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Serum-free spheroid suspension culture maintains high proliferation and differentiation potentials of mesenchymal stem cells

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Abstract

There have been many clinical trials recently using *ex vivo*-expanded human mesenchymal stem cells (MSCs) to treat several indications such as graft-versus-host disease, acute myocardial infarction, Crohn's disease, and multiple sclerosis. However, the conventional 2-dimensional (2D) culture of MSCs is laborious and limited in scale potential. The large dosage requirement for many of the indications further exacerbates this manufacturing challenge. In contrast, spheroid MSC culture does not require a cell attachment surface and is amenable to large-scale suspension cell culture techniques, such as stirred-tank bioreactors. In this present study, we developed and optimized serum free media for culturing MSC spheroids. We used Design of Experiment (DoE)-based strategies to systematically evaluate media mixtures and a panel of different components. The optimization yielded two prototype media that could allow MSCs to form aggregates and proliferate in both static cultures and dynamic cultures. The expanded MSCs expressed the expected surface markers for mesenchymal cells (CD73, CD90 and CD105). In addition, the expanded cells demonstrated multipotency and differentiated to the osteocyte, chondrocyte and adipocyte lineages, which showed similar or enhanced differentiation levels compared with serum-containing adherent cultures.

Keywords

Mesenchymal stem cells; spheroid culture; serum free media; differentiation

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Introduction

Different cell sources such as embryonic stem cells or induced pluripotent stem cells (iPS) have the potential to be used in regenerative medicine because they can be differentiated into any cell type such as neurons or hepatocytes. However, the use of embryonic stem cells is limited by ethical consideration and the application of iPS is limited by the use of viral material^{1–5}. Furthermore, they impose safety concerns because they may form teratoma when implanted⁶. In contrast, it has been shown that adult stem cells have tremendous therapeutic potential in regenerative medicine and show excellent safety record in clinical application^{7–13}. There are multiple sources of adult stem cells located in bone marrow or in hair follicle^{10, 14, 15}. MSCs have unique therapeutic benefits with multiple underlying mechanisms, including differentiation potential, immunomodulatory effect, secretory tropic factors, and the capability of homing to injured tissues¹⁶. MSCs can differentiate into osteogenic, adipogenic, chondrogenic, and myogenic lineage under appropriate conditions^{17–19}, so they have been used as a therapeutic tool to treat bone and cartilage disorders^{20, 21}. In addition, MSCs have been used in many preclinical models²² of diseases including myocardial infarction, stroke^{23, 24}, rheumatoid arthritis²⁵, acute lung injury^{26, 27}, graft-versus-host disease²⁸, and skin-graft rejection²⁹.

Since adult mesenchymal stem cells (MSCs) have such tremendous potential for regenerative medicine, it is important to generate MSCs in large scale while maintaining their desirable characteristics^{20, 30}. The traditional method of culturing MSCs is on a two dimensional (2D) adherent culture surface in serum-containing media. However, the 2D culture is labor-intensive and limited in scaling up. With recent development, microcarriers can be used in stirred tank bioreactors to provide a feasible way for large-scale MSC production^{31, 32}, but the process is highly technical and there can be challenges for downstream cell purification.

Previous studies have shown that culturing MSCs as three dimensional (3D) aggregates is a simple and reproducible method, which can avoid the disadvantages, associated with MSCs culture as a monolayer^{33–35}. However, the media used in these studies all contained serum. From a manufacturing perspective, serum may carry adventitious agents, risking viral or mycoplasma contamination. In addition, serum is subject to lot-to-lot variability with regional and seasonal supply changes. The cost associated with qualifying serum for consistent performance is also prohibitive for commercial scale bio-therapeutic production. Therefore, the development of serum-free media is critical for large-scale MSC spheroid cultures.

Different techniques for MSC spheroid culture have been developed. They can be broadly divided into 2 categories: (1) dynamic cultures using spinner flasks, rotating wall vessel bioreactor or hollow fiber capillary membrane-based bioreactor³⁶, and (2) static cultures that use non adhesive surface to encourage cell suspension or hanging drop. Each developed method has its own benefits and disadvantages. The general consideration for MSC production is the cost production efficiency, and the size of the spheroids. In particular, dynamic cultures in spinner flasks, rotary systems perform long term and dynamic controlled culture. However, the major disadvantage of this method is the fact that

specialized equipment is required and there is a variation in size and cell number of the spheroids^{37–41}. Static cultures are inexpensive and the size of spheroid is well-controlled. However, both the hanging drop and non-adhesive surface approach are not easily scalable and massive production is difficult.^{42–48}

In the present study, we developed and optimized serum-free media for MSC spheroid culture based on a Design of Experiment (DoE) strategy. A series of media mixtures and a panel of different components were systematically evaluated. Static cultures were utilized to screen the media mixtures and the components based on cell proliferation potential. Eventually, we ended up with two prototype media and characterized their performance in both static and dynamic culture by examining the proliferation and differentiation potential of MSC spheroids. The expanded MSCs maintained the mesenchymal phenotype, such as the surface markers CD73, CD90, and CD105, and showed similar or enhanced differentiation potential to osteogenic, chondrogenic or adipogenic lineage compared with serum-containing culture. To our knowledge this is the first study to demonstrate the development of serum-free media for MSC spheroid suspension cultures.

MATERIALS AND METHODS

Adherent Cell Culture

Bone Marrow derived Mesenchymal Stem Cells (BM-MSCs) pooled from 4 donors were cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) MSC-FBS (Life Technologies), 1% (v/v) Antibiotic-Antimycotic (AA; Life Technologies) and 1x GlutaMax-1 (Life Technologies).

Suspension Cell Culture

For static culture, BM-MSCs (0.1 million cells/ml) were seeded in Costar® plate flat bottom ultra low attachment (Corning, NY) using culture media as indicated in each experiment. There were three steps for the serum-free media development: the media mixture study, the component screening, and the optimization of the components. In the media mixture study, 5 serum-free prototype media were used. And the cell growth was evaluated with cells seeded at both a low cell density (LCD) at 5×10^3 cells/mL and a high cell density (HCD) at 5×10^4 cells/mL. In the component screening, 13 different components were evaluated with cells seeded at HCD. The optimization of the components consists of a 2-level 4 factor full factorial design. Passage 4 (P4) cells were cultured for five days and fresh media were added every second day. For dynamic culture, 15 mL of cell suspension at 1.0×10^5 cells/mL was cultured in 125 mL shake flasks (Corning). After aggregates were formed overnight, agitation was started at 80 rpm. 5 mL fresh medium was fed on Day 3 and Day 5 each time to the dynamic culture. After 5 days for the static culture or 7 days for the dynamic culture, the cells were transferred to conical tubes, centrifuged at 500g for 5 min and the majority of the supernatant was removed leaving the cell pellet intact. The cells were treated with 1x TrypLE™ (Life Technologies) and were incubated for 30 min at 37°C until the aggregates were completely dissociated.

Proliferation Assay

Cells were transferred to eppendorf tubes and centrifuged at 500g for 5min. After removal of the supernatant, the cells were frozen overnight at -70°C and the next day, CyQUANT® assay (CQ assay; Life Technologies) was performed according to the manufacturer's protocol. For dynamic cultures in shake flasks, after cell dissociation, cells were quantified with an automatic cell counter Vi-CELL®, which measures viable cell density, viability, and average cell size.

Flow Cytometry

The levels of CD105, CD90, CD73, CD34 in spheroids and adherent culture were measured by flow cytometry. Briefly, the spheroids were dissociated and were re-suspended in 4% (w/v) ice cold paraformaldehyde, permeabilized with PBS containing 0.1% (v/v) Triton X-100 (Sigma) for 10min. Subsequently, cells were incubated in blocking buffer: 5% (v/v) goat serum (Life Technologies) and 0.01% (v/v) Triton X-100 (Sigma, St. Louis, MO) in Phosphate Buffered Saline (PBS). Afterwards, samples were incubated with the following primary antibodies that were diluted 1:100 in blocking buffer for an hour at room temperature: mouse anti-human CD105 (BD Transduction Laboratories, San Jose, CA); mouse anti-human CD90 (BD Transduction Laboratories); mouse anti-human CD73 (BD Transduction Laboratories); mouse anti-human CD34 (BD Transduction Laboratories). Cells were then incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG secondary antibody (1:100 dilution; Life Technologies) for one hour at room temperature. As negative control, cells were incubated with only secondary antibody. Afterwards, the cells were washed three times with PBS and the level of each marker was determined by running the samples through a FACS Calibur flow cytometry (Becton Dickinson, Franklin Lakes, NJ).

Immunostaining

Spheroids were processed in 10% formalin and embedded in agarose and later in paraffin. For immunohistochemistry, 5 μm tissue sections were deparaffinized in xylene and rehydrated. Specimens were washed with PBS and permeabilized. After incubation with blocking buffer, samples were incubated with the following primary antibodies: mouse anti-human PCNA (1:100; Biolegends, San Diego, CA) in blocking buffer overnight at 4°C . After washing with PBS, samples were incubated with Alexa 488-conjugated goat-anti-rabbit IgG (20 $\mu\text{g}/\text{ml}$; Life Technologies), followed by counterstaining of nuclei using Hoechst 33258 (25 $\mu\text{g}/\text{ml}$ in TNE buffer: 10 mmol/l Tris, 2 mol/l NaCl, 1 mmol/l EDTA, pH 7.4; Life Technologies). Fluorescent images were obtained using an inverted fluorescence microscope Zeiss AxioObserver (Zeiss, Thornwood, NY).

Differentiation of Spheroids and Adherent BM-MSCs

After 5 days in culture, the spheroids were dissociated and 5×10^4 cells/cm² was seeded in 24-well tissue culture treated plates under proliferation media. Similarly, the control adherent BM-MSCs was cultured under the same conditions. After overnight incubation, cells were switched to the appropriate differentiation media as indicated below and thereafter cells were replenished with the corresponding differentiation media every three days. For adipogenesis, cells were cultured in adipogenic medium (StemPro® Adipogenesis

Differentiation Kit, Life Technologies) for 2 weeks followed by Oil Red O staining. For chondrogenesis, cells were maintained in chondrogenic medium (StemPro® Chondrogenesis Differentiation Kit, Life Technologies) for 3 weeks before carrying out the Alcian blue staining. For osteogenesis, cells were cultivated in osteogenic differentiation medium (StemPro® Osteogenesis Differentiation Kit, Life Technologies) for 28 days and Von Kossa staining or Alizarin Red S Staining was performed.

Oil Red O Staining

Differentiated BM-MSCs were fixed with 10% formalin at room temperature for 60 min and then incubated with 60% (v/v) isopropanol for 5 min at room temperature. Afterwards, the BM-MSCs were incubated with 0.6% (w/v) filtered Oil Red O (Alfa Aesar, Ward Hill, MA) in 60% isopropanol/40% water at room temperature for 10 min and washed three times with water. The cells were imaged using Zeiss Axio Observer and quantification of images containing oil droplets was performed using Image J software.

Alcian Blue Staining

Differentiated BM-MSCs were fixed with 4% (w/v) paraformaldehyde at room temperature for 10 min and then incubated with 0.5% Alcian Blue (Sigma) in 0.1N HCL overnight. The cells were imaged using Zeiss Axio Observer and quantification of images containing was performed using Image J software.

Von Kossa Staining

Differentiated BM-MSCs were fixed with 4% (w/v) paraformaldehyde at room temperature for one hour and then incubated with 1% silver nitrate solution (Diagnostic Biosystems, Pleasanton, CA) in the dark for 30 min. The cells were washed three times and were exposed to UV light for one hour. Afterwards, the cells were incubated with 5% (w/v) of sodium thiosulfate (Diagnostic Biosystems) for 5 min to remove any unreacted silver nitrate. Images were taken using Zeiss Axio Observer and quantification of images was performed using Image J software.

Alizarin Red S Staining

Differentiated BM-MSCs were fixed with 4% formaldehyde solution for 30 min. After fixation, cells were rinsed twice with distilled water and stained with 2% Alizarin Red S solution for 2 to 3 min. Then cells were rinsed three times with distilled water. Images were taken using Zeiss Axio Observer and quantification of images was performed using Image J software.

Statistical analysis

Statistical analysis of the data was performed using a two-tailed Student's t-test ($\alpha=0.05$) in Microsoft Excel (Microsoft, Redwood, CA). Each experiment was repeated two to three times conducted with at least triplicate samples.

RESULTS

Development of serum-free media for MSC spheroid growth

The development of serum free media was based on the proliferation potential of MSC spheroids. For the media mixture study, media were prepared by a combination of 5 different serum-free media prototypes: medium A, medium B, medium C, medium D, and medium E with different ratios resulting in 24 media (Figure 1A). The highest cell number increase of MSC spheroids was 1.99 ± 0.42 fold in LCD cultures under condition 20. In addition, the cell number increase was 1.31 ± 0.19 fold when the MSC spheroids were cultured with HCD under condition 3 (Figure 1B). Based on the cell proliferation data, the media mixture composition was analyzed using Design-Expert. The optimal statistically-predicted composition was the combination of 50% medium A and 50% medium E. To verify this, we cultured MSCs at HCD and LCD under 50% A: 50% E and captured the formation of spheroids after 5 days using microscopy. As shown in Figure 1C, the HCD cells formed larger diameter aggregates compared with the LCD cells, suggesting that seeding density may play an important role during spheroid formation. Also, a cell growth curve is shown for the spheroid culture with an HCD in the media mixture 50% A: 50% E over a period of 6 days (Figure 1D), indicating that this media mixture is a suitable choice as base medium for further optimization.

Next we supplemented the base medium with 13 components to screen for positive factors that could further boost cell proliferation in suspension. In the experiment, cells were seeded at HCD in 14 different media (C1-C14 with C9 as the control base medium 50% A/50% E), and cell proliferation was evaluated. Cell number increased more than 3-fold in C1, C2, C3, C4, C5, C6, C13, and C14 (Figure 2). In contrast, the components for C7–C12 (with C9 as the control) did not exhibit substantial promotion for cell growth, since they had similar proliferation potential as the cells cultured in the control medium C9. Although 8 of the test media (C1, C2, C3, C4, C5, C6, C13, and C14) promoted cell proliferation in suspension, some of the factors were excluded due to cost considerations, resulting in a total number of 4 selected factors, which were Component 2, Component 4, Component 5, and Component 14.

Based on these factors, a 2-level 4 factor full factorial design was created consisting of 16 medium prototypes (M1-M16) that were based on different combinations of Component 2, Component 4, Component 5, and Component 14 with M12 the same as the non-supplemented control medium C9 in the component screening step (Figure 3A). Then the MSCs were seeded at HCD in the 16 different media, and the proliferation potential were evaluated 5 days later. Cells cultured in M3 and M7 showed the highest proliferation increase (>5 fold) compared to the other conditions (Figure 3B). Also, the MSCs formed spheroids in M3 and M7 as in the control M12 (Figure 3C).

MSC spheroids maintained the MSC phenotype

MSC spheroids were characterized in the optimized media M3 and M7. Cell surface immunophenotype were examined with flow cytometry for positive markers CD73, CD90, and CD105, and for a negative marker CD34. The cells harvested from the spheroids exhibited similar high levels (>99%) of the surface markers as adherent cells cultured in

DMEM+10%FBS for CD73 and CD90. CD105 was slightly lower for cells cultured in serum-free M3 (77.1%) and M7 (85.3%) in suspension than in DMEM+10%FBS (98.9%) (Figure 4A).

In addition, MSCs in the static serum-free spheroid cultures exhibited similar or better differentiation potential compared to the adherent culture in serum-containing medium. To this end, MSC spheroids were differentiated towards the osteogenic, adipogenic, and chondrogenic lineages. The degree of differentiation was measured by functional assays specific for each lineage, namely Alcian Blue staining for chondrogenesis, Von Kossa staining for osteogenesis, and Oil Red O staining for adipogenesis. In particular, the spheroids were grown in M3 and M7 and cells were dissociated 5 days later. The cells were plated in adherent 2D culture and induced towards the chondrogenic, osteogenic and adipogenic lineages. As shown in Figure 4B, the spheroids cultured in M3 or M7 showed higher osteogenic differentiation levels as indicated by integrated intensity of calcium deposition than the adherent culture. Also, the spheroids exhibited higher intensity of stained Alcian Blue-GAGs than the adherent culture under the chondrogenic condition (Figure 4C). Furthermore, the spheroids demonstrated higher level of differentiation under adipogenic condition in M3 and M7 than the adherent culture (Figure 4D). Taken together, these results showed that the spheroids statically cultured in the serum-free media M3 and M7 exhibited higher differentiation potentials in the trilineage differentiation assay than the cells in adherent culture.

Dynamic culture of MSC spheroids

We then used M3 and M7 to grow MSC spheroids in agitated shake flasks to demonstrate the scalability of the serum-free MSC expansion method. The proliferation assay exhibited more than 6 fold increase in cell growth in M3 and M7, while there was no cell growth as spheroids in the serum-containing control medium (Figure 5A). Also, a larger number and a larger size of MSC spheroids were formed in M3 and M7 than the cells cultured in serum-containing control medium (Figure 5B). Although the spheroids were larger in M3 and M7, the cells exhibited high viability during harvest (Figure 5C). Similarly, immunostaining based on proliferating cell nuclear antigen (PCNA) and Hematoxylin and Eosin (H&E) stain showed that the cells were actively growing in the center of the spheroid independent of the media type, indicating that M3 and M7 enhanced the proliferation of the cells (Figure 5D&E).

For characterization of the dynamically cultured cells, the spheroids cultured in M3 and M7 exhibited similar high levels of the positive surface markers as cells cultured in the control medium in agitated shake flasks, indicating that these cells retained the MSC phenotypes (Figure 6A). Finally, as the static culture, the MSCs with M3 or M7 in the agitated shake flask cultures exhibited similar or better differentiation level for adipogenesis, chondrogenesis, and osteogenesis, compared to the MSCs cultured in the serum-containing control medium. In particular, a higher level of osteogenesis (Figure 6B) and adipogenesis (Figure 6D) was seen with cultures in M3 and M7 than the serum-containing condition. Interestingly, during the chondrogenic differentiation, 3D structures were formed (Figure

6C), and a similar or higher level of staining for Alcian Blue was observed under M7 and M3, respectively.

Although the spheroid serum-free culture performed high proliferation and differentiation potential, this culture can generate unique properties for MSCs. In particular, the size of dissociated cells was smaller with serum-free spheroid suspension culture by 70% than the 2D or 3D serum-containing culture (Figure 6D). To this end, their small size makes them eligible for direct in vivo application since these cells cannot get trapped after infusion.

Discussion

In this study, we developed serum-free media for culturing human MSC spheroids in suspension. MSCs have the ability to self-renew and differentiate into many different cell types. Due to this fact, MSCs have an enormous potential for use in tissue engineering and regenerative medicine therapies. In most of the clinical studies 1–5 million cells/kg have been used^{30, 49} and therefore, the expansion of MSCs to high numbers is crucial. However, conventional adherent culture, such as using cell factory, is laborious and limited in scale. Therefore, culturing MSCs as spheroids in suspension, with the amenability to bioreactor operation and the elimination of culture surface, is a compelling alternative to the 2D cultures for scaling-up. In most of the studies, the media used for MSCs expansion are supplemented with FBS at 10–20 % (v/v) since FBS contains a high amount of growth factors and other compounds required for cell growth. Other studies have used serum reduced conditions, such as EGM2 media or knockout conditions^{50–52}. However, the use of FBS is not desirable to culture cells for clinical applications as several problems can rise. One potential problem associated with the use of FBS is the risk of contamination with adventitious agents. Also, the cells manufactured with FBS may be immunogenic when applied in patients. In addition, FBS can contain growth inhibitors or cytotoxic molecules that can interfere with the hormones and growth factors for the cells being used in cellular therapy⁵³. To overcome these limitations, it is necessary to develop serum-free conditions to generate large amount of viable cells for therapeutic purpose. Some studies showed that MSC culture in serum-free media may provide additional advantages. In particular, defined formulations of serum free media can sustain the generation of a “pure” population of MSCs avoiding the culture of undesired cells. Also, serum free media can increase the clonogenic potential of MSC compared to FBS-based cultures⁵⁴ and enhance the production of cells with desired attributes such as high anti-inflammatory properties⁵⁵. Currently, there is still no serum-free medium for MSC spheroid suspension culture commercially available or reported in the literature.

For these reasons, we developed the serum free media for human MSC spheroid cultures. A DoE-based strategy was employed for the systematic development, consisting of three steps: media mixture studies, component screening, and the optimization of selected components. The evaluation of the culture media was based on the proliferation and differentiation potential of the MSC spheroids in static cultures. The MSCs cultured with these optimized media yielded higher proliferation rate and differentiation potential than the regular serum-containing conditions for spheroid cultures. Since large quantity of MSCs is desirable for therapeutic use, we also demonstrated the expansion of MSCs in a dynamic culture setting,

which is more favorable for scaling up. Similar to our static culture, when we applied the optimal media in shaking flasks, the cells showed higher proliferation and differentiation potential compared to serum-containing media in spheroid suspension cultures.

In addition, the spheroid culture can generate unique properties for MSCs. For example, the smaller cell sizes of MSCs, as we observed in our work, may prevent the cells from getting trapped in the lung after i.v. infusion, thus allowing a better distribution to the site needed⁵¹. Furthermore, based on literature, when cultured as spheroids, MSCs did not only possess high proliferation potential but also exhibited high anti-inflammatory properties⁵¹. In particular, it has been shown that interleukin 24, a tumor suppressor^{17, 56}, or TSG-6, an anti-inflammatory protein that have beneficial effects on myocardial infarction⁵⁷, were up regulated in spheroid culture⁵¹.

Similar to other studies, human MSC spheroids cultured in our serum free conditions maintain their characteristic surface markers, such as CD90, CD105, and CD73. Interestingly, the CD105 levels in MSCs from spheroids were actually maintained higher in M3 (77.1%) and in M7 (85.3%) than that in a serum-containing medium from the literature (~11%)¹⁷.

Conclusion

In the present study, we utilized a systematic approach to develop serum free media for human MSC spheroid culture in suspension. We showed that MSCs cultured under serum-free conditions either in static or dynamic cultures exhibited high proliferation and comparable or higher differentiation potential comparing to the serum-containing culture in spheroids. The developed serum-free media present a highly value-added alternative to the current MSC expansion paradigm. It is expected that with further process development, the serum-free media can be used in a more clinically-relevant cell manufacture setting, such as using stirred tank bioreactors.

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Figure 1. Medium Mixture Study for MSC Growth in Suspension

(A) Design of medium prototype mixtures. (B) The proliferation of MSC spheroids was measured by CQ assay and the values were normalized to the initial cell density (n=2). (C) Representative images for MSC spheroids in suspension on day 0 or day 5. (D) Growth curve of MSCs cultured in suspension over a period of 6 days.

A

Figure 2. Component Screening for Serum-Free Media

Media mixture 50%A/50%E was supplemented with appropriate concentration of 13 factors (C1–C14 with C9 as the control). The proliferation of MSC spheroids was measured by CQ assay and the values were normalized to the control media (C9).

Figure 3. DOE-based Component Optimization

(A) A 2-level full factorial DOE study was designed to evaluate experimental components. The base medium without a test factor served as control medium (M12). (B) Proliferation of pooled donor BM-MSCs spheroids was measured by CQ assay and the values were normalized to the initial cell density (n=2). The asterisk (*) denotes significant difference ($p < 0.05$) between the indicated sample and control medium (M12). (C) Morphology of BM-MSCs in suspension in M3, M7 and M12 media. Bar=1000 μ m.

Figure 4. MSCs Cultured as Spheroids Maintained Their Phenotypes
(A) MSCs cultured as spheroids were dissociated by TrypLE, stained with antibodies for MSC surface markers (positive: CD105, CD90, and CD73; negative: CD34), and analyzed by flow cytometry. (B) Von Kossa staining for osteogenic lineage. The intensity was measured by Image J and normalized to the total area. (C) Alcian Blue staining for chondrogenic lineage. The intensity was measured by Image J and normalized to the total area. (D) Oil red staining for adipogenic lineage. The intensity was measured by Image J and normalized to the field area (n=3). The asterisk (*) denotes significant difference ($p<0.05$) between the indicated sample and cells cultured in 10% FBS. Bar=500 μ m.

Figure 5. Dynamic Culture of MSC Spheroids

(A) MSCs were seeded at 1.0×10^5 cells/mL in 15 mL of media in 125 mL shake flasks with duplicate for each condition on a platform agitated at 80 rpm. The proliferation of MSC spheroids was measured 5 days later and the values were normalized to the control medium (10% FBS). The asterisk (*) denotes significant difference ($p < 0.05$) between the indicated sample and 10% FBS. (B) Morphology of BM-MSCs in suspension under M3, M7 and 10% FBS media (Bar: 1000 μ m). (C) Cell viability assay for MSCs spheroids growing in shake flasks in M3, M7 and in DMEM supplemented with 10% FBS. (D) Immunostaining for PCNA (Red); the nuclei were counterstained with Hoechst dye (blue). Bar=100 μ m. (E) Hematoxylin and Eosin (H&E) staining

Figure 6. Dynamic Culture of MSC Spheroids Maintained MSC Phenotype and High Differentiation Potential

(A) MSCs cultured as spheroids were dissociated by TrypLE, stained with antibodies for MSC surface markers (positive: CD105, CD90, and CD73; negative: CD34), and analyzed by flow cytometry. (B) Alizarin red staining for osteogenic lineage, (C) Alcian Blue staining for chondrogenic lineage, (D) Oil Red O staining for adipogenic lineage. The intensity was measured by Image J and normalized to the field area (n=3). The asterisk (*) denotes significant difference ($p<0.05$) between the indicated sample and 10% FBS. Bar=500 μm. (E) MSCs cultured as spheroids and after 5 days, they dissociated by TrypLE. The cell size was measured by using Vi-CELL® automatic cell counter.