Short-term exposure of human minor salivary gland stem cells to hypergravity enhances stemness

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Abstract

Radiation therapy for head and neck cancer damages local stem cells and epithelial cells in salivary glands (SG), leading to irreversible SG dysfunction. Biological understanding of the responses of tissue-resident stem cells to gravity is required to develop therapeutic strategies for damaged tissue regeneration. In this study, we successfully isolated human minor salivary gland stem cells (huMSGSCs), which have high proliferation rates, express multiple stem markers, and can be differentiated into mesenchymal cell types. Cell proliferation, sphere-forming ability, stemness marker expressions, and epithelial differentiation potentials were checked after exposing huMSGSCs short-term to hypergravity (HyperG) or microgravity (MicroG). Proliferation after exposure to HyperG (40 G) was greater than exposure to 1G, but no difference was observed between MicroG (10^{-3} G) and 1G. Numbers of large spheres were significantly higher post-HyperG and lower post-MicroG than at 1G, whereas numbers of small spheres were significantly lower post-HyperG and higher post-MicroG. The expressions of stemness markers (CD90, LGR5, CD29, and CD24) and junction markers (ZO-1 and ZO-2) were increased post-HyperG. Furthermore, increases in PAS staining and the gene expressions of albumin and CK19 confirmed that HyperG enhances the epithelial differentiation potential of huMSGSCs. This study shows that cultivating huMSGSCs under HyperG conditions enhances stemness and that a gravity control system could be applied to modulate huMSGSC functions.

Introduction

Salivary glands (SGs) are exocrine glands that secrete saliva and play important roles in oral health, swallowing, digestion, and speaking. On the other hand, SG dysfunction causes fissured tongue, taste loss, swallowing difficulty, and xerostomia and may be caused by aging, drug abuse, sialadenitis, or Sjögren's syndrome[1,2]. More specifically, radiotherapy for head and neck cancer reduces SG stem cell numbers and, therefore SG cell numbers. Thus, restoring SG numbers using stem cells has been suggested as a potential treatment for radiotherapy-induced SG cell loss[3–5].

Previous studies have focused mainly on the use of adipose tissue, bone marrow, and umbilical cord-derived mesenchymal stem cells to treat injured SG tissues. Recent studies have attempted to isolate an appropriate source of stem/progenitor cells for this purpose[6–8]. Although major SG-derived stem cells have been well studied, their clinical applications are limited by biopsy-associated difficulties [9]. However, minor salivary glands in oral mucosa have been suggested as an alternative because they can be readily harvested in large numbers [10–13].

Culture-related factors that enhance cell functions and therapeutic effects include soluble factors, scaffolds, hypoxia, and gravity [14–18]. Several studies have reported that hypergravity (HyperG) enhances the growth rate of adipose stem cells [19]. However, others have suggested that microgravity (MicroG) is favorable for stem cell culture [20,21] or that MicroG limits stem cell growth [22,23]. Nonetheless, little is known of the effects of gravity on human minor salivary gland stem cells (huMSGSCs).

The purpose of this study was to isolate and characterize huMSGSCs and evaluate changes in their properties after exposure to different gravities.

Results

Cell morphologies, proliferation activities, and differentiation potentials of huMSGSCs

Isolated huMSGSCs had a fibroblast-like appearance and proliferated well to passage 10 without any morphological changes (Figs. 1a and 1b). We tested whether hMSGSCs could differentiate into many cell types by examining their potentials to differentiate to adipocytes, chondrocytes, or osteocytes. When huMSGSCs were induced to differentiate into these lineages, they exhibited fat, bone, and cartilage phenotypes, respectively. The expressions of lineage-specific molecular markers were also confirmed. huMSGSCs treated with adipogenic, chondrogenic, or osteogenic differentiation media exhibited significantly stronger expressions of adipogenic (PPARγ and FABP4), chondrogenic (COL1 and SOX9), and osteogenic (SP7 and GBGLAP) genes, respectively, than those in the undifferentiated state (Fig. 1c).

Stem Cell Marker Expression

The expressions of mesenchymal stem cell markers (CD29, CD44, CD90, and CD105) and pluripotent stem cell markers (Nanog, Oct3/4 and SOX2) on huMSGSCs were analyzed by flow cytometry. huMSGSCs were positive for mesenchymal markers such as CD44, CD90, CD105, and CD29 with positivities of 95.7%, 72.4%, 32.1%, and 19.7%, respectively. Interestingly, huMSGSCs expressed pluripotent markers such as Nanog and Oct3/4 at 20.7% and 31.6% but rarely expressed SOX2 (3.0%) (Fig. 1d).

Proliferation Activities Of Humsgscs After Exposure To Microg Or Hyperg

We next tested whether short-term exposure to MicroG or HyperG affected huMSGSC proliferation. After culturing for 24 hr, cell proliferations were similar in the 1G, MicroG, and HyperG groups. However, after 48 and 72 hr, cells in the HyperG group proliferated more than cells in the 1G group in a time-dependent manner. Proliferation in the MicroG group was significantly higher than in the 1G group at 48 hr, but no difference was observed at 24 or 72hr (Fig. 2a).

Sphere Forming Abilities Of Humsgscs Exposed To Different Gravities

To determine whether exposure to gravity altered stemness, we compared the size and number of spheres generated in the 1G, MicroG, and HyperG groups. Comparisons showed mean sphere size was no

different in the HyperG and 1G groups, but that sphere size was significantly smaller in the MicroG group than in the 1G group (Figs. 2b and 2c). The effect of sphere size was examined by subgroup analysis using a cut-off of 1000 pixels. The number of large spheres was significantly greater in the HyperG group and lower in the MicroG group than in the 1G group (54.3 ± 1.5 spheres in the HyperG group, 45 ± 4.3 in the 1G group, and 5.6 ± 0.5 in the MicroG group), whereas the mean numbers of small spheres in the HyperG, MicroG, and 1G groups were 80.3 ± 1.5, 142.6 ± 2.5, and 86.6 ± 1.5, respectively (Fig. 2d).

Expressions Of Stem Cell Markers In Humsgscs Exposed To Different Gravities

We also tested stem cell marker expressions in huMSGSCs exposed to MicroG and HyperG. At the gene expression level, HyperG exposed cells had higher mesenchymal stem cell marker (CD24 and CD29) and salivary stem cell marker (CD90 and LGR5) expressions than cells in the 1G group. MicroG exposed cells expressed CD24 less than cells in the 1G group, but CD90, LGR5, and CD29 gene expressions were similar in the MicroG and 1G groups (Fig. 3a). Western blot showed the protein expressions of CD90, LGR5, and SOX2 were significantly more expressed in HyperG exposed cells than in 1G cells. SOX2 expression was higher in the MicroG group than in the 1G group, but the expressions of CD90 and LGR5 were similar (Fig. 3b). Immunofluorescence analysis showed the expressions of CD90, LGR5, and SOX2 were higher in HyperG exposed cells than in 1G cells (Fig. 3c).

Cell-cell Junction Gene And Protein Expression Changes After Exposure To Different Gravities

The gene expressions of ZO-1 and ZO-2 were significantly higher in the HyperG group than in the 1G group but similar in the MicroG and 1G groups (Fig. 4a). The protein expressions of ZO-1 and ZO-2 were higher in the HyperG group but lower in the MicroG group than in the 1G group (Fig. 4b). Immunofluorescence staining confirmed that ZO-1 and ZO-2 expressions were higher in the HyperG group than in the 1G and MicroG groups (Fig. 4b).

Hyperg Promoted Humsgscs Differentiation

To evaluate the epithelial differentiation potentials of huMSGSCs after HyperG exposure, we assessed their abilities to differentiate into hepatocytes *in vitro*. Upon inducing hepatogenic differentiation, cells in the 1G and HyperG groups adopted a round form. PAS staining, which specifically stains glycogen in liver hepatocytes, showed the HyperG group had a greater percentage of PAS positive cells than the 1G group (Fig. 5a). Hepatocyte marker genes, including albumin, CK14, and CK19, were significantly more expressed in the HyperG group than in the 1G group (Fig. 5b). Collectively, these results show short-term exposure to HyperG enhances the epithelial differentiation potential of huMSGSCs.

Discussion

Stem cell therapy is a treatment option in several areas in the biomedical field, but the origins of stem cells are commonly overlooked. Many studies have been conducted on the applications of adipose tissue, bone marrow, and umbilical cord-derived stem cells for tissue repair [24–27], and interest in tissue-specific stem cells is increasing. Major salivary gland-derived stem cells have been well-studied and successfully differentiated into salivary glands *in vivo*[8]. However, the major salivary glands are not considered an ideal or safe stem cell source due to the invasiveness of the procedure at donor sites. On the other hand, there are hundreds of minor salivary glands in human oral mucosa, and they are considered a promising candidate for cell therapy because of their accessibility and abundance [10–13].

In this study, we isolated human minor salivary gland stem cells (huMSGSCs) and examined their stem cell properties, including proliferation, stemness marker expression, and differentiation potentials. Isolated huMSGSCs had a fibroblast-like appearance and proliferated well to passage 10 without any morphological changes (data not shown). Flow cytometry showed huMSGSCs expressed mesenchymal stem cell markers such as CD44, CD29, CD73, CD90, and CD105 and pluripotent/embryonic stem cell markers such as Nanog and Oct3/4.

In addition, when huMSGSCs were induced to differentiate into adipogenic, chondrogenic, osteogenic, and hepatogenic lineages, they exhibited fat, cartilage, bone, and hepatocyte phenotypes. Furthermore, huMSGSCs treated with adipogenic, chondrogenic, osteogenic, or hepatogenic differentiation medium exhibited significantly stronger expressions of adipogenic (PPARγ and FABP4), chondrogenic (COL1 and SOX9), osteogenic (SP7 and GBGLAP), and hepatogenic (Albumin) genes, respectively, than cells in the undifferentiated state. These results suggest that huMSGSCs could be classified as multipotent, as previously reported [11].

Stem cells reside in a specialized microenvironment, a niche, that provides support and signals them to maintain themselves and self-renew as required by local cellular dynamics [28]. Stem cells communicate with the local microenvironment via mechanical signals to regulate cell fate and cell behavior and guide developmental processes [29].

It has long been established that external mechanical forces modulate response to injury. Mechanical unloading of bone in a MicroG environment favors the maintenance and expansion of mesenchymal and hematopoietic stem cells in bone marrow while limiting their differentiation [30]. Moreover, proliferation rates and microfilaments and microtubule densities of human adipose-derived stem cells were improved under HyperG conditions [19]. Many reports have been issued on focal adherence complex, proliferation, differentiation, and growth behavior changes of human cells exposed to different gravities [31–35], but no study has investigated the effects of gravity on salivary gland stem cells. Accordingly, we investigated the effects of short-term gravity exposure (30 min) on the physiologies and molecular make-ups.

Regarding the effects of gravity on huMSGSC proliferation, after culture for 24 hr, cell proliferation rates were similar in the HyperG, MicroG, and 1G groups. However, cells in the HyperG proliferated more rapidly than those in the 1G group in a time-dependent manner, which concurs with that reported for adipose-derived stem cells [19]. On the other hand, proliferation in the MicroG group was significantly greater than in the 1G group at 48 hr but similar at 24 and 72 hr.

Sphere formation assays are widely used to assess stemness[36]. We compared the sizes and numbers of spheres in the HyperG, MicroG, and 1G groups. Sphere size was significantly smaller in the MicroG group than in the 1G and HyperG groups, and large sphere numbers were greater in the HyperG group than in the 1G group. On the other hand, small sphere numbers were greater in the MicroG group than in the 1G group. According to the literature, stem cells give rise to large spheres while progenitor cells without the ability to self-renew generate small spheres, and sphere size reflects the proliferative ability of sphere-forming cells [37]. Therefore, our observation of higher numbers of large spheres in the HyperG group suggests that HyperG increased stemness.

When we investigated the expressions of mesenchymal stem markers (CD24 and CD29), salivary stem markers (CD90 and LGR5), and a pluripotent stem marker (SOX2) in the HyperG, MicroG, and 1G groups, we found the mRNA levels of CD24, CD29, CD90, and LGR5 and the protein expressions of CD90, LGR5, and SOX2 were significantly higher in the HyperG group than in the 1G group. However, stem marker expressions at the mRNA and protein levels (except those of SOX2) were similar in the MicroG and 1G groups. Immunofluorescence analysis showed that the expressions of LGR5, CD90, and SOX2 were higher in the HyperG group but lower in MicroG group than in the 1G group. Our results confirm that the expression of stemness markers is significantly greater for large spheres, which concurs with a previous report [38].

Several recent reports have concluded that cell junctions are critical for maintaining stem cell function [39], niche homeostasis [40], and neural stem cell differentiation [41], and Alvarez R. reported that simulated MicroG causes epithelial barrier dysfunction and delayed junction localization of tight junction proteins, occluding, and ZO-1 in RWV cells[42]. We investigated how the expressions of tight junctions in huMSGSCs are altered by MicroG and HyperG exposure. The gene expressions of ZO-1 and ZO-2 were significantly higher in the HyperG group than that in the 1G group, but expressions were similar in the MicroG and 1G groups. Western blot showed the protein expressions of ZO-1 and ZO-2 were significantly higher in the HyperG group than in the MicroG group and lower in the MicroG group than in the 1G group. Fluorescence analysis confirmed that ZO-1 and ZO-2 were expressed at higher levels in the HyperG group than in the other two groups.

According to previous studies, the differentiation ability of stem cells is closely related to stemness [44], and the results of our study show that cells exposed to HyperG had better stemness than cells in the 1G group but that cells exposed to MicroG group were similar to cells in the 1G group. Therefore, we compared the differentiation abilities of HyperG and MicroG exposed huMSGSC. Interestingly, cells in the HyperG group showed better epithelial differentiation than cells in the 1G group based on increased

percentages of PAS-positive cells and higher hepatogenic gene expressions (albumin CK14 and CK19), which concurs with a previous report [43].

In conclusion, we found that established huMSGSCs exposed short-term to HyperG (30 min at 40G) exhibited higher cell proliferation rates and stemness marker expressions and greater sphere forming abilities and epithelial differentiation potentials. To the best of our knowledge, this is the first report that HyperG affects the stemness and epithelial differentiation potential of huMSGSCs. Further investigations are needed to identify the mechanisms initiated by exposing huMSGSCs to high gravity and understand their biological roles.

Materials And Methods

Isolation of human minor salivary gland stem cells (huMSGSCs)

A human minor salivary gland tissue was carefully excised from a minor salivary gland after obtaining informed patient consent and Inha University Hospital IRB approval (INHA 18 0503–560). All experimental procedures were performed according to the guidelines and regulations issued by Inha University. The tissue sample was then isolated and cultured as follows. The minor salivary gland sample was washed, chopped, and minced, incubated with 0.05% trypsin-EDTA solution (Gibco, USA) in a 5% CO_2 atmosphere for 30 min at 37℃, filtered, and centrifuged. Collected cells were then cultured in mesenchymal stem cell media (ATCC, USA) in a humidified 5% CO_2 atmosphere at 37℃.

Proliferation Assessment

After exposure to normal (1G), micro (10^{-3} G), or hyper-gravity (40G), cells were incubated for 3 hrs in CELLOMAX™ solution (PreCareGene, Korea). The absorbances of the purple solution of the cells were measured at 450 nm using a 96-well plate reader (Dynex Revelation, Dynex Ltd., UK).

Differentiation

The differentiation abilities of four types of huMSGSCs (adipogenic, chondrogenic, osteogenic, and hepatogenic) were investigated. For adipogenesis differentiation, huMSGSCs were seeded in a 12-well plate at $1x10^5$ cells/mL and cultured in adipogenesis medium in 5% CO_2 atmosphere for 3 days at 37℃. Differentiated huMSGSCs were then fixed with 4% formaldehyde and stained with Oil Red O. For chondrogenic differentiation, a micromass culture of huMSGSCs was generated by seeding a 5 uL droplet of a cell solution containing $1X10^7$ cell/mL in the center of a 24-well plate. After micromass culture for 2 hrs, chondrogenesis media was added and cells were incubated in 5% CO_2 for 14 days at 37℃. Differentiated huMSGSCs were washed with DPBS, fixed with 4% formaldehyde solution, and

stained with 1% Alcian Blue Solution in 0.1N HCl (Gibco, USA). Blue staining (indicating the synthesis of proteoglycans by chondrocytes) was detected under a light microscope (Nikon, Japan). For osteogenic differentiation, $1X10^5$ cell/mL of huMSGSCs were seeded into a 12-well plate containing osteogenesis media and cultured in 5% CO_2 for 21 days at 37°C. After fixation, huMSGSCs were stained with 2% Alizarin Red S solution (pH 4.2), rinsed with distilled water, and visualized under a light microscope. For hepatogenic differentiation, cells were seeded in 6-well plates, cultured in hepatocyte differentiation media for 14 days, and stained with periodic acid–Schiff (PAS).

Flow Cytometric Analysis For Stem Cell Marker Expression

huMSGSCs in passages 4–8 were subjected to flow cytometry to analyze cell surface marker expressions. Briefly, huMSGSCs were washed with DPBS, harvested using trypsin/EDTA, and incubated with isothiocyanate (FITC) fluorescent conjugated antibodies. Cells were then analyzed using a CytoFlex unit (Beckman Coulter, USA), and data were analyzed using Flowjo software v.10 (BD Biosciences, USA). The monoclonal antibodies (all from Santa Cruz, USA) used were; CD29, CD44, CD90, and CD105 (mesenchymal stem cell markers) and Nanog and OCT3/4 (pluripotent stem cell markers).

Exposure To Microg

An omnidirectional gravity control device "Gravite®" (Space Bio-Laboratories, Japan) was used to generate MicroG. By controlled rotation about two axes, the device minimizes the accumulated gravity vector at the center of the device and produces an average gravity of 10^{-3} G. huMSGSCs were seeded in 25T flask at $5x10^5$ cells/plate containing culture medium and maintained in 5% CO_2 for 24h at 37°C and then placed inside the Gravite device for 30 min. After exposure to microgravity, cells were incubated for up to 3 days.

Hyperg Exposure By Centrifuge

A centrifuge (Eppendorf, Germany) was used to apply HyperG (40 G) to cultured huMSGSCs [11]. In brief, huMSGSCs were seeded in a 25T flask at $5X10^5$ cells/plate containing culture media and maintained in 5% CO_2 for 24h at 37°C. They were then exposed to Hyper G at 40G for 30 min using a centrifuge and incubated for up to 3 days.

Creation Of Humsgsc Spheres

To generate spherical huMSGSCs, cells were seeded in an ultra-low attachment flask (Corning, USA) at $1x10^5$ cells/plate, exposed to 1G, MicroG, or HyperG, and cultured in 5% CO_2 for 14 days at 37°C. Images of huMSGSCs spheres were captured using an optical microscope, and sphere sizes and numbers were calculated using Image J (NIH, USA). Spheres were collected after culture, fixed with 4%

paraformaldehyde solution, embedded in paraffin, cryosectioned at 5 μm, stained with Hematoxylin, and examined under an optical microscope.

Gene Expression Analysis

Total RNA was isolated from cells using the RNeasy Mini kit (Qiagen, Germany). Complementary DNA (cDNA) was synthesized from total RNA using the Tetro cDNA synthesis kit (Bioline, USA) by incubating reaction mixtures for 30 min at 45℃, 5 min at 85℃, and then cooling to 4℃. Using cDNA as template, real-time PCR (real-time polymerase chain reaction) was performed in 96-well plates (Applied Biosystems, USA) using SYBR green II Master Mix (Takara Bio Inc., Japan) in a StepOne unit (Applied Biosystems, USA) using the following profile: 95 ℃ for 20 s followed by 40 amplification cycles of 95℃ for 5 s and 60℃ for 20 s. Albumin, CD24, CD29, CD90, CK14, CK19, LGR-5, SOX9, ZO-1, and ZO-2 were amplified, and β-actin was used as the endogenous control.

Protein Expression Analysis

Protein samples were isolated from huMSGSCs (40 μg), mixed in reduction buffer, boiled, resolved by SDS-PAGE, and transferred to PVDF membranes by electroporation. Membranes were incubated overnight at 4°C with antibodies to the following antigens: CD90, LGR5, SOX2, ZO-1, ZO-2, and GAPDH (Santa Cruz, USA), washed, and incubated with secondary antibody conjugated HRP (Santa Cruz, USA) corresponding to each primary antibody. Protein bands were enhanced by chemiluminescence (Healthcare Life Science, USA), and protein band intensities were quantified as relative ratios versus GADPH using Image J.

Immunofluorescence Analysis

After culture for 14 days, huMSGSC spheres were collected, fixed in 4% paraformaldehyde solution, dehydrated using an ethanol series, embedded in paraffin, and sectioned at 5 μm. Sections were blocked with 5% BSA in PBS, incubated with CD90, LGR5, SOX2, ZO-1, and ZO-2 antibodies (Santa Cruz, USA, 1:50) overnight at 4°C, then with secondary antibody conjugated with Texas Red (Santa Cruz, USA, 1:250) for 2 h at room temperature in the dark, and observed using a fluorescence microscope (Nikon, Japan).

Statistical analysis

The significances of differences between group means were determined by one-way analysis of variance (ANOVA) and the student's t-test. All experiments were performed at least three times in triplicate. The analysis was performed using GraphPad Prism software, and P values of < 0.05 were considered significant.

Declarations

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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AUTHOR CONTRIBUTIONS

Conceptualization: J.M.K., T.H.M., and J-S.C.; data curation: E.J.J., M.E.C., and J.M.P.; formal analysis, funding acquisition: Y-M.K; investigation: J.M.K., M.E.C, E.J.J, and J.M.P.; methodology: J.M.K., T.H.M., and S.Y.K.; project administration: S.Y.K.; supervision: J-S.C. : J.M.K. and T.H.M. contributed equally to this work. These two authors were considered co-first author. All the authors proofread and approved the final manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

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Figures

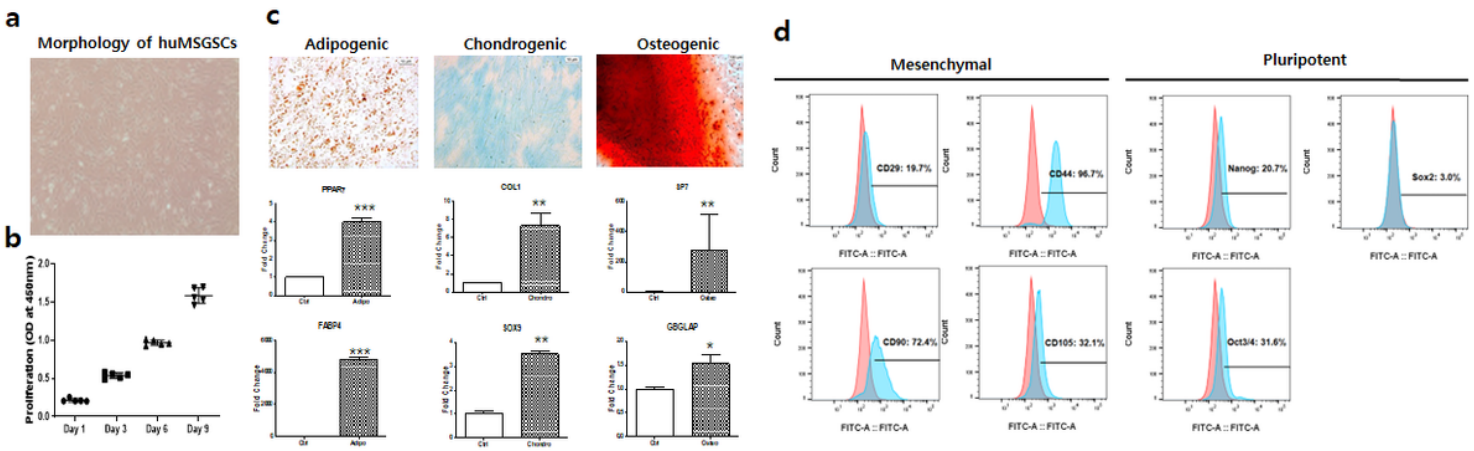

Figure 1

Isolation and characterization of huMSGSCs. a) Cell morphology of huMSGSCs, **b)** Cell growth curve of huMSGSCs, and **c)** Mesenchymal differentiation potential of huMSGSCs. The huMSGSCs were differentiated into three mesenchymal cell types. After each differentiation, the cells were stained with oil red O, Alcian blue, or alizarin red S to evaluate differentiation to the adipogenic, chondrogenic, and osteogenic lineages, respectively. In addition to cytochemical staining, molecular marker expressions for each lineage were analyzed by real-time PCR. **d)** Analysis of multi-lineage cell marker expressions. Numbers above bracketed lines indicate the percentage of cells positive for each marker; Blue histograms, each marker indicated; Red histograms, isotype-matched control antibody. Results are

presented as means±SDs as determined by one-way ANOVA and Turkey's post hoc multiple comparison test: * versus controls ***p<0.001, **p<0.01, *p<0.05. GAPDH was used as the internal standard. *Abbreviations*: huMSGSCs: human minor salivary gland stem cells.

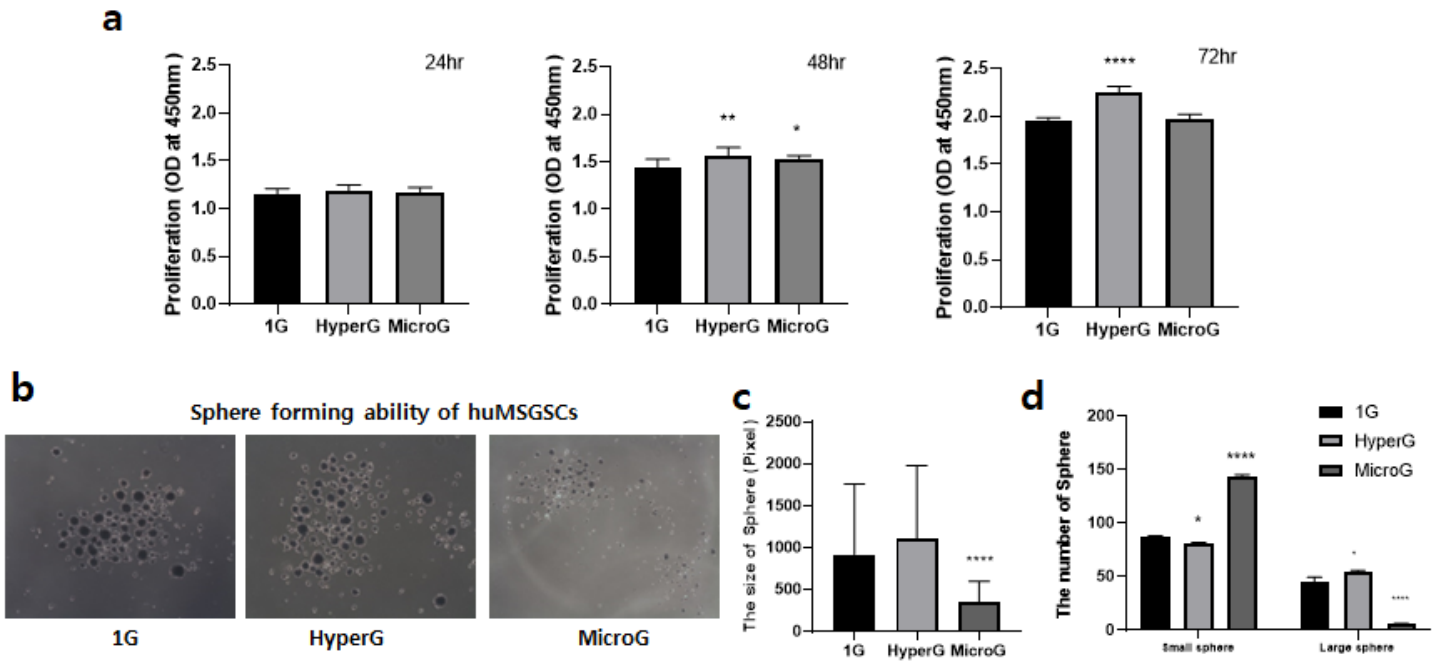

Figure 2

Proliferation and sphere formation by huMSGSCs after exposure to different gravities. a) Proliferations of huMSGSCs exposed to 1G, HyperG, or MicroG. huMSGSC proliferation was confirmed using CELLOMAX^{TM} solution at 24, 48, or 72 hr after short-term exposure to MicroG or HyperG. **b)** huMSGSC sphere morphologies. Spheres were generated from the huMSGSCs exposed to 1G, MicroG, or HyperG and cultured for 14 days **c)** Total numbers of spheres in 1G, MicroG, and HyperG treated cells. **d)** Number of spheres in subgroups divided by size; large spheres >1000 pixels and small spheres <1000 pixels. Results are presented as means±SDs. The analysis was performed using One-way ANOVA and Turkey's post hoc multiple comparison test: * versus 1G ****p<0.0001, **p<0.01, *p<0.05. *Abbreviations*: huMSGSCs: human minor salivary gland stem cells, 1G: normal gravity, MicroG: microgravity, HyperG: hypergravity.

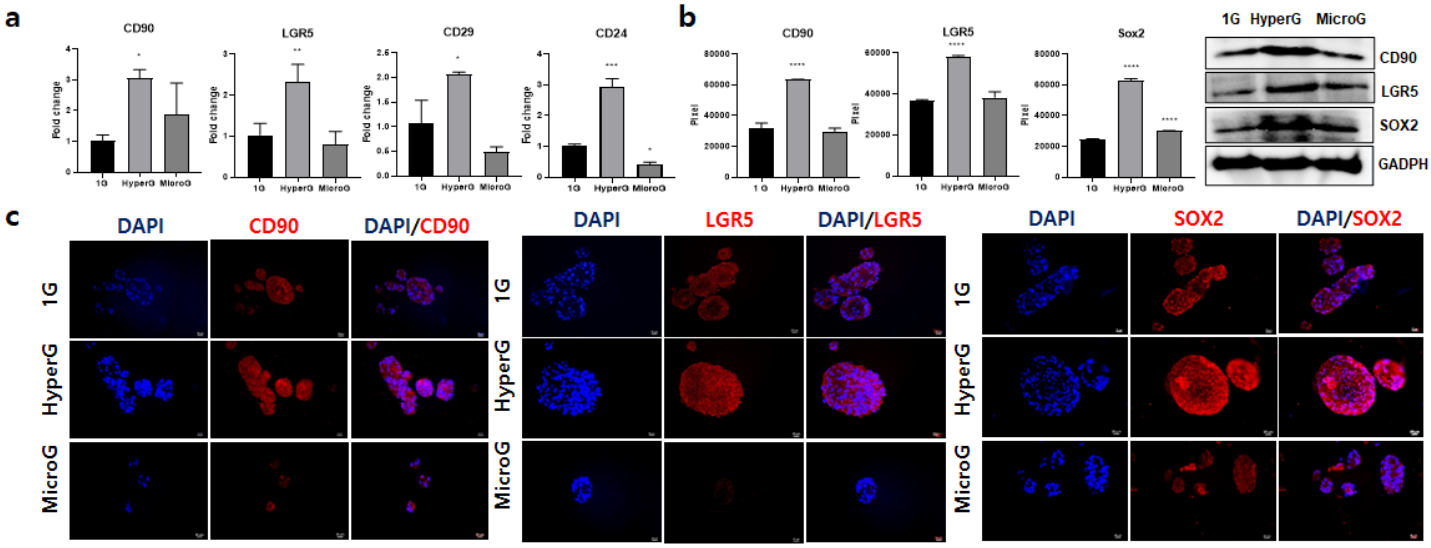

Figure 3

Stem cell marker expressions in huMSGSCs exposed to different gravities. (a) Real-time PCR and **(b)** Western blot analyses of stemness markers in huMSGSCs exposed to 1G, MicroG, or HyperG. **c)**Immunofluorescence staining of huMSGSCs, scale bar = 20 μm. Results are presented as means±SDs. The analysis was performed using One-way ANOVA and Turkey's post hoc multiple comparison test: * versus 1G ****p<0.0001, ***p<0.001, **p<0.01, *p<0.05. GAPDH was used as the internal standard. *Abbreviations*: huMSGSCs: human minor salivary gland stem cells, 1G: normal gravity, MicroG: microgravity, HyperG: hypergravity.

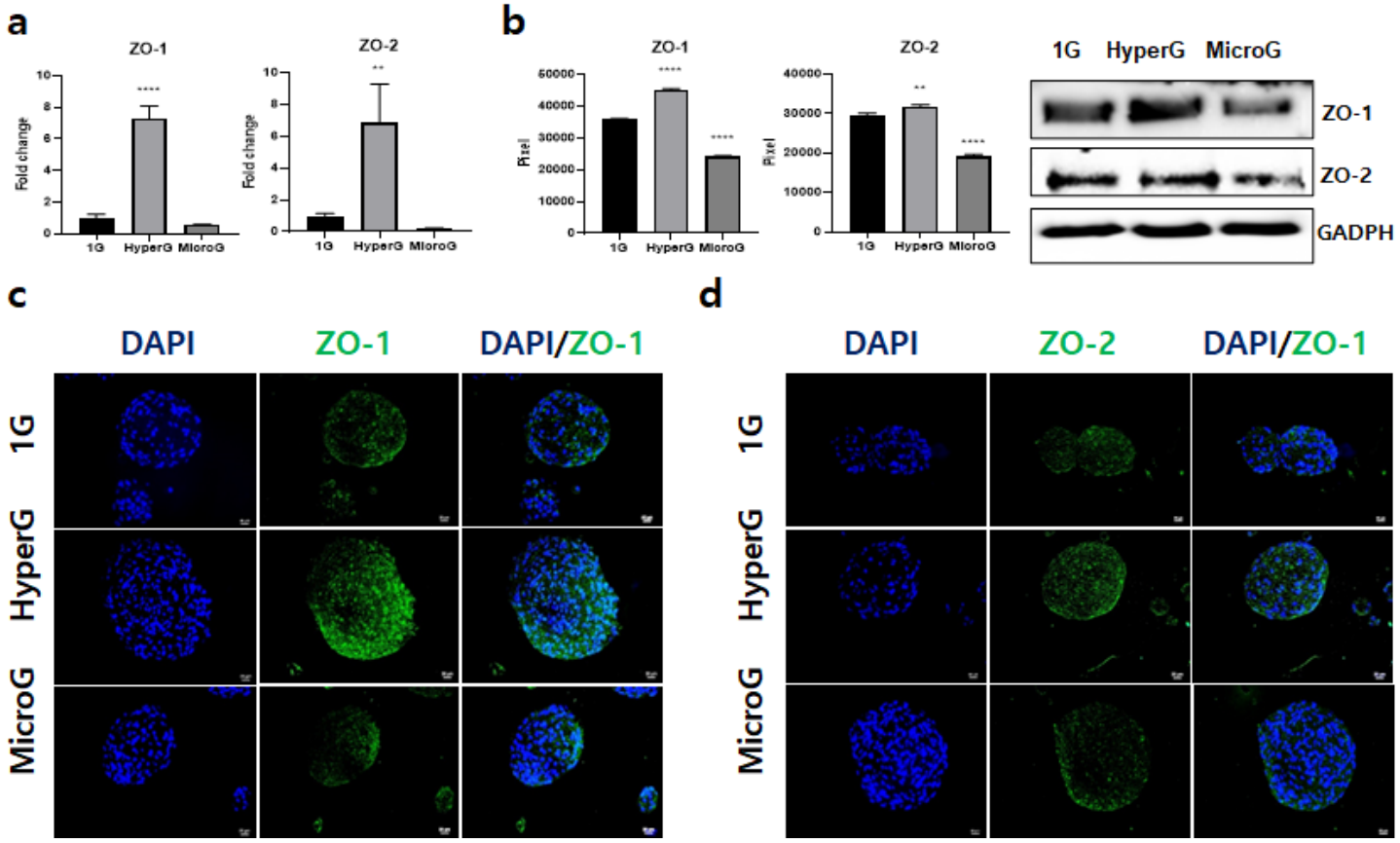

Figure 4

Cell junction expressions on huMSGSCs exposed top different gravities. (a) Real-time PCR and **(b)** Western blot analysis and quantification (left) of ZO-1 and ZO-2 in huMSGSCs after 30 minutes of 1G, MicroG, or HyperG. **c)**Immunofluorescence staining of huMSGSCs; scale bar = 20 μm. Results are presented as means±SDs. The analysis was performed using One-way ANOVA and Turkey's post hoc multiple comparison test: * versus 1G ****p<0.0001, ***p<0.001, **p<0.01. GAPDH was used as the internal standard. *Abbreviations*: huMSGSCs: human minor salivary gland stem cells, 1G: normal gravity, MicroG: microgravity, HyperG: hypergravity.

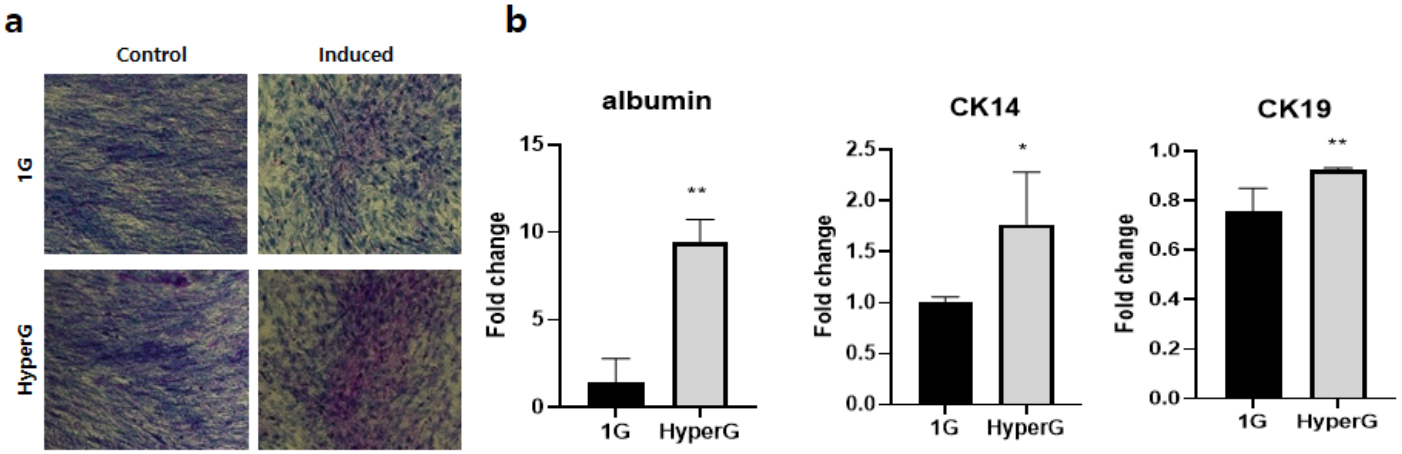

Figure 5

Enhanced epithelial differentiation potential of huMSGSCs exposed to HyperG. a) To assess the effect of HyperG on the differentiations of huMSGSCs, hepatocyte differentiation was induced and evaluated by PAS staining. **b)** Expressions of hepatocyte differentiation markers, including albumin, CK14, and CK19 were analyzed by real-time PCR. Results are presented as means±SDs. The analysis was performed using One-way ANOVA and Turkey's post hoc multiple comparison test: * versus 1G ** $p<0.01$, * $p<0.05$. GAPDH was used as the internal standard. *Abbreviations*: huMSGSCs: human minor salivary gland stem cells, 1G: normal gravity, HyperG: hypergravity.

Supplementary Files

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- supplementaryfigures.pdf