Original Article

Optimizing growth media for *in vitro* expansion of human bone marrow-derived mesenchymal stem cells

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Human Bone marrow-derived mesenchymal stem cells (hMSCs) are widely used in skeletal tissue regeneration and have shown success in the clinical uses for various diseases. Besides the age-dependent decrease in proliferation potential as we reported in hMSCs, during the requisite two-dimensional monolayer expansion to achieve a clinically relevant number of cells, hMSCs exhibit profound degeneration in progenitor potency. To harness the robust therapeutic potential of hMSCs, a consistent and rapid expansion method is necessary. In this study, we investigated the effects of various growth media on the proliferation potential of hMSCs. Our data demonstrated that the investigated expansion media have significant different influences on the proliferation potential of hMSCs; comparing with regular used growth medium, MEM-plus FBS, the media of MEM-plus MSC-FBS, MEM-plus ESC-FBS, MEM-plus FBS and E8 supplement, MEM-plus FBS and STEMPRO hESC supplement, or Essential 8 plus FBS have better potentials for *in vitro* expansion of hMSCs. Our study may be useful to determine the optimization of *in vitro* expansion of hMSCs for skeletal tissue regeneration and their clinical uses.

Key Words: Human mesenchymal stem cells (hMSCs), growth media, growth characteristics, proliferation, Cell Counting Kit 8 (CCK8), WST-8.

INTRODUCTION

Human adult bone marrow-derived mesenchymal stem cells a.k.a. marrow stromal cells (MSCs), have been identified as precursors of several different cellular lineages, including osteoblasts, chondrocytes, myoblasts, adipocytes, and fibroblasts, as well as non-mesenchymal lineages, including neurons and glial cells.^{1-5,20} MSCs or MSC-like cells are not a unique feature of the bone marrow, as they also are found in non-marrow tissues such as fat, umbilical cord blood, amniotic fluid, placenta, dental pulp, tendons, synovial membrane, and skeletal muscle, though the complete equivalency of such populations has not been formally demonstrated using robust scientific methods.^{1,6} Besides human MSCs, MSCs have been isolated from different species including mice, rats, rabbits, sheep, pigs, and dogs.⁷⁻¹²

Mesenchymal stem cells (MSCs) are considered of the most promising cells for gene therapy and tissue engineering.¹³⁻¹⁷ MSC therapy is poised to establish a new clinical paradigm; although MSCs are originally considered to treat connective tissue defects,

preclinical studies revealed potent immunomodulatory properties that prompted the use of MSCs to treat numerous inflammatory conditions.¹⁸ The capability of MSCs to differentiate into multiple skeletal cell lineages under specific *in vitro* and *in vivo* conditions provides the potential for their clinical applications for repairing, bone, cartilage and joint damage by autologous stem cell transplantation or genetic tissue engineering.^{15,21-25,68} One of the

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challenges in the cell and gene therapy uses of hMSCs for a wide range of human conditions is to quickly obtain an adequate number of cells and meanwhile maintain their suitable differentiation potential to achieve therapeutic outcomes. Many *in vivo* and *in vitro* factors influence monolayer expansion to achieve a clinically relevant number of hMSCs, including the clinical attributes of subjects from whom the hMSCs were obtained, *e.g.*, age,^{2, 61} serum PTH and vitamin D levels,¹⁹ Chronic Kidney Disease,^{61, 34} exercises²⁷ and chronic ethanol intake²⁸ etc. as well as variations of *in vitro* culture media and supplements.^{66, 67}

Serum is most commonly used as a low-cost supplement in cell culture medium to expand MSCs. It contains a wide range of growth factors, hormones, vitamins, amino acids, fatty acids and trace elements required for cell growth. Serum provides carriers or chelators for labile or water-insoluble nutrients, hormones and growth factors.³⁷ The use of animal serum in culture medium of hMSCs confers several advantages and also some risks especially used in clinical applications; for example, the extensive usage of supplements containing xenogeneic components in growth media might pose a risk to the post-transplantation safety of patients.²⁶ Several categories of serum-free medium have been developed³⁷ including (a) serum-free medium, with additional mammalian hormones, growth factors, proteins, and polyamines; (b) chemically defined medium, it contains contamination-free ultra-pure inorganic and organic ingredients, and may also contain pure protein additives, like growth factors such as Fibroblast Growth Factor 2 (FGF2) and platelet-derived growth factor (PDGF) AA/AB that enhance MSC proliferation without altering MSC characteristics;^{39, 40} (c) protein-free medium, containing peptide fragments from the enzymatic or acid hydrolysis of proteins derived from animals or plants. Besides the age-dependent decrease in proliferation potential as we reported in hMSCs,² during the requisite two-dimensional monolayer expansion to achieve a clinically relevant number of cells, hMSCs exhibit profound degeneration in progenitor potency.^{66, 67} In this study, we investigated the effects of various growth media on the proliferation potential of hMSCs.

MATERIALS AND METHODS

Isolation of human mesenchymal stem cells (hMSCs)

Bone marrow samples were obtained, with IRB approval and annual review, as fresh discarded excess iliac crest marrow used for the repair of alveolar cleft from an 8-year-old female subject. Low-density marrow mononuclear cells were isolated by centrifugation in Ficoll/Histopaque 1077 (Sigma, MO).^{2, 4} This procedure removes differentiated marrow cells and enriches undifferentiated, low-density marrow mononuclear cells that include a population of non-adherent hematopoietic cells and a fraction capable of adherence and differentiation into musculo-skeletal cells. Adherent hMSCs were expanded in monolayer culture in growth medium (phenol red-free α -MEM medium (Invitrogen, Carlsbad, CA), 10% Fetal Bovine Serum (FBS) (Atlanta Biologicals, Flowery Branch, GA), 100 U/mL penicillin, and 100 μ g/mL streptomycin (Invitrogen, Carlsbad, CA). Human MSCs were analyzed for expression of CD166, CD90, CD105, STRO-1, CD34, and CD45 with flow cytometry. All the samples expressed CD166, CD90, CD105, and STRO-1.^{2, 4} The level of expression of CD34 and CD45 (markers of hematopoietic lineage) was very dim in all samples, and in some cases, negative when

compared to controls.^{2, 4}

Proliferation assays of hMSCs in different growth media

Human MSCs were sub-cultured in an incubator until passage 2 before being planted into 96-well cell microplates. For the planting process, the medium was vacuumed out from the 25-cm² cell culture flasks or 100-mm dishes containing the hMSCs and 1-2 mL of trypsin enzyme was added to the flask/dish before it was placed in an incubator. After about 5 minutes, the cells had detached from the flask/dish. MEM- α with 10% FBS was added to the mixture, which was then pipetted into a 10 mL plastic test tube to be centrifuged. After centrifugation, the liquid in the test tube was vacuumed out and replaced with 5 mL of MEM- α with 10% FBS. Cell number was determined by a hemocytometer and the solution was diluted accordingly to 1,000 cells per 100 μ L. 100 μ L of the diluted solution was transferred to each of 60 wells in a 96-well plate and incubated for 3 days. After the incubation period, the sixty samples of hMSCs were treated with various media, growth factors or BCI (Table 1 and Fig. 2&3) and allowed to proliferate over the course of 3-4 days.

Cell proliferation is not clearly displayed.

Cell Counting Kit-8 (Dojindo Molecular Technologies, Rockville, Maryland) was used to assess the proliferation of hMSCs. After 3-4 days with different growth media in 96-well plates, each well was given a mixture of 10 μ L WST-8 and 100 μ L MEM α and incubated for 1 hour before the optical density (OD) was measured at a 450nm wavelength using an ELISA reader.

Statistical analysis

All experiments were performed at minimum in triplicate. All test parameters were quantified as continuous data and used standard statistical tests. The Kolmogorov-Smirnov tool was used to test whether data sets were distributed normally. Group data are presented as mean $\pm$ SEM. Unless otherwise indicated, quantitative data were analyzed with non-parametric tools, either the Mann-Whitney test for group comparisons or Spearman correlation test. If data allowed, parametric tools were used, either t-test for two groups or one-way ANOVA for multiple group comparisons or Pearson correlation test. Multivariable regressions were performed with GraphPad Instat (GraphPad Software, La Jolla, CA) to determine the significant contribution variable(s). A value of $p < 0.05$ was considered significant.

RESULTS

Calibration between the number of hMSCs and optical density

Cell Counting Kit-8 allows sensitive colorimetric assays for the determination of cell viability in cell proliferation and cytotoxicity assays (CCK-8, Dojindo Molecular Technologies). Water-soluble tetrazolium salt, WST-8, is reduced by dehydrogenase activities in hMSCs to give a yellow-color formazan dye (WST-8 formazan), which is soluble in the tissue culture media and the optical density (OD) can be measured at a 450nm wavelength using an ELISA reader (Fig. 1A). To calibrate whether the amount of the formazan dye, generated by the activities of dehydrogenases in hMSCs, is directly proportional to the number of living cells, hMSCs

obtained from an 8-year-old female subject were seeded from 0-10,000 cells per well in 96-well plates (5 wells per group). Spearman Rank correlation assays showed significantly

correlations between OD at 450nm and cell numbers of hMSCs in two different assays (Fig. 1B: $r=0.98$, $p=0.007$; Fig. 1C: $r=0.98$, $p=0.009$; $n=5$ wells per group).

Table 1 The different growth media for *in vitro* expansion of hMSCs

Medium No.	Growth medium composition	Note
#1	MEM- α + 10% FBS	MEM- α - Minimum Essential Medium α (Invitrogen/Thermo Fisher Scientific); FBS - Fetal Bovine Serum (Atlanta Biologicals).
#2	MEM- α + 10% MSC-FBS	MSC-FBS - Mesenchymal stem cell (MSC) Qualified FBS (Gibco/ Thermo Fisher Scientific); Performance-tested using standard MSC/colony forming unit-fibroblast (CFU-F) assay.
#3	MEM- α + 10% ESC-FBS	Embryonic stem cells - Qualified FBS (Gibco/ Thermo Fisher Scientific).
#4	MEM- α + 1xE8s	E8s - E8 Supplement (STEMCELL Technologies). E8 supplement includes L-ascorbic acid-2-phosphate magnesium (64 mg/L), sodium selenium (14 μ g/L), FGF2 (100 μ g/L), insulin (19.4 mg/L), NaHCO ₃ (543 mg/L) and transferrin (10.7 mg/L), TGF β 1 (2 μ g/L) or NODAL (100 μ g/L). ³²
#5	MEM- α + 10% FBS + 1xE8s	
#6	MEM- α + 1xSTEMPRO	STEMPRO - StemPro [®] hESC Supplement (Gibco/ Thermo Fisher Scientific).
#7	MEM- α + 10% FBS + 1xSTEMPRO	
#8	Essential 8 + 10% FBS Medium	Essential 8 [™] Medium is a low-protein, feeder-free maintenance medium for human ES and iPS cells (STEMCELL Technologies).
#9	MEM- α + 10% FBS + 2 ng/mL FGF-2 + 10 μ g/mL Heparin.	Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium with FGF-2. ³³
#10	MEM- α + 10% FBS + 25 μ M BCI	BCI - (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one. BCI enhances FGF signaling by blocking dual specificity phosphatase 6 activity. ³⁶

Figure 1 Cell proliferation was determined with Cell Counting Kit-8 (CCK-8). (A) Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies) allows sensitive colorimetric assays for the determination of cell viability in cell proliferation and cytotoxicity assays. Water-soluble tetrazolium salt, WST-8, is reduced by dehydrogenase activities in hMSCs to give a yellow-color formazan dye (WST-8 formazan), which is soluble in the tissue culture media and the optical density (OD) can be measured at a 450nm wavelength using an ELISA reader. (B&C) The amount of the formazan dye, generated by the activities of dehydrogenases in hMSCs obtained from an 8-year-old female subject, is directly proportional to the number of living cells as shown by Spearman correlations between OD (A450nm) and cell numbers of hMSCs (B: $r=0.98$, $p=0.007$; C: $r=0.98$, $p=0.009$; $n=5$ wells per cell number).

The effects of various growth media on the proliferation of hMSCs.

Human MSCs obtained from an 8-year-old female subject (8F) were seeded 1,000 cells per well in 96-well plates ($n=5$ wells per medium). After the incubation period of 3 days in MEM- α with 10% FBS, hMSCs were treated with various growth media (Table 1) and allowed to proliferate over the course of 3 days before CCK-8 assays. The experiments were performed twice and the representative results from one of the experiments were shown in Figure 2. Our data showed that 10% Mesenchymal Stem Cell (MSC)-qualified FBS (MSC-FBS, Gibco/ Thermo Fisher Scientific) in MEM- α is significant better serum for the proliferation of hMSCs than FBS and ESC-FBS (Fig. 2A, $***p<0.001$, #2 vs. #1, $n=5$; $##p<0.01$, #2 vs. #3, $n=5$; ANOVA); ESC-FBS is slight better serum than regular FBS ($*p<0.5$, #3 vs. #1, $n=5$, ANOVA). Essential 8[™] Medium is a low-protein, feeder-free maintenance medium for human ES and iPS cells (STEMCELL Technologies). E8 Supplement (STEMCELL Technologies) includes L-ascorbic acid-2-phosphate magnesium (64 mg/L), sodium selenium (14 μ g/L), FGF2 (100 μ g/L), insulin (19.4 mg/L), NaHCO₃ (543 mg/L) and transferrin (10.7 mg/L), TGF- β 1 (2 μ g/L) or NODAL (100 μ g/L).³² Our data (Fig. 2B) showed that there is no different between E8 supplement (1xE8s, #4) and 10% FBS (#1) (NS, not significant); but E8 supplement plus 10% FBS (#5) has a synergistic effect on the proliferation of hMSCs ($**p<0.01$, #5 vs. #1, $n=5$; $##p<0.01$, #5 vs. #4, $n=5$; ANOVA). STEMPRO is a supplement for hESCs (Gibco/ Thermo Fisher Scientific). Our data (Fig. 3C) showed that STEMPRO (#6) plus 10 % FBS has a synergistic effect on the proliferation of hMSCs (NS, not significant, #6 vs. #1; $**p<0.01$, #7 vs. #1, $n=5$; $##p<0.01$, #7 vs. #6, $n=5$; ANOVA). Our

data (Fig. 2D) showed that Essential 8™ Medium plus 10% FBS is a better medium than MEM- α plus 10% FBS for hMSCs (** $p=0.0048$, Unpaired t test with Welch correction).

Figure 2 Effects of various media on proliferation of hMSCs. hMSCs obtained from an 8-year-old female subject were seeded 1,000 cells per well in 96-well plates. After the incubation period of 3 days, hMSCs were treated with various media (Table 1) and allowed to proliferate over the course of 3 days. (A) MSC-FBS is significant better serum for the proliferation of hMSCs than FBS and ESC-FBS (** $p<0.001$, #2 vs. #1, $n=5$; ## $p<0.01$, #2 vs. #3, $n=5$; ANOVA); ESC-FBS is slight better serum than regular FBS (* $p<0.5$, #3 vs. #1, $n=5$, ANOVA). (B) There is no different between E8 supplement (1xE8s, #4) and 10% FBS (#1) (NS, not significant); but E8 supplement plus 10% FBS (#5) has a synergistic effect on the proliferation of hMSCs (** $p<0.01$, #5 vs. #1, $n=5$; ## $p<0.01$, #5 vs. #4, $n=5$; ANOVA). (C) STEMPRO (#6) plus 10% FBS has a synergistic effect on the proliferation of hMSCs (NS, not significant, #6 vs. #1; * $p<0.01$, #7 vs. #1, $n=5$; ## $p<0.01$, #7 vs. #6, $n=5$; ANOVA). (D) Essential 8™ Medium plus 10% FBS is a better medium than MEM- α plus 10% FBS for hMSCs (** $p=0.0048$, Unpaired t test with Welch correction).

Effects of FGF signaling on proliferation of hMSCs.

FGF-2 is a growth factor for many type stem cells including hMSCs²⁹ and human embryonic stem cells.³⁰ Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium with FGF-2.³³ Our data (Fig. 3A) showed that 2 ng/mL of FGF plus 10 μ g/mL of Heparin in MEM- α with 10% FBS significantly stimulated the proliferation of hMSCs (8F) (** $p<0.01$, #9 vs. #1, $n=5$). BCI, (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one, enhances FGF signaling by blocking dual specificity phosphatase 6 activity.³⁶ Our data (Fig. 3A) showed that 25 μ M of FGF signaling activator, BCI, is toxic for hMSCs (** $p<0.01$, #10 vs. #1, $n=5$; ANOVA). A second round of experiments was performed with hMSCs taken from the same bone marrow samples, after 4 days of treatments [but not 3 days as

in Fig. 2 and Fig. 3A] with 1 nM, 10 nM, 100 nM, 1 μ M, 5 μ M, 10 μ M, or 25 μ M BCI (DMSO was used as a control), the cells were given a mixture of MEM- α containing 10% FBS with WST-8 and incubated for one hour before the plate was placed into a microplate reader for cell counting. Our data (Fig. 3B) showed that low doses of BCI (1-100 nM) had no effect and 1 μ M of BCI significantly reduced the proliferation potential of hMSCs; high doses of BCI (>5 μ M) are toxic to hMSCs (** $p<0.001$, BCI vs. DMSO, $n=4$, ANOVA).

Figure 3 Effects of FGF signaling on proliferation of hMSCs. (A) After 3 days, FGF plus Heparin in MEM- α with 10% FBS significantly stimulated the proliferation of hMSCs (8F) (** $p<0.01$, #9 vs. #1, $n=5$); 25 μ M of FGF signaling activator, BCI, is toxic for hMSCs (** $p<0.01$, #10 vs. #1, $n=5$; ANOVA). (B) The dose-response effects of BCI on the proliferation of hMSCs. hMSCs were treated with 1nM, 10 nM, 100 nM, 1 μ M, 5 μ M, 10 μ M, or 25 μ M BCI for 4 days; DMSO was used as a control. Low doses of BCI (1-100 nM) had no effect and 1 μ M of BCI significantly reduced the proliferation potential of hMSCs; high doses of BCI (>5 μ M) are toxic to hMSCs (** $p<0.001$, BCI vs. DMSO, $n=4$, ANOVA).

DISCUSSION

Mesenchymal stem cells play an increasingly important role in tissue engineering, gene therapy, and immunotherapy. Many *in vivo* and *in vitro* factors influence monolayer expansion to achieve a clinically relevant number of hMSCs, including variations of culture media and supplements.^{66,67} The different expansion media had a significant influence on the expression of CD10, CD90, CD105, CD140b CD146 and STRO-1.³⁵ The proliferative capacity of mesenchymal stem cells is a key factor affecting the efficacy of its clinical application. In this study, we investigated the effects of by various growth media on the proliferation capability of hMSCs.

Our data demonstrated that serum is necessary for these media to proliferate hMSCs. Fetal bovine serum (FBS) comes from the blood drawn from a bovine fetus *via* a closed system of collection at the slaughterhouse. Fetal bovine serum is the most widely used serum-supplement for the *in vitro* cell culture of eukaryotic cells. This is due to it having a very low level of antibodies and containing more growth factors, allowing for versatility in many different cell culture applications.³¹ FBS contains a complex array of protein components that are required by many cells to grow which is why it has been successfully used in cell culture. Unfortunately, large quantities of undefined proteins can lead to unwanted stimulation of cells. The precise composition of FBS is unknown because this additive comprises various unidentified, naturally occurring small molecules and proteins in fluctuating

proportions.⁴¹ Bovine serum albumins are the major component of FBS. Growth factors in FBS are essential for the maintenance and growth of cultured cells.³⁹ FBS also contains a variety of small molecules like amino acids, sugars, lipids, and hormones. The concentration of FBS (10%) is commonly used growth media; Kwon *et al* showed that high concentrations (20% and 30%) increased the proliferation of hADSCs by approximately two-fold.⁴² Therefore, searching appropriate FBS concentration may be one of the directions for promoting cells proliferation. MSC-Qualified FBS is specially tested for the ability to support the expansion and clonal enumeration (Colony Forming Unit Fibroblast Assay) of mesenchymal stem cells (Gibco/ Thermo Fisher Scientific). Our data showed that MSC-qualified FBS in MEM- α is significant better serum for the proliferation of hMSCs than FBS and ESC-FBS, demonstrated that lot-to-lot variation in FBS is a very important factor for *in vitro* expansion of hMSCs.

FBS is a component used for the “classical” method of culturing hMSCs for many years. However, because of many unknown elements in FBS, such as growth factors, apoptotic factors, nutrients and other undefined proteins that can cause lot-to-lot variation, lead to serious immune response and potential risk for disease infection when they were used for clinical therapy. To avoid undesirable complications, other types of media like serum-free media and xeno-free media have been evaluated as promising alternatives for the manufacturing of clinical-grade hMSCs. Recently, many researches have focused on the development of animal serum-free culture media with the utilization of human derived growth supplements for hMSC expansion, such as human platelet lysates.⁴³⁻⁴⁶ Human serum contains abundant bioactive compounds and growth factors such as platelet-derived growth factors that enhance MSC proliferation without alteration of surface epitopes and differentiation potentials.⁴⁰ Additionally, serum-free media and xeno-free media have been developed for the expansion of hMSC towards clinical applications. Naung *et al.* demonstrate that palatal-derived MSCs could become adipocytes, chondrocytes, and osteoblasts under the culture of serum-free media and xeno-free media.⁴⁷ A comparative study's results showed that Wharton's jelly derived mesenchymal stem cells cultured in serum-free, xeno-free medium exhibit superior growth kinetics and functional angiogenesis, alongside other MSC characteristics.⁴⁸ In this study, StemPro supplement (group #6) and Essential 8 supplement (E8s, group #4) belong to serum-free and xeno-free media respectively. StemPro MSCs media can provide superior efficiency of human MSC expansion at high cell densities, requiring less medium, surface area, and time compared with classical medium (DMEM low glucose + 10% FBS).⁴⁹⁻⁵¹ Essential 8 Medium has been extensively tested and proved to maintain pluripotency in multiple iPSC lines. In addition, Essential 8 Medium has been used to scale up production of iPSCs and shown to support iPSC growth for >50 passages without any signs of karyotypic abnormalities, along with maintaining the ability of iPSCs to differentiate into all three germ line lineages.⁵² Our results showed that expansion of hMSCs in StemPro (#6) and E8 media (#4) are comparable to classical medium with serum (MEM- α + 10% FBS, group #1); after added 10% FBS (group #5 and #7), these media significantly improve the proliferation of hMSCs compared with FBS alone group (#1). Although previous studied have tested that serum-free media/xeno-free culture media can augment the growth potential of MSCs.⁴⁶⁻⁴⁸ However, additional studies demonstrated that

serum-free media result in reduced the proliferative capacity of MSCs,⁵³ and cannot promote hMSC growth without the addition of cytokines and/or growth factors, possibly since serum induces intracellular calcium oscillations, which are vital to stem cell proliferation and differentiation.⁵⁴

Studies have demonstrated that cytokines and/or growth factors influence the proliferation of MSCs.^{39, 40, 56} Fibroblast Growth Factors (FGFs) are members of a large family of secreted glycoproteins that serve important functions in development, proliferation and cellular homeostasis.⁶³ In humans, 22 members of the FGF family have been identified, all of which are structurally related signaling molecules.^{55, 63-65} FGF2, also known as basic fibroblast growth factor (bFGF), is a growth factor and signaling protein encoded by the FGF2 gene that has been demonstrated to enhance various kind of tissue repair³⁸ and a growth factor for many type stem cells including hMSCs²⁹ and human embryonic stem cells.³⁰ Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium with FGF-2.³³ In this study, we tested the influence of FGF signaling on proliferation of hMSCs and showed that FGF-2 plus Heparin in MEM- α with FBS significantly stimulated the proliferation of hMSCs; FGF-2 alone has no additional effect on the proliferation of hMSCs comparing with 10% FBS (Data not shown). Small molecules have great potential as substitutes for recombinant cytokines and unknown factors in serum that control stem cell fate have been focus on interest in recent years, as the critical roles of pathways mediating stem cell proliferation and self-renewal have become better understood,⁵⁷⁻⁵⁹ suggesting that small molecules can replace or enhance the effects of morphogen factors that would be suitable for making defined media for large-scale culture. BCI, (E)-2-benzylidene-3-(cyclohexylamino)-2,3-dihydro-1H-inden-1-one, is a small molecule of FGF-2 activator, enhances FGF signaling by blocking dual specificity phosphatase 6 activity.³⁶ Molina G *et al* demonstrated FGF signaling activation by BCI treatment (5 to 10 μ M) during zebrafish embryo development is reported to expand myocardial progenitor population at the expense of other progenitor cell types, resulting in enlarged heart size.³⁶ Our study demonstrated that BCI cannot instead of FGF-2 because its toxic for hMSCs when concentrations of BCI is over 1 μ M and low concentration of BCI has no influence on hMSCs.

In summary, our data demonstrated that the investigated growth media have significant different influences on the proliferation potential of hMSCs; comparing with regular growth medium, MEM- α plus FBS, the media of MEM- α plus MSC-FBS, MEM- α plus ESC-FBS, MEM- α plus FBS and E8 supplement, MEM- α plus FBS and STEMPRO hESC supplement, or Essential 8 plus FBS have better potential for *in vitro* expansion of hMSCs. Our study may be useful to determine the optimization of *in vitro* expansion of hMSCs for skeletal tissue regeneration and their clinical applications.

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Conflicts of Interest

The authors declare no conflict of interest.

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