Single clonal glandular stem cells derived from human parotid glands do not attain malignant phenotype during long-term in vitro culture

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Mesenchymal stem cells (MSCs) are being intensively investigated as future therapeutics for various human diseases. One of the most important challenges to the clinical application of MSCs is the possibility of malignant transformation during long-term in vitro culturing. However, there have been no reports on the tumorigenicity of salivary gland-derived MSCs following long-term in vitro culturing. Here, we isolated a single clonal glandular stem cells from human parotid gland stem cells (hpGSCs) using a modified sub-fractionation culturing method. The possibility of malignant transformation of these cells following long-term culturing was evaluated under in vitro and in vivo culture conditions. Single clonal glandular stem cells from the human parotid gland have unique multipotent MSCs traits. hpGSCs at passage 18 stained strongly for β-galactosidase expression and the long-term culture of hpGSCs led to a reduction in telomerase activity. hpGSCs could not survive in a soft agar environment and did not cause tumor formation in a xenograft mouse model. In addition, the expression of salivary cancer-related oncogenes was not elevated in hpGSCs following the long-term culture. In conclusion, we demonstrated that there is no possibility of acquiring a malignant transformation during long-term in vitro cell expansion of hpGSCs.

Key words: mesenchymal stem cell, parotid gland, neoplastic processes

Functionally, MSCs are capable of self-renewal, proliferation, and in particular, have anti-inflammatory and immunosuppressive functions [5]. Also, they can differentiate into multifunctional organs, and regenerate damaged tissue. This has resulted in extensive research being conducted worldwide to apply them to various diseases, such as graft versus host-, blood-, cardiovascular-, neurological- and congenital diseases, as future therapeutic modalities [6]. In fact, allogeneic and autologous MSCs therapies have been attempted in some patients as a clinical application [7].

So far, several researchers have isolated MSCs from human salivary gland tissues and reported their characteristics [8, 9]. Several distinct markers including CD29, CD49f, CD90, CD105, CD166, and CD117 have been reported in human salivary MSCs [10]. However, the unique and specific markers of human salivary glands derived-MSCs have not yet been identified. These salivary MSCs have been proven to be effective in increasing saliva production in several preclinical animal models. In fact, a recent study reported the clinical application of adipose tissue-derived MSC therapy in radiation-induced xerostomia with promising results [11]. However, there are still several limitations to the clinical application of MSCs, especially because studies on the side
effects and biosafety of MSCs are limited. One of the most important safety concerns related to the use of MSCs in clinical application is the possibility of tumorigenesis of these cells following patient administration because MSCs can differentiate into a complex bio-tissue, similar to tumor tissue which is also derived by proliferation from a single cell [12]. Also, mouse MSCs derived from bone marrow have been reported to undergo malignant transformation after long-term culture in vitro [13]. Moreover, to increase the clinical efficacy of MSCs, a very large number of cells are required as less than 1% of blood vessel-injected MSCs localize to the target tissue [14]. Unfortunately, the number of viable MSCs obtained from patients is very small, and increasing the cell number artificially through in vitro methods to obtain a sufficient number of stem cells is crucial. Such in vitro cell culture processes may however induce changes in the molecular biological and physiological functions of the cells, which could result in tumorigenesis. Thus, preclinical validation of the possibility of tumorigenicity and biosafety following long-term laboratory culture of MSCs is essential.

We previously developed and characterized single clonal glandular stem cells from the human parotid gland (hpGSCs) using a modified sub-fractionation culture method and revealed that these cells possessed both epithelial and mesenchymal characteristics, as well as multi-faceted differentiation ability unlike the salivary stem cells established previously by other researchers that contained a mixed and heterogeneous cell population [10]. Furthermore, we identified that these hpGSCs enhanced the function of the salivary gland in vivo, suggesting the possibility of potential clinical application in the near future.

Thus in the present study, we investigated the possibility of malignant transformation of single clonal hpGSCs during in vitro expansion culture prior to their clinical application.

Patients and methods

Ethical statement. Written informed consent was obtained from all participants prior to experiments, and approval for this study was obtained from the Institutional Review Board (2015-10-011-002).

Cell culture. MC3 (human salivary mucoepidermoid carcinoma) cells and adipose mesenchymal stem cells (MSCs) were generously gifted by Prof. SD Cho (Chonbuk National University, Jeonju, Korea) and JM Yoo (Cha University, Pocheon, Korea), respectively and cultured in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (DMEM/F12) supplemented with 10% fetal bovine serum and 1% antibiotics. Single clonal glandular stem cells from the human parotid glands (hpGSCs) were isolated from normal tissue samples collected from parotidectomized patients diagnosed with benign parotid tumors. A subfractionation culturing method for generating clonal stem cells was used for this purpose, as previously described [10]. hpGSCs were also maintained in low-glucose Dulbecco’s modified Eagle’s medium (low-DMEM) supplemented with 10% FBS and 1% antibiotics at 37°C in an incubator with 5% CO₂.

Cell morphology. To observe the cell morphology during continuous subculturing, hpGSCs were plated in a 6-well plate and examined under an optical microscope (Axiovert200; Carl Zeiss). Images were captured using a digital camera.

Cell proliferation assay. The proliferation capacity of MC3 cells and hpGSCs was evaluated using a Cell Proliferation Assay kit (G5421; Promega). Briefly, cells were plated in a 96-well culture plate at 5 x 10⁴ cells/well and proliferation was analyzed on days 1 through 4 following cell plating following the addition of 20 μl of cell proliferation reagent and incubation at 37°C for 30 min. The proliferation of the cells was measured at 490 nm using a plate reader (SpectraMax® 190; Molecular Devices).

β-galactosidase staining. To assess cell senescence, a β-Galactosidase Staining Kit (9860S; Cell Signaling) was used according to the manufacturer’s manuals, followed by the observation under an optical microscope (Axiovert200; Carl Zeiss) and the images were obtained with a digital camera.

Telomerase activity. Cell pellets were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.5; 1 mM MgCl₂, 1 mM EGTA, 0.1 mM PMSF; 0.5% CHAPS, and 10% Glycerol) and incubated on ice for 30 min. A total of 4 μg of the quantified protein for each cell lysate was treated with TRAP solution (20 mM Tris-HCl, pH 8.3; 1.5 mM MgCl₂, 63 mM KCl, 0.005% Tween 20, and 1 mM EGTA), 0.2 μg of telomerase primer TS (5’-AATCCGTTCGAGCAGAGTT-3’), 2.5μg of BSA, and 1.25 mM dNTP to a final volume of 20 μl, followed by incubation at 30°C for 30 min. Anchored return primer ACX (5’-GCGCGGCTTACCCTTACCCTTACCCTAACC-3’; 0.1 μg) and 25 μl of SYBR Green I PCR Master Mix (23724620, Roche) were added to 10 μl of the previous reaction mix. The samples were pre-incubated for 10 min at 95°C, and amplified for 40 cycles of 20 s at 95°C, 30 s at 60°C, and 90 s at 72°C, followed by a melting curve of 5 s at 95°C and 60 s at 65°C using the LightCycler 480 Real-Time PCR cycler (Roche). Each sample was analyzed in triplicate.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Total RNA was isolated from the cells using TRI REAGENT (TR 118; Molecular Research Centre) and cDNA was synthesized using a ReverTra Ace® qPCR RT Master Mix with gDNA Remover kit (FSQ-301; TOYOBO), according to the manufacturer’s instructions. Real-time RT-PCR analysis was performed on a LightCycler 480 II real-time PCR cycler (Roche), using 2x LightCycler 480 SYBR Green I Master Mix (23724620; Roche). Quantification of GAPDH, the housekeeping gene, expression was performed as a control. Each experiment was conducted in triplicate. The sequences of all the primers used for qPCR are listed in Supplementary Table S1.

Cell apoptosis assay. Cell apoptosis was determined using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection kit (BD Biosci-
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ences, Franklin Lakes, NJ, USA). Briefly, the cell suspension was then incubated with 5 μl Annexin V-FITC solution in the dark at room temperature for 15 min, followed by incubation with 5 μl PI, according to the manufacturer's protocol. Stained cells were distinguished as viable (Annexin V−/PI−), early apoptotic (Annexin V+/PI−), or late apoptotic (Annexin V+/PI+) using a FACSCalibur flow cytometer.

**Soft agar colony formation assay.** Briefly, 1.2% bottom layer agar was prepared and mixed with 2× medium (containing 20% FBS and 2% antibiotics) in the ratio of 1:1. A total of 1.5 ml of the mix was added to a 6-well plate and allowed to solidify at room temperature for 20 min. To prevent drying of the agar, 800 μl of complete medium was added and the plates were incubated at 37 °C in an incubator with or without 5% CO2. After 2 weeks, images of the colonies formed were photographed in dark-field using an optical microscope (Axiovert 200; Carl Zeiss).

**In vivo tumorigenicity.** Animal experiments were approved by the Institutional Animal Care and Use Committee of the Konkuk University. To assess the tumorigenicity of the hpGSCs, 4×10^4 cells were mixed in 100 μl Matrigel (354248; BD Biosciences) and injected subcutaneously in the flank of 8-week-old female BALB/c nude mice (n=6) using a 22-gauge needle. Mice were raised in a specific pathogen-free facility. Visual observation and palpation were performed until tumors appeared in the engrafted mice per 3 days. Forty days following the injections, the mice were sacrificed with CO2 gas (5 seconds with 10 PSI pressure).

**RNA-sequence analysis.** For RNA isolation, total RNA was isolated using TRIzol reagent (Invitrogen). RNA quality was assessed by Agilent 2100 bioanalyzer using the RNA 6000 Nano Chip (Agilent Technologies, Amstelveen, Netherlands), and RNA quantification was performed using ND-2000 Spectrophotometer (Thermo Inc., DE, USA). For library preparation and sequencing, the construction of the library was performed using QuantSeq 3’ mRNA-Seq Library Prep Kit (Lexogen, Inc., Austria) according to the manufacturer’s instructions. In brief, each 500 ng total RNA were prepared and an oligo-dT primer containing an Illumina-compatible sequence at its 5’ end was hybridized to the RNA and reverse transcription was performed. After degradation of the RNA template, second-strand synthesis was initiated by a random primer containing an Illumina-compatible linker sequence at its 5’ end. The double-stranded library was purified by using magnetic beads to remove all reaction components. The library was amplified to add the complete adapter sequences required for cluster generation. The finished library was purified from PCR components. High-throughput sequencing was performed as single-end 75 sequencing using NextSeq 500 (Illumina, Inc., USA). For data analysis, QuantSeq 3’ mRNA-Seq reads were aligned using Bowtie2 (Langmead and Salzberg, 2012). Bowtie2 indices were either generated from the genome assembly sequence or the representative transcript sequences for aligning to the genome and transcriptome. The alignment file was used for assembling transcripts, estimating their abundances, and detecting differential expression of genes. Differentially expressed genes were determined based on counts from unique and multiple alignments using coverage in Bedtools (Quinlan AR, 2010). The RC (Read Count) data were processed based on the quantile normalization method using EdgeR within R (R development Core Team, 2016) using Bioconductor (Gentleman et al., 2004). Gene classification was based on searches done by DAVID (http://david.abcc.ncifcrf.gov/) and Medline databases (http://www.ncbi.nlm.nih.gov/). These RNA-seq data are available in a public database (geo@ncbi.nlm.nih.gov, accession number:GSE160439).

**Statistical analysis.** All data are presented as the mean ± standard deviation. The statistical significance of the experimental results was calculated using paired or unpaired Student’s t-test. Statistical significances were considered at *p<0.05; **p<0.001; ***p<0.0005.

**Results**

hpGSCs have unique multipotent MSC characteristics. We previously developed single colony-forming unit-derived clonal cells from the human parotid gland using a modified sub-fractionation culturing method [10] and showed that the cells possessed concurrent epithelial and mesenchymal stem cell phenotype characterized by the following: 1/ a fibroblast-like cell morphology (Figure 1A); 2/ the multi-faceted cell phenotype characterized by the following: 1/ a fibroblast-like cell morphology (Figure 1A); 2/ the multi-faceted potential to differentiate into various cell types. As shown in Figure 1B, the cells could be induced to undergo multi-functional differentiation, including adipogenic, osteogenic, and chondrogenic differentiation, as judged by staining with oil red O, alizarin red S, and safranin O, which are putative stains for adipogenic, osteogenic, and chondrogenic differentiation, respectively. In addition, expression of lineage-specific markers, including adipogenic markers, PPARγ2, FABP4, and LPL; osteogenic markers, RUNX2 and BGLAP; and chondrogenic markers, COL2, COL10, and ACAN was also induced (Figure 1C). When these cells were plated into the Matrigel-containing culture plates and cultured in serum-free Hepato-STIM medium, they formed spheroid cell aggregates with markedly increased expression of the salivary acinar markers, AQP5 and α-amylase, suggesting that these cells had the capacity to differentiate into salivary epithelial cells (Figure 1D). Finally, the clones also expressed positive mesenchymal markers (CD90, CD29, CD73, and CD105), as well as the pluripotent marker, OCT4. However, it was negative for endothelial markers of hematopoietic cells (CD45 or HLA-DR) (Figure 1E). Finally, we compared transcriptome expression levels between adipose MSCs and hpGSCs using RNA-sequence analysis, showing 17,703 similarly expressed genes (68.8%), but 3,635 genes (14.1%) and 4,399 genes (17.1%) elevated in MSCs and hpGSCs,
respectively (Figure 1F). All these results indicated that the hpGSCs have unique multipotent MSC characteristics.

**hpGSCs undergo cell senescence in *in vitro* long-term culture.** While normal cells are programmed to undergo senescence, cancer cells have the capacity to proliferate continuously [15]. The characteristics of the senescent cell include loss of original cell architecture, such as larger flattened cytoplasm, increased vacuoles and cytoplasmic filaments, bigger nucleus and nucleoli, and multinucleated lysosomes and increased number of Golgi. The hpGSCs showed a spindle-shaped morphology in the early passages (<P7; Figure 2A, left). However, in higher passages (>P20), they showed an irregular morphology as the cytoplasm became more granular with debris formation, as well as the exfoliation of some cells (Figure 2A, right), indicative of cellular senescence of the hpGSCs during long-term expansion culture. One of the features of senescence is the increased expression of various lysosomal proteins and contents [16]. The activity of β-galactosidase (β-gal), a lysosomal enzyme, is often used as an indicative marker of enhanced lysosomal content of aging cells with senescence [17]. Senescent cells are characterized by the activity of lysosomal β-gal detectable at pH 6, which is not found in non-senescent, or immortal cells. Thus, we evaluated β-gal activity in the MC3s and hpGSCs. We found that hpGSCs at P18 displayed strong β-gal expression, but not MC3s or hpGSCs at P12 (Figure 2B). In addition, we found that the long-term culture of hpGSCs led to a reduction in telomerase activity (Figure 2C). In addition, we evaluated apoptosis rates of hpGSCs at passages 12 and 14. As a result, the apoptotic rate of hpGSCs at passage 14 (21.4%) was significantly increased compared to hpGSCs at passage 12 (11.8%) (Figure 2D). All these results suggest that hpGSCs undergo cell senescence in long-*in vitro* culture.

**hpGSCs lack tumorigenicity during the long-term culture.** One of the key features of malignant transformation is the ability of continuous proliferation, resulting in the establishment of bulk mass in both *in vitro* and *in vivo* environments. Thus, we explored whether hpGSCs have tumorigenic capacity following the long-term culture. MC3 cells maintained a high growth rate with increasing cell number, whereas the hpGSCs showed a very low growth rate (Figure 3A). Anchorage-independent growth is another feature of cancer cells. MC3 cells formed growing cell clusters with the spheroid formation in a soft agar environment, whereas the hpGSCs could not survive in this environment (Figure 3B). Furthermore, MC3 cells formed tumors when they were inoculated into the flank of the immunodeficient mouse, but hpGSCs could not (Figure 3C). All these results indicated that hpGSCs do not possess tumorigenicity during the long-term culture.

**Expression of oncogenic genes is not elevated in the long-term cultured hpGSCs.** Finally, we analyzed the differences in gene expressions between MSCs and hpGSCs focusing on oncogenic pathway. As a result, analysis of
Figure 2. Senescence-associated changes in the hpGSCs in late passages. A) Morphological changes in hpGSCs in late passages, showing representative senescent cells. 10× magnification, scale bar: 100 μm. B) β-galactosidase staining of MC3 cells or 12th and 18th passage hpGSCs. 10× magnification, and scale bar: 100 μm. C) Specific activity of telomerase was determined at the indicated passages. Data represent the mean ± standard deviation (SD; n=3). **p<0.01; D) Apoptosis assay of hpGSCs at passage 12 or 14 using the Annexin V method.

Figure 3. hpGSCs do not undergo tumorigenic change during the long-term culture. A) Proliferation ability of MC3 or late passage hpGSCs at the indicated time points. Data represent the mean ± SD (n=3). ***p<0.001. B) Representative images of the colonies in soft agar on day 14. 4× magnification, scale bar: 100 μm. C) Tumor growth 40 days after the subcutaneous injection of nude mice with MC3 or late passage hpGSCs.
transcriptome profiles of hpGSCs compared to MSCs showed that 851 genes (86.8%) were similarly expressed, but 60 genes (6.1%) and 70 genes (7.1%) were elevated in MSCs and hpGSCs, respectively (Figure 4A). Especially, expression levels of BRAF, c-Myc, HRAS, KRAS, and MYB in hpGSCs, known as oncogenic drivers of salivary cancers [18], did not increase compared to those in MSCs (Figure 4B). In addition, we compared the transcriptional level of these oncogenes in MC3 cells and hpGSCs and found that the transcriptional level of the oncogenes analyzed was significantly lower in hpGSCs compared to the MC3 cells (Figure 4C).

Discussion

One of the characteristics of MSCs is that they secrete proangiogenic and anti-apoptotic factors to promote the regeneration of viable tissue [19]. These characteristics are consistent with the characteristics of tumor cells. Embryonic stem cells and induced stem cells, that are similar to MSCs, are known to form tumors in vivo [20]. Therefore, MSCs are also likely to have this potential in the context of the chromosomal aneuploidy that was observed in mouse bone marrow-derived MSCs in the third generation of the culture, which led to the proliferation and contact inhibition of the cells [21]. In addition, in an in vivo mouse model, injection of mouse bone marrow-derived MSCs into the leg muscles of immunosuppressed mice led to the formation of locally invasive sarcoma capable of distant metastasis to the lungs [22]. The ex vivo expansion of cells has been reported to cause the conversion of these cells to spontaneous tumor cells.

In general, unlike mouse MSCs, human MSCs are known to exhibit no chromosomal abnormality or tendency of cell immortalization even when continuously passaged in culture ex vivo. In addition, in the recent studies that reported the possibility of tumorigenesis of human MSCs, it was found to be due to the cross-contamination between human MSCs and other existing cancer cells [23]. Nevertheless, it has been reported that normal human MSCs from myelodysplastic syndrome (MDS) patients when cultured with myelodysplastic cells and then injected into mice, led to the formation of tumors [24]. Therefore, studies on the possibility of the tumorigenicity of each organ-derived MSCs are indispensable prior to their clinical application.

Cell culturing in vitro is a complex process that involves the culture of cells in an artificial environment, and unlike in the body, inappropriate processing may result in changes in the inherent properties of the cells. In vitro culture of mouse MSCs has been reported to cause genomic instability following malignant transformation of MSCs [25]. Nevertheless, prior studies on whether in vitro expansion of human salivary gland-derived MSCs results in malignant transformation are limited. To our knowledge, no study has reported the probability of tumor development of salivary gland-derived MSCs. Ma et al. reported that MSCs possessed the capacity to stimulate the invasion of salivary cancer cells under a chemokine CXCL12 gradient [26].

Figure 4. Comparison of salivary oncogene expression in hpGSCs and MSCs. A) Bendiagram of gene expression changes in hpGSCs and MSCs using RNA-sequence analysis. B) Comparison of expression of putative salivary cancer oncogenes (BRAF, c-Myc, HRAS, KRAS, and MYB) in hpGSCs and MSCs using RNA-sequence analysis. C) RT-PCR analysis of putative salivary cancer oncogenes (BRAF, c-Myc, HRAS, KRAS, and MYB) in the MC3 or hpGSCs (passage 13). Data represent the mean ± SD (n=3). *p<0.05, **p<0.01.
In our study, hpGSCs were found to undergo senescence, and lack proliferation, anchorage-independent growth, and in vivo tumorigenicity during the long-term culture. These observations collectively suggest that hpGSCs do not undergo a malignant transformation during in vitro culture for cell expansion. This enabled us to establish the non-tumorigenic nature of single clonal parotid stem cells, which is particularly relevant in the context of their clinical applications. This is the first report examining the potential for tumorigenicity in single clonal salivary stem cells derived from the human parotid gland. However, although these cells are likely to be oncologically safe, further research is needed in the future to study the possibility of malignant transformation of MSCs in various environments in vivo or enhancement of the tumorigenicity of other resident tumor cells prior to their ultimate clinical application.

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Supplementary information is available in the online version of the paper.

References


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Supplementary Information

Supplementary Table S1. Primers used for RT-PCR.

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