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Vitamin C stimulates human gingival stem cell proliferation and expression of pluripotent markers

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Abstract Gingival stem cells (GSCs) are a novel source of mesenchymal stem cells (MSCs) that are easily accessed from the oral cavity. GSCs were considered valuable autograft MSCs with particular characteristics. However, the limitation in the number of available GSCs remains an obstacle. Therefore, this study aimed to stimulate GSC proliferation by ascorbic acid (AA) and determined the effects of AA on GSC pluripotent potential-related gene expression. GSCs were isolated from gum tissue by explant culture and continuously subcultured before analysis of stemness and effects of AA on pluripotent-related gene expression. GSCs cultured with various concentrations of AA showed increased proliferation in a dose-dependent manner. AA-treated GSCs showed significantly higher expression of SSEA-3, Sox-2, Oct-3/4, Nanog, and TRA-1-60 compared with control cells. More importantly, GSCs also maintained their stemness with MSC phenotypes and failed to cause tumors in nude athymic mice. Our results show that AA is a suitable factor to stimulate GSC proliferation.

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Introduction

Mesenchymal stem cells (MSCs) are a widely used source of stem cells in both basic research and clinical applications. To date, MSCs have been used in the treatment of more than 50 different diseases, included knee osteoarthritis (Emadedin *et al.* 2015; Kim *et al.* 2015), wound healing (Isakson *et al.* 2015; Li *et al.* 2015b), autoimmune disease (Munir and McGettrick 2015), bone regeneration (Padial-Molina *et al.* 2015), femoral head necrosis (Daltro *et al.* 2015; Wang *et al.* 2015), foot ulcer (Sener and Albeniz 2015), severe systolic heart failure (Zhao *et al.* 2015), sequelae of traumatic brain injury (Wang *et al.* 2013), stroke (Gutierrez-Fernandez *et al.* 2015), cardiac diseases (Karantalis and Hare 2015), Crohn's disease (Labidi *et al.* 2014), intervertebral disc repair (Mochida *et al.* 2015), diabetes mellitus (Kong *et al.* 2014; Liu *et al.* 2014), and graft-versus-host disease (Introna and Rambaldi 2015). Thus far, according to ClinicalTrials.gov (www.clinicaltrials.gov), more than 500 clinical trials have used MSCs in disease treatment. Although many MSC sources have been discovered, such as bone marrow (Phadnis *et al.* 2011), adipose tissue (Estes *et al.* 2010), peripheral blood (Kassis *et al.* 2006), umbilical cord blood (Zhang *et al.* 2011), banked umbilical cord blood (Phuc *et al.* 2011), umbilical cord (Cutler *et al.* 2010; Farias *et al.* 2011), umbilical cord membrane (Deuse *et al.* 2011), umbilical cord vein (Santos *et al.* 2010), Wharton's jelly of the umbilical cord (Zeddou *et al.* 2010; Peng *et al.* 2011), placenta (Semenov *et al.* 2010; Pilz *et al.* 2011), decidua basalis (Lu *et al.* 2011), ligamentum flavum (Chen *et al.* 2011), amniotic fluid (Gucciardo *et al.* 2013), amniotic

membrane (Chang *et al.* 2010), and dental pulp (Yalvac *et al.* 2010), researchers continue to search for new sources.

Gingival gum tissue currently serves as a novel source of MSCs, and gingival stem cells (GSCs) are considered important MSCs for jawbone and teeth regeneration as well as gum disease. While teeth implantation is a commonly used technique for patients with missing teeth, this technique is only performed when the jawbone is responsive to the procedure. Therefore, some studies have attempted to regenerate the jawbone using MSCs from bone marrow before performing implants. However, the major limitation for wide use of GSCs as a source of MSCs is the relatively low number of GSCs that can be isolated from gum tissue.

Ascorbic acid (AA) is a commonly used vitamin in medicine. Previous studies revealed AA as an essential agent in stem cell proliferation as well as triggering pluripotent markers in both adult stem cells (Kim *et al.* 2014; Wei *et al.* 2014; Yu *et al.* 2014; Bae *et al.* 2015; Li *et al.* 2015a) and embryonic stem cells or induction of pluripotent stem cells (Esteban and Pei 2012; Gao *et al.* 2013).

In this study, we investigated the effects of AA as a proliferating agent for GSCs with the potential aim to increase proliferation without changes in GSC stemness.

Materials and Methods

Gum tissue collection. Gum tissues were collected from gum tissue disease patients. Informed consent was obtained from all patients. The collection was performed in accordance with the ethical standards of the local ethics committee.

GSC isolation and culture. Gum tissues were washed three times with phosphate-buffered saline (PBS) (Invitrogen-Gibco, Carlsbad, CA) supplemented with antibiotic-antimycotic 1× solution. Samples were cut into 0.5–1 cm^3 pieces and suspended in Dulbecco's modified Eagle's medium

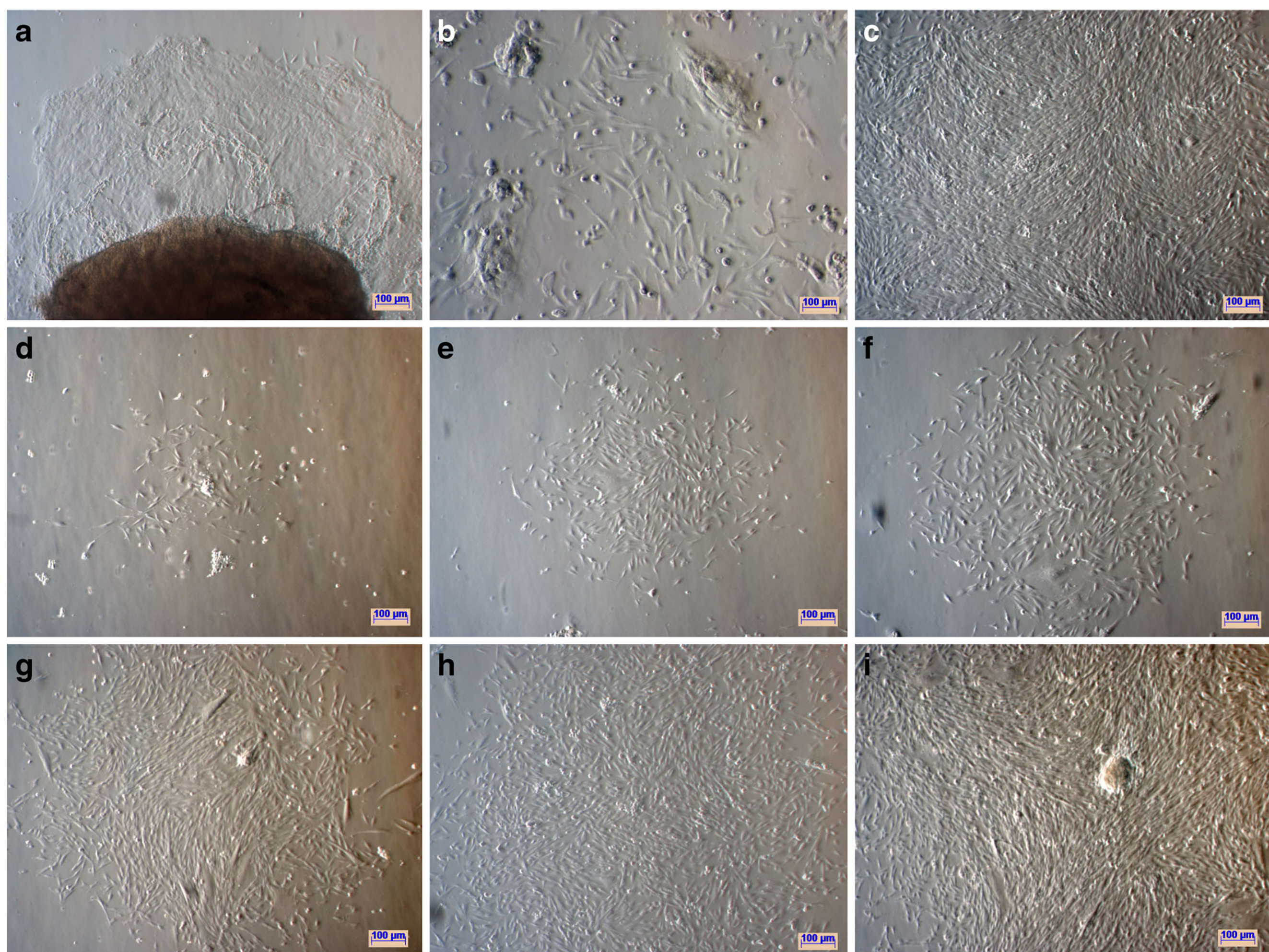

Figure 1 Isolation of GSCs by tissue explant culture. *a* Cell growth from tissues at day 5. *b* GSC candidates at first subculture (*b*) and after five passages (*c*). GSC candidate form colony after 3 d (*d*), 5 d (*e*), 7 d (*f*), 9 d (*g*), 12 d (*h*), and 15 d (*i*).

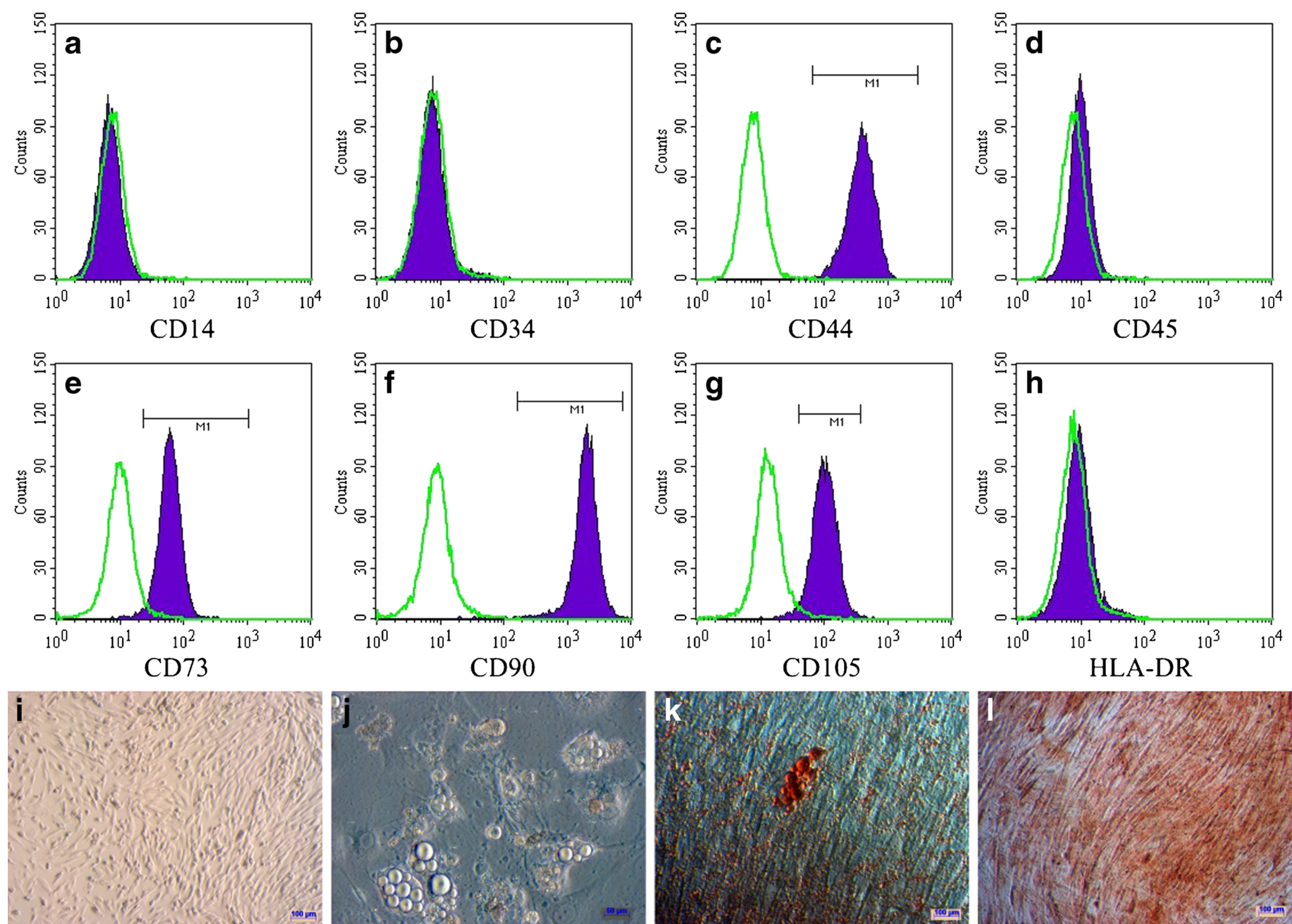

Figure 2 GSCs exhibited the MSC phenotype. GSCs were positive for CD44 (*c*), CD73 (*e*), CD90 (*f*), and CD105 (*g*) expression and negative for CD14 (*a*), CD34 (*b*), CD45 (*d*), and HLA-DR (*h*). GSCs successfully differentiated into adipocytes (*j*, *k*), osteoblasts (*l*), and control (*i*).

(DMEM)/F12, 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic solution (all from Sigma-Aldrich, St. Louis, MO). The tissue was left undisturbed for 4 d in a 37°C humidified incubator with 5% CO_2. Culture medium was replaced every 3 d after this time. The cells were passaged using 0.25% trypsin/EDTA solution (Sigma-Aldrich) when cells reached 80–90% confluence. GSC candidates were subcultured to the fifth passage, and these cells were used for further experiments.

Phenotype analysis. Primary antibodies against human antigen CD13, CD14, CD34, CD44, CD45, CD73, CD90, and CD105 and histocompatibility antigen DR alpha chain (HLA-DR) were purchased from BD Biosciences (San Jose, CA). MSCs (5×10^5 cells) were re-suspended in 500 μL PBS and stained with fluorescein-isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated primary antibodies for 20 min at room temperature. FITC- or PE-conjugated human IgGs were used as isotype controls at the same concentration as the specific primary antibodies. The fluorescence intensity of the cells was evaluated by flow cytometry (FACSCalibur; BD Biosciences). Data were analyzed using CELLQUEST software (BD Biosciences).

Differentiation assays. For differentiation into adipogenic cells, GSCs (fifth passage) were plated at 1×10^4 cells/well in 24-well plates. At 70% confluence, the cells were cultured for 21 d in DMEM containing 0.5 mmol/L 3-isobutyl-1-methyl-xanthine, 1 nmol/L dexamethasone, 0.1 mmol/L indomethacin, and 10% FBS (all from Sigma-Aldrich). Adipogenic differentiation was evaluated by observing lipid droplets in cells under a microscope.

For differentiation into osteogenic cells, GSCs were plated at 1×10^4 cells/well in 24-well plates. At 70% confluence, the cells were cultured for 21 d in DMEM/F12 containing 10% FBS, 10^{-7} mol/L dexamethasone, 50 μmol/L ascorbic acid-2 phosphate, and 10 mmol/L β-glycerol phosphate (all purchased from Sigma-Aldrich). Osteogenic differentiation was confirmed by Alizarin red staining.

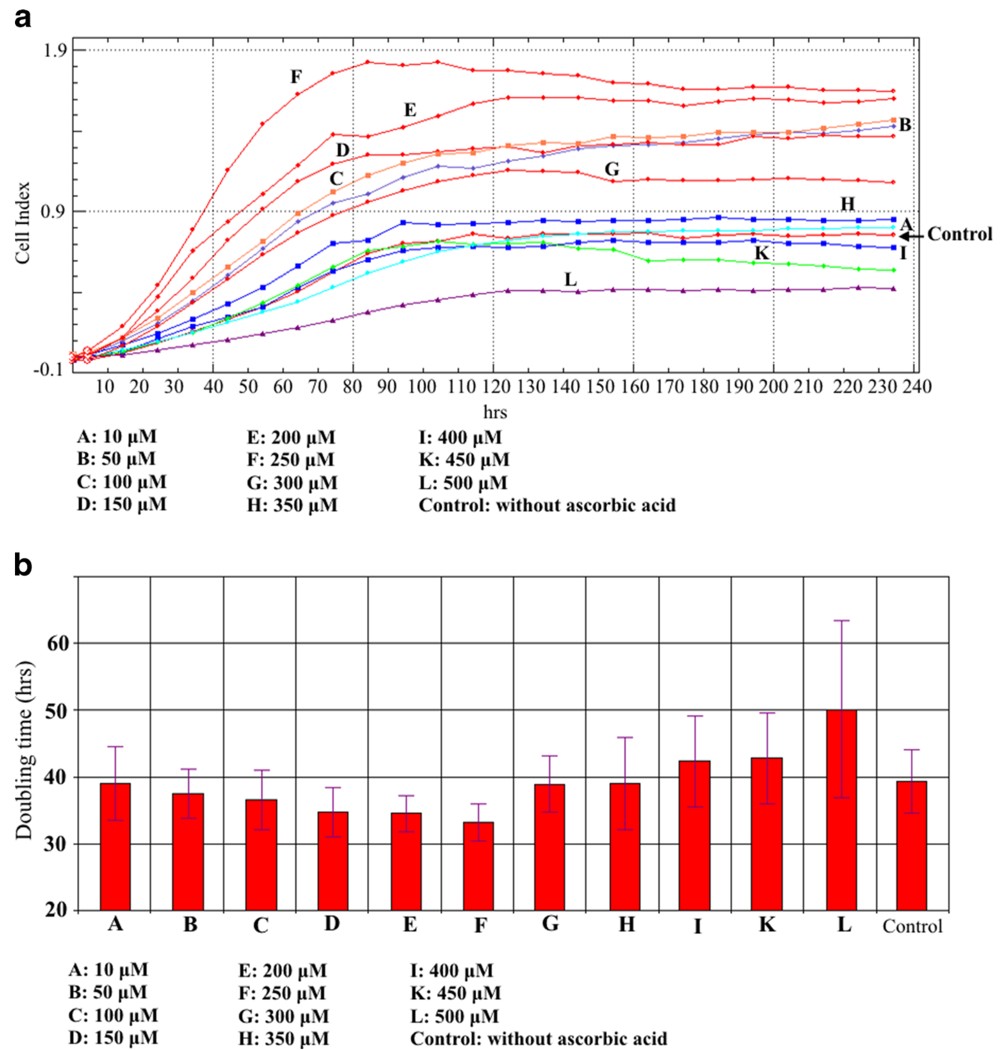

Figure 3 GSC proliferation was evaluated by xCELLigence assay. GSCs were cultured in E-plate and treated with 10, 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 µM of AA and control (without AA). The GSC proliferation in all groups was recorded by xCELLigence machine (*a*). The doubling time of GSCs in different groups were calculated (*b*).

For differentiation into chondrogenic cells, GSCs were induced to differentiate by a commercial medium for chondrogenesis (StemPro Chondrogenesis Differentiation Kit, A10071-01; Life Technologies, Carlsbad, CA). GSCs were differentiated in pellet form according to the manufacturer's guidelines. After 21 d, the cell pellets were stained with an anti-aggrecan monoclonal antibody (BD Biosciences).

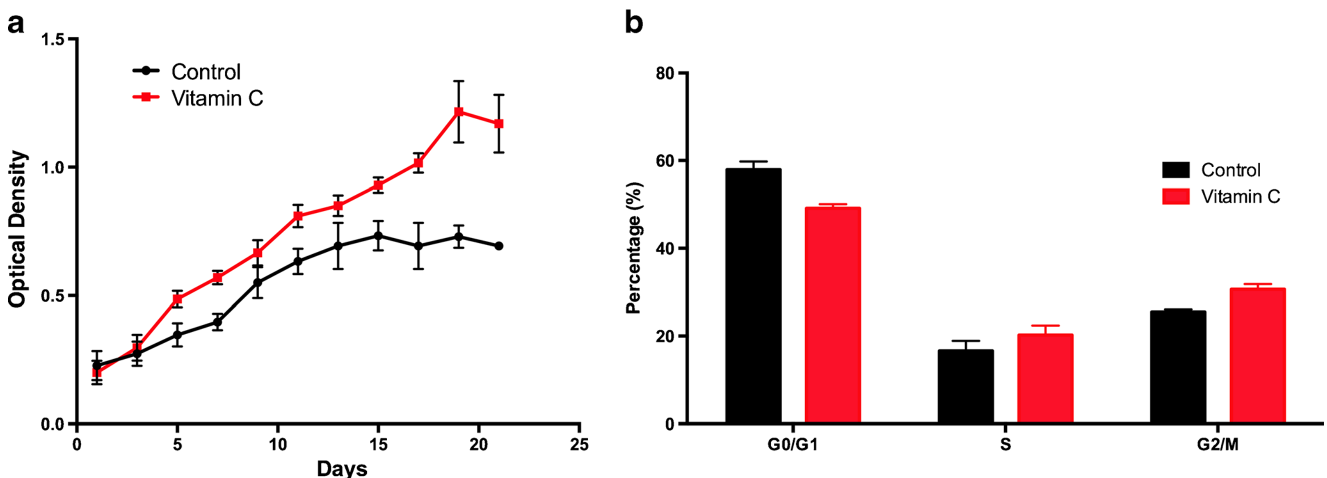

Figure 4 AA triggered GSC proliferation at 250 µM AA. *a*, Cell proliferation curve and *b*, doubling time.

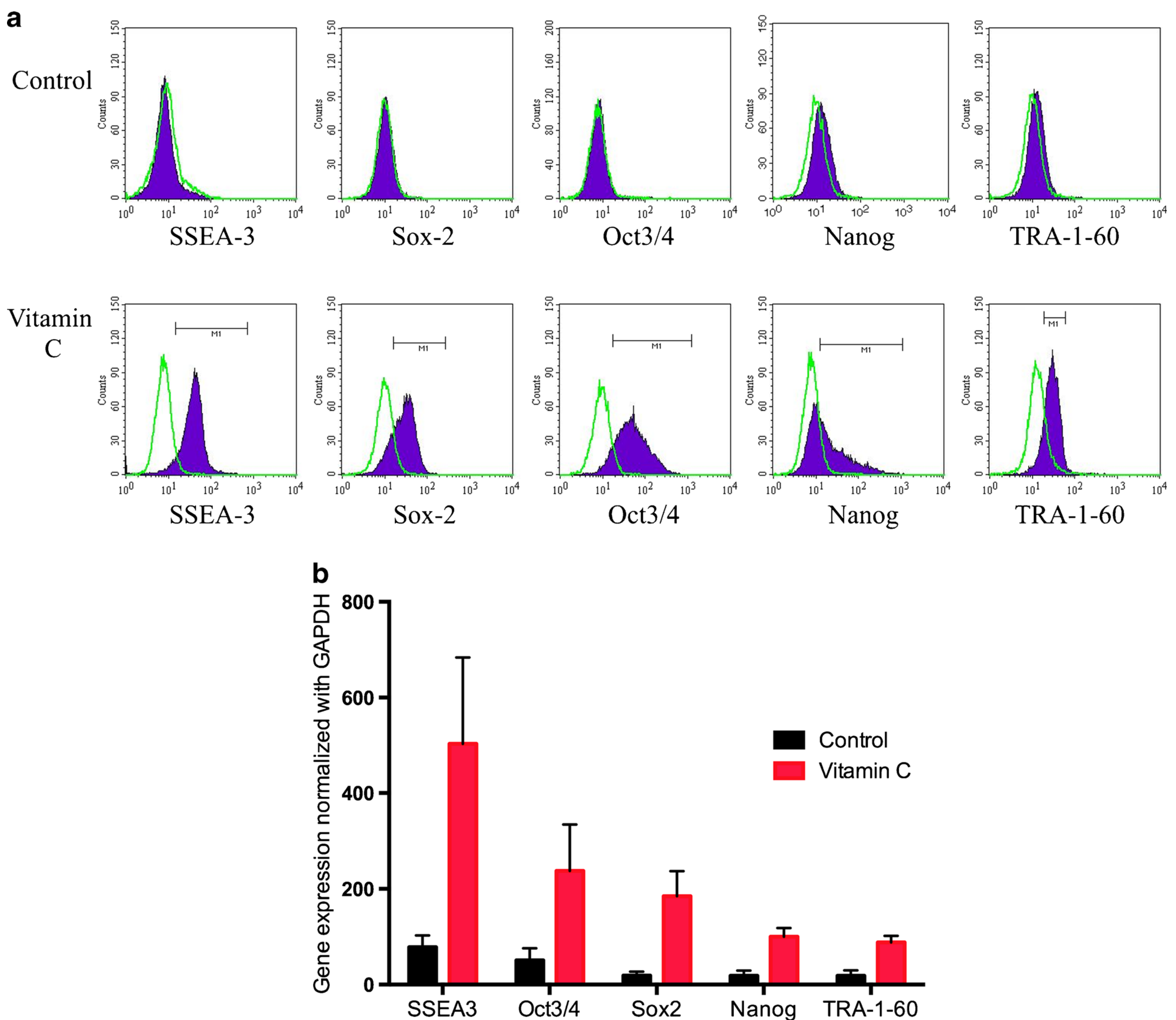

Figure 5 Expression of pluripotent markers in GSCs treated with AA. Upon treatment with 250 μM AA, GSCs strongly expressed SSEA-3 and highly expressed Sox-2, Oct-3/4, Nanog, and TRA-1-60 as shown by flow cytometry analysis (*a*) and RT-PCR (*b*).

MTT assays. Cells were grown in culture medium DMEM/F12 supplemented with 10% FBS, 1% antibiotic-antimycotic solution, and with different concentrations of AA (10, 50, 100, 150, 200, 250, 300, 350, 400, 450, or 500 μM). AA stock solution was prepared by diluting 14.45 g L ascorbic acid 2-phosphate in sterile 1 L H_2O to get 50 mM solution, filtered, and stored at 4°C. This solution was added into culture medium to get the suitable concentration of AA. The effects of AA on GSC proliferation were evaluated by 3-(4,5-dimethylthiazol-2-Yl)-2,5-diphenyltetrazolium bromide (MTT) assay and xCELLigence assay.

About MTT assay, briefly, 20 μL of MTT (5 g/L; Sigma-Aldrich) was added to each well of 96-well plates, followed by incubation for 4 h and addition of 150 μL of DMSO (Sigma-Aldrich). Plates were then mixed well for 10 min until the crystals dissolved completely. Absorption values (*A* value) for each well was measured at a wavelength of 490 nm using the microplate reader DTX 880 (Beckman Coulter, GmbH, Krefeld, Germany). An offset value of *A* and absorption value of the control group reflect GSC proliferation.

xCELLigence assay. To confirm the effects AA on GSC proliferation from MTT assay, we used another assay, so-called xCELLigence assay. This assay used xCELLigence Real-Time Cell Analyzer equipment (Roche Applied Science, Indianapolis, IN). xCELLigence Real-Time Cell Analyzer was used to evaluate cell proliferation and cytotoxicity based

Figure 6 AA-treated GSCs maintained the MSC phenotype. AA-treated GSCs expressed MSC markers such as CD44, CD73, CD90, and CD105 and were negative for CD14, CD34, CD45, and HLA-DR (*a–h*). GSCs treated with AA also successfully differentiated into adipocytes (*j*, *k*) and osteoblasts (*l*).

on changes in electric impedance at the surface of the E-plate, a specific plate with electric nodes on the surface allowing measurement of changes in impedance. This method was used to evaluate cell proliferation for adherent cells.

A total of 1×10^3 cells were seeded into each well of a 96-well E-plate in triplicate. The culture plates were placed into the eXCELLigence system and incubated at 37°C with 5% CO_2. Cell proliferation was monitored for 240 h with fresh

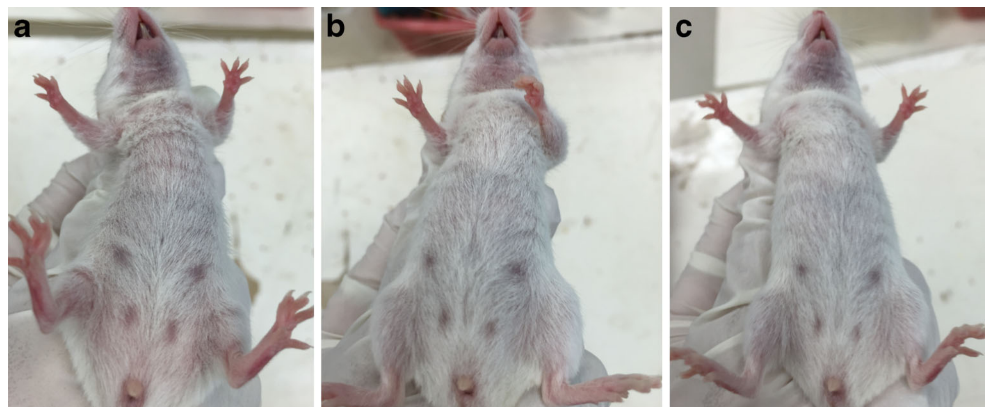

Figure 7 AA-induced GSCs failed to cause tumor in NOD/SCID mice (three mice). Each mouse was injected subcutaneously with 5×10^6 GSCs. Tumor formation in mice was followed up for 3 mo. The results showed that all three mice were free with tumors at injection sites. (*a*) Mice 1, (*b*) mice 2, and (*c*) mice 3.

medium changes every third day. The cell doubling time was determined by the software of the eXCELLigence system.

Flow cytometry of marker expression. To analyze MSC marker expression, cultured GSCs were deattached by trypsin/EDTA and washed twice with cold PBS. Cells were stained with primary antibodies against human antigen CD13, CD14, CD34, CD44, CD45, CD73, CD90, CD105, and HLA-DR and then stained with FITC- or PE-conjugated secondary antibodies. The fluorescence intensity of the cells was evaluated by flow cytometry, and data were analyzed using CELLQUEST software.

To evaluate pluripotent stem cell marker expression, GSCs treated with AA were evaluated for SSEA-3, Sox-2, Oct-3/4, Nanog, and TRA-1-60 expression by flow cytometry. GSCs in untreated medium were used as a control. Anti-SSEA-3, anti-Sox-2, anti-Oct-3/4, anti-Nanog, and anti-TRA-1-60 antibodies were purchased from Santa Cruz Biotechnology, Canada.

Real-time RT-PCR. To evaluate pluripotent stem cell markers, GSCs were examined for SSEA-3, Sox-2, Oct-3/4, Nanog, and TRA-1-60 messenger RNA (mRNA) expression. GSCs in experimental groups were collected by trypsinization, and mRNA was isolated using the Trizol protocol. Obtained total RNA was used as a template for one-step real-time RT-PCR. Real-time RT-PCR reaction was performed using the SYBR® Green Quantitative RT-qPCR Kit (Sigma-Aldrich).

Tumorigenicity assay. The tumorigenicity of GSCs was examined in nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. All experiments and handling of mice were approved by the Local Ethics Committee of Stem Cell Research and Application, University of Science (Ho Chi Minh City, Vietnam). Each mouse was injected subcutaneously with 5×10^6 GSCs (three mice per group). Tumor formation in mice was followed up for 3 mo.

Statistical methods. The results are expressed as the mean± SD. One-way ANOVA and two-tailed tests were used for all statistical analyses and performed with GraphPad Prism software, version 4.0. P values less than 0.05 were considered statistically significant.

Results

Gingival stem cell isolation. Similar to methods described in previous publications, in this study, we cultured isolated GSCs by tissue expansion culture. Three samples were collected and cultured, and all samples appeared as single cells adhering to the flask surface from day 5 after seeding (Fig. 1*a*). However, at this time, there were two kinds of cells appearing: epithelial-like cells and mesenchymal-like cells (Fig. 1*b*). After 10 d of cell migration from tissue, tissues were removed and cells were subcultured at a dilution factor of 1:3. The GSC candidates rapidly proliferated after the first subculture. The cell population became homogenous in shape after the fifth subculture with a fibroblast-like shape (Fig. 1*c*). These cells could strongly form colony (Fig. 1*d–i*).

Gingival stem cell characterization. GSC candidates at the fifth passage were examined for expression of surface markers for MSC phenotype as well as differentiation potential into adipocytes, osteoblasts, and chondrocytes. The results demonstrated that GSC candidates expressed the MSC-specific marker profile, including positive CD13, CD44, CD73, CD90, and CD105 expression and negative CD14, CD34, CD45, and HLA-DR expression (Fig. 2*a–h*).

More importantly, the cells also successfully differentiated into two kinds of mesoderm cells including adipocytes and osteoblasts (Fig. 2*j*, *k*, *l*). In fact, before differentiation, GSCs exhibited fibroblast morphology (Fig. 2*i*), and after differentiation, they showed storage of lipid drops inside the cytoplasm to become adipocytes (Fig. 2*j*, *k*) and calcium matrix accumulation (alizarin red positive) to become osteoblasts (Fig. 2*l*).

AA stimulated GSC proliferation in a concentration-dependent manner. We next examined GSC proliferation in response to various concentrations of AA. Proliferation was enhanced in a concentration-dependent manner; at the lowest concentration of AA (10 μM), AA stimulated GSC proliferation, and proliferation of GSCs gradually increased from 10 to 250 μM AA. At 250 μM, the proliferation rate of GSCs was at maximum levels and significantly higher compared with other concentrations ($p<0.5$). At this concentration, the doubling time was clearly reduced from 40 h (in control group) to 32 h (in 250 μM AA). However, at concentrations higher than 250 μM, GSC growth was partially or totally inhibited. At 300 and 350 μM, GSCs slightly inhibited with nonsignificant difference to 250 μM but from 250 to 500 μM, GSCs strongly inhibited the sudden decrease in proliferation rate and extended the doubling time (Figs. 3 and 4).

These observations were confirmed by cell cycle analysis. The results showed that 10–250 μM AA treatment of GSCs increased the percentage of cells in S and G2/M phases. In particular, treatment with 250 μM AA significantly increased the percentage of cells in G2/M + S phases compared with other groups (Fig. 4) ($p<0.5$). At concentrations higher than 250 μM AA, almost all cells were at G0/G1 and the percentage of cells at G0/G1 was significantly increased in response to 500 μM AA ($p<0.5$).

AA triggered the expression of pluripotent markers. After 2 wk of treatment with 250 μM AA, GSCs showed increased expression of pluripotent markers including SSEA-3, Sox-2, Oct-3/4, Nanog, and TRA-1-60 (Fig. 5). Both real-time RT-PCR and flow cytometry showed that these markers were increased in AA-treated GSCs compared with controls. SSEA-3 showed the highest expression, and nearly 100% of treated cells strongly expressed this marker after 2 wk. Expressions of Sox-2, Oct-3/4, Nanog, and TRA-1-60 were 32.41, 45.31, 31.03, and 19.79% higher than controls, respectively ($p<0.5$). Expression of SSEA-3 mRNA was approximately 503.33±180.09 times higher than controls, while Sox-2, Oct-3/4, Nanog, and TRA-1-60 mRNA levels were 237.33 ±97.44, 184.67±52.60, 99.67±18.48, and 88.00±13.53 times higher, respectively, compared with controls ($p<0.5$).

Despite expression of pluripotent stem cell markers, GSCs maintained the MSC phenotype. GSCs treated with AA still maintained the MSC phenotype and showed a MSC marker profile that was positive for CD44, CD73, CD90, and CD105 expression and negative for CD14, CD34, CD45, and HLA-DR (Fig. 6*a–h*). GSCs also successfully differentiated into adipocytes, osteoblasts, and chondrocytes in the inducing medium. In the adipocyte-inducing medium, GSCs stored lipid drops and these drops gradually grew larger after 1 mo of induction (Fig. 6*j*, *k*). In the osteoblast-inducing medium, cells strongly produced matrix with high calcium that was positive for alizarin red staining (Fig. 6*l*). More importantly, although these cells showed upregulation of the pluripotent markers, they could not cause tumors in NOD/SCID mice (Fig. 7).

Discussion

Gum tissue plays essential roles in gum disease and mandibular defects. Some studies have hypothesized that GSCs in gum tissue play a role in the overall regeneration of bone augmentation. These cells were considered as a stem cell source for bone regeneration in mandibular defects. In fact, these cells have high potential for osteoblast differentiation. Moreover, they can produce a suitable microenvironment for bone development. These stem cells are also used in craniofacial trauma reconstruction or jawbone before teeth implantation. However, different to umbilical cord blood, umbilical cord gum tissue is a limited tissue source. Therefore, the main obstacle for wide use of GSCs is the limited number of available cells. In this study, we examined stimulation of GSC proliferation by AA.

We isolated GSCs via tissue explant culture and successfully cultured and isolated GSCs with MSC phenotype. These obtained cells satisfied the minimum standard for MSCs according to (Dominici *et al.* 2006). The isolated cells showed three major characteristics of MSCs, including the following: (1) exhibited fibroblast-like cells that well adhered to the plastic flask surface; (2) expressed specific markers of MSCs including CD44, CD73, CD90, and CD105 positive expression and negative expression for CD14, CD34, CD45, and HLA-DR; and (3) successfully differentiated into adipocytes, osteoblasts, and chondroblasts. These results agreed with previously published studies (Tang *et al.* 2011; Ge *et al.* 2012; Hsu *et al.* 2012; Zhang *et al.* 2012; Fournier *et al.* 2013; Gao *et al.* 2014; El-Sayed *et al.* 2015; Jin *et al.* 2015).

We found that GSCs treated with concentrations of AA from 10 to 250 μM showed a concentration-dependent increase of growth proliferation. This conclusion was confirmed by doubling time and cell cycle assay. However, with concentrations higher than AA 250 μM, AA could intoxicate with GSCs that caused them to go to apoptosis. In fact, in this group, almost GSCs go to the G0/G1 phase. In this experiment, we recognized that AA effected to phase S and G2/M. By this chemical, the S and G2/M time clearly reduced.

To the best of our knowledge, this is the first study on the effects of AA on GSC proliferation and stemness. However, the effects of AA on MSCs were confirmed in many previously published studies. AAAA also maintained differentiation potential in bone marrow-derived MSCs through expression of hepatocyte growth factor (Bae *et al.* 2015). In the adipose-derived stem cell (ADSC) models, AA helps ADSCs against mitoptosis, necroptosis, and apoptosis (Padial-Molina *et al.* 2015). Vitamin C increased ERK1/2 phosphorylation, and inhibition of the mitogen-activated protein kinase pathway attenuated the proliferation of ADSCs (Kim *et al.* 2014). By microarray and quantitative polymerase chain reaction analysis, Kim *et al.* (2014) showed that AA upregulated expression of proliferation-related genes, including Fos, E2F2, Ier2, Mybl1, Cdc45, JunB, FosB, and Cdca5. It also caused increases in the mRNA expression of HGF, IGFBP6, VEGF, bFGF, and KGF (Kim *et al.* 2014).

Previous studies showed that AA significantly enhanced expression of pluripotent markers such as Sox-2, Oct-4, and Nanog in ADSCs (Yu *et al.* 2014). In a recent publication, Wei *et al.* (2014) demonstrated that AA was an essential factor to maintain stemness of mADSCs in vitro (Wei *et al.* 2014). AA can interact with the C-terminal catalytic domain of Tet enzymes, which likely promotes folding and/or recycling of the cofactor Fe (2+). In mouse embryonic stem cells, AA significantly increases the levels of all 5 mC oxidation products, particularly 5-formylcytosine and 5-carboxylcytosine, leading to a global loss of 5 mC (~40%) (Yin *et al.* 2013).

AA also plays an essential role in inducing a pluripotent state in mouse embryonic stem cells by modulating microRNA expression (Gao *et al.* 2015). In fact, some reports suggest that AA can enhance somatic reprogramming efficiency to produce pluripotent stem cells (Esteban and Pei 2012). The underlying mechanism is related to the increase

of promoter activity of pluripotent genes and enhanced protein level (Gao *et al.* 2014).

However, a few studies have reported AA as a differentiating factor. AA can promote the direct conversion of mouse fibroblasts into beating cardiomyocytes (Talkhabi *et al.* 2015), induce cardiac differentiation of murine pluripotent stem cells (Ivanyuk *et al.* 2015), and cause adipocyte differentiation of embryonic stem cells into adipocytes (Cuaranta-Monroy *et al.* 2014).

Conclusion

GSCs are a new source of stem cells. These stem cells play important roles for teeth as well as jawbone growth. This study showed that AA is a proliferating factor that can trigger GSC proliferation. The proliferation mechanism of AA is related to upregulation of some pluripotent stem cell markers such as SSEA-3, Oct-3/3, Nanog, Sox-2, and TRA-1-60. However, AA-treated GSCs still maintained the MSC phenotype with MSC marker expression as well as mesoderm cell differentiation. More importantly, these cells cannot cause tumors in athymic mice. These results showed that AA can be a suitable factor to stimulate proliferation of GSCs for clinical applications.

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Conflict of interest The authors declare that they have no competing interests.

References

Bae SH, Ryu H, Rhee KJ, Oh JE, Baik SK, Shim KY, Kong JH, Hyun SY, Pack HS, Im C, Shin HC, Kim YM, Kim HS, Eom YW, Lee JI (2015) l-Ascorbic acid 2-phosphate and fibroblast growth factor-2 treatment maintains differentiation potential in bone marrow-derived mesenchymal stem cells through expression of hepatocyte growth factor. Growth Factors 33:71–78

Chang YJ, Hwang SM, Tseng CP, Cheng FC, Huang SH, Hsu LF, Hsu LW, Tsai MS (2010) Isolation of mesenchymal stem cells with neurogenic potential from the mesoderm of the amniotic membrane. Cells Tissues Organs 192:93–105

Chen YT, Wei JD, Wang JP, Lee HH, Chiang ER, Lai HC, Chen LL, Lee YT, Tsai CC, Liu CL, Hung SC (2011) Isolation of mesenchymal stem cells from human ligamentum flavum: implicating etiology of ligamentum flavum hypertrophy. Spine (Phila Pa 1976) 36:E1193–E1200

Cuaranta-Monroy I, Simandi Z, Kolostyak Z, Doan-Xuan QM, Poliska S, Horvath A, Nagy G, Bacso Z, Nagy L (2014) Highly efficient differentiation of embryonic stem cells into adipocytes by ascorbic acid. Stem Cell Res 13:88–97

Cutler AJ, Limbani V, Girdlestone J, Navarrete CV (2010) Umbilical cord-derived mesenchymal stromal cells modulate monocyte function to suppress T cell proliferation. J Immunol 185:6617–6623

Daltro GC, Fortuna V, de Souza ES, Salles MM, Carreira AC, Meyer R, Freire SM, Borojevic R (2015) Efficacy of autologous stem cell-based therapy for osteonecrosis of the femoral head in sickle cell disease: a five-year follow-up study. Stem Cell Res Ther 6:110

Deuse T, Stubbendorff M, Tang-Quan K, Phillips N, Kay MA, Eiermann T, Phan TT, Volk HD, Reichenspurner H, Robbins RC, Schrepfer S (2011) Immunogenicity and immunomodulatory properties of umbilical cord lining mesenchymal stem cells. Cell Transplant 20:655–667

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317

El-Sayed KM, Paris S, Graetz C, Kassem N, Mekhemar M, Ungefroren H, Fandrich F, Dorfer C (2015) Isolation and characterisation of human gingival margin-derived STRO-1/MACS(+) and MACS(-) cell populations. Int J Oral Sci 7:80–88

Emadedin M, Ghorbani Liastani M, Fazeli R, Mohseni F, Moghadasali R, Mardpour S, Hosseini SE, Niknejadi M, Moeininia F, Aghahossein Fanni A, Baghban Eslaminejhad R, Vosough Dizaji A, Labibzadeh N, Mirazimi Bafghi A, Baharvand H, Aghdami N (2015) Long-term follow-up of intra-articular injection of autologous mesenchymal stem cells in patients with knee, ankle, or hip osteoarthritis. Arch Iran Med 18:336–344

Esteban MA, Pei D (2012) Vitamin C improves the quality of somatic cell reprogramming. Nat Genet 44:366–367

Estes BT, Diekman BO, Gimble JM, Guilak F (2010) Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. Nat Protoc 5:1294–1311

Farias VA, Linares-Fernandez JL, Penalver JL, Paya Colmenero JA, Ferron GO, Duran EL, Fernandez RM, Olivares EG, O'Valle F, Puertas A, Oliver FJ, Ruiz de Almodovar JM (2011) Human umbilical cord stromal stem cell express CD10 and exert contractile properties. Placenta 32:86–95

Fournier BP, Larjava H, Hakkinen L (2013) Gingiva as a source of stem cells with therapeutic potential. Stem Cells Dev 22:3157–3177

Gao Y, Han Z, Li Q, Wu Y, Shi X, Ai Z, Du J, Li W, Guo Z, Zhang Y (2015) Vitamin C induces a pluripotent state in mouse embryonic stem cells by modulating microRNA expression. FEBS J 282:685–699

Gao Y, Yang L, Chen L, Wang X, Wu H, Ai Z, Du J, Liu Y, Shi X, Wu Y, Guo Z, Zhang Y (2013) Vitamin C facilitates pluripotent stem cell maintenance by promoting pluripotency gene transcription. Biochimie 95:2107–2113

Gao Y, Zhao G, Li D, Chen X, Pang J, Ke J (2014) Isolation and multiple differentiation potential assessment of human gingival mesenchymal stem cells. Int J Mol Sci 15:20982–20996

Ge S, Mrozik KM, Menicanin D, Gronthos S, Bartold PM (2012) Isolation and characterization of mesenchymal stem cell-like cells from healthy and inflamed gingival tissue: potential use for clinical therapy. Regen Med 7:819–832

Gucciardo L, Ochsenbein-Kolble N, Ozog Y, Verbist G, Van Duppen V, Fryns JP, Lories R, Deprest J (2013) A comparative study on culture conditions and routine expansion of amniotic fluid-derived mesenchymal progenitor cells. Fetal Diagn Ther 34:225–235

Gutierrez-Fernandez M, Otero-Ortega L, Ramos-Cejudo J, Rodriguez-Frutos B, Fuentes B, Diez-Tejedor E (2015) Adipose tissue-derived mesenchymal stem cells as a strategy to improve recovery after stroke. Expert Opin Biol Ther 15:873–881

Hsu SH, Huang GS, Feng F (2012) Isolation of the multipotent MSC subpopulation from human gingival fibroblasts by culturing on chitosan membranes. Biomaterials 33:2642–2655

Introna M, Rambaldi A (2015) Mesenchymal stromal cells for prevention and treatment of graft-versus-host disease: successes and hurdles. Curr Opin Organ Transplant 20:72–78

Isakson M, de Blacam C, Whelan D, McArdle A, Clover AJ (2015) Mesenchymal stem cells and cutaneous wound healing: current evidence and future potential. Stem Cells Int 2015:831095

Ivanyuk D, Budash G, Zheng Y, Gaspar JA, Chaudhari U, Fatima A, Bahmanpour S, Grin VK, Popandopulo AG, Sachinidis A, Hescheler J, Saric T (2015) Ascorbic acid-induced cardiac

differentiation of murine pluripotent stem cells: transcriptional profiling and effect of a small molecule synergist of Wnt/beta-catenin signaling pathway. Cell Physiol Biochem 36:810–830

Jin SH, Lee JE, Yun JH, Kim I, Ko Y, Park JB (2015) Isolation and characterization of human mesenchymal stem cells from gingival connective tissue. J Periodontal Res 50:461–467

Karantalis V, Hare JM (2015) Use of mesenchymal stem cells for therapy of cardiac disease. Circ Res 116:1413–1430

Kassis I, Zangi L, Rivkin R, Levdansky L, Samuel S, Marx G, Gorodetsky R (2006) Isolation of mesenchymal stem cells from G-CSF-mobilized human peripheral blood using fibrin microbeads. Bone Marrow Transplant 37:967–976

Kim JH, Kim WK, Sung YK, Kwack MH, Song SY, Choi JS, Park SG, Yi T, Lee HJ, Kim DD, Seo HM, Song SU, Sung JH (2014) The molecular mechanism underlying the proliferating and preconditioning effect of vitamin C on adipose-derived stem cells. Stem Cells Dev 23:1364–1376

Kim YS, Choi YJ, Koh YG (2015) Mesenchymal stem cell implantation in knee osteoarthritis: an assessment of the factors influencing clinical outcomes. Am J Sports Med 43(9):2293–2301

Kong D, Zhuang X, Wang D, Qu H, Jiang Y, Li X, Wu W, Xiao J, Liu X, Liu J, Li A, Wang J, Dou A, Wang Y, Sun J, Lv H, Zhang G, Zhang X, Chen S, Ni Y, Zheng C (2014) Umbilical cord mesenchymal stem cell transfusion ameliorated hyperglycemia in patients with type 2 diabetes mellitus. Clin Lab 60:1969–1976

Labidi A, Serghini M, Ben Mustapha N, Fekih M, Boubaker J, Filali A (2014) Stem cell transplantation as rescue therapy for refractory Crohn's disease: a sytematic review. Tunis Med 92:655–659

Li CJ, Sun LY, Pang CY (2015a) Synergistic protection of N-acetylcysteine and ascorbic acid 2-phosphate on human mesenchymal stem cells against mitoptosis, necroptosis and apoptosis. Sci Rep 5:9819

Li M, Zhao Y, Hao H, Han W, Fu X (2015b) Mesenchymal stem cell based therapy for non-healing wounds: today and tomorrow. Wound Repair Regen 23(4):465–482

Liu X, Zheng P, Wang X, Dai G, Cheng H, Zhang Z, Hua R, Niu X, Shi J, An Y (2014) A preliminary evaluation of efficacy and safety of Wharton's jelly mesenchymal stem cell transplantation in patients with type 2 diabetes mellitus. Stem Cell Res Ther 5:57

Lu GH, Zhang SZ, Chen Q, Wang XF, Lu FF, Liu J, Li M, Li ZY (2011) Isolation and multipotent differentiation of human decidua basalis-derived mesenchymal stem cells. Nan Fang Yi Ke Da Xue Xue Bao 31:262–265

Mochida J, Sakai D, Nakamura Y, Watanabe T, Yamamoto Y, Kato S (2015) Intervertebral disc repair with activated nucleus pulposus cell transplantation: a three-year, prospective clinical study of its safety. Eur Cell Mater 29:202–212, discussion 212

Munir H, McGettrick HM (2015) Mesenchymal stem cells therapy for autoimmune disease: risks and rewards. Stem Cells Dev 24(18): 2091–2100

Padial-Molina M, O'Valle F, Lanis A, Mesa F, Dohan Ehrenfest DM, Wang HL, Galindo-Moreno P (2015) Clinical application of mesenchymal stem cells and novel supportive therapies for oral bone regeneration. Biomed Res Int 2015:341327

Peng J, Wang Y, Zhang L, Zhao B, Zhao Z, Chen J, Guo Q, Liu S, Sui X, Xu W, Lu S (2011) Human umbilical cord Wharton's jelly-derived mesenchymal stem cells differentiate into a Schwann-cell phenotype and promote neurite outgrowth in vitro. Brain Res Bull 84:235–243

Phadnis SM, Joglekar MV, Dalvi MP, Muthyala S, Nair PD, Ghaskadbi SM, Bhonde RR, Hardikar AA (2011) Human bone marrow-derived mesenchymal cells differentiate and mature into endocrine pancreatic lineage in vivo. Cytotherapy 13:279–293

Phuc PV, Nhung TH, Loan DT, Chung DC, Ngoc PK (2011) Differentiating of banked human umbilical cord blood-derived mesenchymal stem cells into insulin-secreting cells. In Vitro Cell Dev Biol Anim 47:54–63

Pilz GA, Ulrich C, Ruh M, Abele H, Schafer R, Kluba T, Buhring HJ, Rolauffs B, Aicher WK (2011) Human term placenta-derived mesenchymal stromal cells are less prone to osteogenic differentiation than bone marrow-derived mesenchymal stromal cells. Stem Cells Dev 20:635–646

Santos TM, Percegona LS, Gonzalez P, Calil A, Corradi Perini C, Faucz FR, Camara NO, Aita CA (2010) Expression of pancreatic endocrine markers by mesenchymal stem cells from human umbilical cord vein. Transplant Proc 42:563–565

Semenov OV, Koestenbauer S, Riegel M, Zech N, Zimmermann R, Zisch AH, Malek A (2010) Multipotent mesenchymal stem cells from human placenta: critical parameters for isolation and maintenance of stemness after isolation. Am J Obstet Gynecol 202:193, **e191-193 e113**

Sener LT, Albeniz I (2015) Challenge of mesenchymal stem cells against diabetic foot ulcer. Curr Stem Cell Res Ther 10(6):530–534

Talkhabi M, Pahlavan S, Aghdami N, Baharvand H (2015) Ascorbic acid promotes the direct conversion of mouse fibroblasts into beating cardiomyocytes. Biochem Biophys Res Commun 463(4):699–705

Tang L, Li N, Xie H, Jin Y (2011) Characterization of mesenchymal stem cells from human normal and hyperplastic gingiva. J Cell Physiol 226:832–842

Wang C, Wang Y, Meng HY, Yuan XL, Xu XL, Wang AY, Guo QY, Peng J, Lu SB (2015) Application of bone marrow mesenchymal stem cells to the treatment of osteonecrosis of the femoral head. Int J Clin Exp Med 8:3127–3135

Wang S, Cheng H, Dai G, Wang X, Hua R, Liu X, Wang P, Chen G, Yue W, An Y (2013) Umbilical cord mesenchymal stem cell transplantation significantly improves neurological function in patients with sequelae of traumatic brain injury. Brain Res 1532:76–84

Wei C, Liu X, Tao J, Wu R, Zhang P, Bian Y, Li Y, Fang F, Zhang Y (2014) Effects of vitamin C on characteristics retaining of in vitro-cultured mouse adipose-derived stem cells. In Vitro Cell Dev Biol Anim 50:75–86

Yalvac ME, Ramazanoglu M, Rizvanov AA, Sahin F, Bayrak OF, Salli U, Palotas A, Kose GT (2010) Isolation and characterization of stem cells derived from human third molar tooth germs of young adults: implications in neo-vascularization, osteo-, adipo- and neurogenesis. Pharmacogenomics J 10:105–113

Yin R, Mao SQ, Zhao B, Chong Z, Yang Y, Zhao C, Zhang D, Huang H, Gao J, Li Z, Jiao Y, Li C, Liu S, Wu D, Gu W, Yang YG, Xu GL, Wang H (2013) Ascorbic acid enhances Tet-mediated 5-methylcytosine oxidation and promotes DNA demethylation in mammals. J Am Chem Soc 135:10396–10403

Yu J, Tu YK, Tang YB, Cheng NC (2014) Stemness and transdifferentiation of adipose-derived stem cells using L-ascorbic acid 2-phosphate-induced cell sheet formation. Biomaterials 35: 3516–3526

Zeddou M, Briquet A, Relic B, Josse C, Malaise MG, Gothot A, Lechanteur C, Beguin Y (2010) The umbilical cord matrix is a better source of mesenchymal stem cells (MSC) than the umbilical cord blood. Cell Biol Int 34:693–701

Zhang QZ, Nguyen AL, Yu WH, Le AD (2012) Human oral mucosa and gingiva: a unique reservoir for mesenchymal stem cells. J Dent Res 91:1011–1018

Zhang X, Hirai M, Cantero S, Ciubotariu R, Dobrila L, Hirsh A, Igura K, Satoh H, Yokomi I, Nishimura T, Yamaguchi S, Yoshimura K, Rubinstein P, Takahashi TA (2011) Isolation and characterization of mesenchymal stem cells from human umbilical cord blood: re-evaluation of critical factors for successful isolation and high ability to proliferate and differentiate to chondrocytes as compared to mesenchymal stem cells from bone marrow and adipose tissue. J Cell Biochem 112:1206–1218

Zhao XF, Xu Y, Zhu ZY, Gao CY, Shi YN (2015) Clinical observation of umbilical cord mesenchymal stem cell treatment of severe systolic heart failure. Genet Mol Res 14:3010–3017