LA, Penick KJ. Simplification of

aggregate culture of human mesenchymal stem cells as a chondrogenic screening assay. Biotechniques. 2007;42:732, 734-737.

26. Desbordes SC, Placantonakis DG, Ciro A, Socci ND, Lee G, *et al*. High-throughput screening assay for the identification of compounds regulating self-renewal and differentiation in human embryonic stem cells. Cell Stem Cell. 2008;2:602-612.
27. Brey DM, Motlekar NA, Diamond SL, Mauck RL, Garino JP, *et al*. High-throughput screening of a small molecule library for promoters and inhibitors of mesenchymal stem cell osteogenic differentiation. Biotechnol Bioeng. 2011; 108:163-174.
28. Ding S, Wu TY, Brinker A, Peters EC, Hur W, *et al*. Synthetic small molecules that control stem cell fate. Proc Natl Acad Sci U S A. 2003;100:7632-7637.
29. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods. 1983;65:55-63.
30. Woessner RD, Mattern MR, Mirabelli CK, Johnson RK, Drake FH. Proliferation- and cell cycle-dependent differences in expression of the 170 kilodalton and 180 kilodalton forms of topoisomerase II in NIH-3T3 cells. Cell Growth Differ. 1991;2:209-214.