

Isolation and characterization of stem cells derived from human third molar tooth germs of young adults: implications in neo-vascularization, osteo-, adipo- and neurogenesis

ME Yalvac^{1,7}, M Ramazanoglu^{2,7},
AA Rizvanov^{1,3,4}, F Sahin¹,
OF Bayrak¹, U Salli⁵, A Palotás⁶
and GT Kose¹

¹Department of Genetics and BioEngineering, College of Engineering and Architecture, Yeditepe University, Kayisdagi, Istanbul, Turkey;

²Department of Oral Surgery, College of Dentistry, Istanbul University, Capa, Istanbul, Turkey; ³Department of Genetics, Faculty of Biology and Soil Sciences, Kazan State University, Kazan, Russia; ⁴Core Research Laboratory, Kazan State Medical University, Kazan, Russia;

⁵Department of Pharmacology, College of Medicine, Pennsylvania State University, Hershey, PA, USA and ⁶Asklepios-Med Bt. (Private Practice and Research Center), Szeged, Hungary

Correspondence:

AA Rizvanov, Department of Genetics, Faculty of Biology and Soil Sciences, Kazan State University, ul. Kremlevskaya 18, R-420008 Kazan, Russia. or Dr A Palotás, Asklepios-Med Bt, H-6722 Szeged, Kossuth Lajos sgt. 23, Szeged H-6722, Hungary.
E-mail: rizvanov@gmail.com or palotas@asklepios-med.eu

⁷These authors contributed equally to this work.

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A number of studies have reported in the last decade that human tooth germs contain multipotent cells that give rise to dental and peri-odontal structures. The dental pulp, third molars in particular, have been shown to be a significant stem cell source. In this study, we isolated and characterized human tooth germ stem cells (hTGSCs) from third molars and assessed the expression of developmentally important transcription factors, such as *oct4*, *sox2*, *klf4*, *nanog* and *c-myc*, to determine their pluri-potency. Flow-cytometry analysis revealed that hTGSCs were positive for CD73, CD90, CD105 and CD166, but negative for CD34, CD45 and CD133, suggesting that these cells are mesenchymal-like stem cells. Under specific culture conditions, hTGSCs differentiated into osteogenic, adipogenic and neurogenic cells, as well as formed tube-like structures in Matrigel assay. hTGSCs showed significant levels of expression of *sox2* and *c-myc* messenger RNA (mRNA), and a very high level of expression of *klf4* mRNA when compared with human embryonic stem cells. This study reports for the first time that hTGSCs express developmentally important transcription factors that could render hTGSCs an attractive candidate for future somatic cell re-programming studies to differentiate germs into various tissue types, such as neurons and vascular structures. In addition, these multipotential hTGSCs could be important stem cell sources for autologous transplantation.

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Introduction

Besides presenting an important potential for developing stem cell-based therapies, adult stem cells present less ethical controversy compared with embryonic stem cells (ESCs). The mesenchymal stem cell, primarily obtained from bone marrow stroma, is one of the most promising adult stem cell types for regenerative medicine.^{1,2} Bone marrow-derived mesenchymal stem cells have been shown to differentiate into various cell types.^{3–7} However, for various reasons, such as surgical trauma caused by bone marrow isolation procedures or bone marrow-related diseases, much of the stem cell research has focused on finding alternative resources of adult stem cells that require non-invasive or

minimally invasive collection procedures. Studies have shown that these pluri-potent adult stem cells are present in various tissues and organs, such as the nerve, skin, adipose, tendon, synovial membrane and liver.^{8–12}

Tooth germs (that is, pulp and surrounding tissues) form during embryonic development as a result of ecto-mesodermal interactions that give rise to neural crest cells.¹³ These progenitor cells differentiate into the dental organ, dental papilla and dental follicle.¹⁴ As a result of delayed stages of tooth development, some of these progenitor cells still reside in dental tissues, such as dental pulp, periodontal ligament and dental papilla.^{15–18} For example, the dental follicle that surrounds the developing tooth germ contains progenitor cells for the development of the peri-odontium.¹⁹ These progenitor (stem) cells have been shown to differentiate into osteocytes, adipocytes and chondrocytes.^{20–23} We have earlier isolated dental follicle cells and found that they can be efficiently transfected with non-viral methods, suggesting a potential for future gene-based cell therapies.²⁴ Similarly, the dental pulp cells also differentiate into several cell types, such as osteocytes, neurons, adipocytes and chondrocytes.^{25,26} In humans, tooth germ tissues derived from third molars are quite unique as they undergo organo-genesis to give rise to dental structures at around age 6. It means that until this time, embryonic tissues remain quiescent and undifferentiated. In other words, organo-genesis occurs in third molars (wisdom teeth) well after birth.¹³ Multipotent tooth germ progenitor cells derived from the dental papilla of developing third molars have been successfully differentiated into ecto-dermal and meso-dermal cells and have shown a therapeutic effect on damaged liver.²⁷ In a recent study, Takeda *et al.*²⁸ reported that human dental pulp stem cells isolated from earlier stage (crown-completed) tooth development showed higher proliferation rates than those isolated at a later stage (root-completed). However, both cell types showed similar osteo/odonto-blastic differentiation efficiency. Third molar tooth germs are routinely extracted for prophylactic reasons as an orthodontic treatment, and the enucleated tooth germs are usually discarded. As these cast-off tooth germs contain undifferentiated ecto-dermal and meso-dermal components, it is an attractive approach to isolate stem cells from this source and assess their potential for therapeutic purposes for adult stem cell-based therapies. In addition, harvesting stem cells from this surgical waste tissue causes no ethical controversy.

As an alternative individual stem cell resource, Takahashi and Yamanaka²⁹ have re-programmed somatic cells into induced pluri-potent stem cells by retro-virus-mediated transfection of *oct4*, *klf4*, *sox2* and *c-myc*. The first re-programming of human cells used these four developmentally important transcription factors to re-write cells;³⁰ however, recent studies report triumphant re-programming with the use of fewer factors. For example, neural stem cells were successfully re-programmed by using only *oct4* and *klf4* transcription factors upon determining that these cells already present *sox2* and *c-myc*.³¹ It seems that endogenous expression levels of transcription factor genes such as *oct4*,

klf4, *sox2* and *c-myc* in adult cells are very important for re-programming adult cells to induced pluri-potent stem cells.²⁹ The expression levels of these genes have not yet been characterized in human tooth germ stem cells (hTGSCs).

In this study, stem cells from human tooth germs were isolated, characterized and referred to as hTGSCs—the term first used by the authors. They were analyzed for the expression of:

- (i) stem cell markers;
- (ii) transcription factors essential to maintain self-renewal of undifferentiated embryonic stem cells: *oct4*, *sox2*, *nanog*;
- (iii) transcription factors involved in cell-growth and differentiation: *klf4*;
- (iv) proteins regulating cell proliferation: *c-myc*, nucleostemin;
- (v) human telomerase reverse transcriptase: of note, telomerases (RNA-dependent polymerases) lengthen telomeres in DNA strands, thereby allowing senescent cells that would otherwise become postmitotic and commit apoptosis to become potentially immortal;
- (vi) de-phosphorylators: alkaline phosphatase; and
- (vii) intermediate filaments (dynamic structures of the cyto-skeleton): vimentin, nestin, neuro-filament.

The plasticity and multipotential capacity of hTGSCs were shown by differentiating them into osteogenic, adipogenic and neurogenic cells. It was also shown that hTGSCs were able to form tube-like structures when cultured on Matrigel (BD Biosciences, San Diego, CA, USA): an *in vitro* assay that shows the angiogenic potential of cells. In addition, the expression levels of *oct4*, *klf4*, *nanog*, *sox2* and *c-myc* genes in hTGSCs were compared with those in ESCs.

Materials and methods

Isolation of human tooth germ stem cells

Human impacted third molar tooth germs (Figure 1) used in this study were surgically removed from eight healthy patients (11–17 years of age) as part of a prophylactic treatment for ortho-dontic reasons. Written informed consent was obtained from the patients and their parents after receiving approval by the Institutional Ethics Committee of Istanbul University, Turkey. Before extraction, each subject was screened for systemic diseases and oral infections. Enucleation of tooth germs was conducted under local anesthesia. After elevation of a full-thickness flap, the maxillar/mandibular bone tissue over the tooth germ was excised by using 023–029 round diamond burs under irrigation with sterile saline to prevent any damage to the tissues. The enucleated tooth germ was immediately placed in sterile physiological saline and transferred to the laboratory within 2 h.

For each cell line, an entire tooth germ tissue, including the dental mesenchyme residing in the developing crown and its surrounding follicle, was taken and placed in 10-mm tissue culture dishes for mechanical disruption. Samples

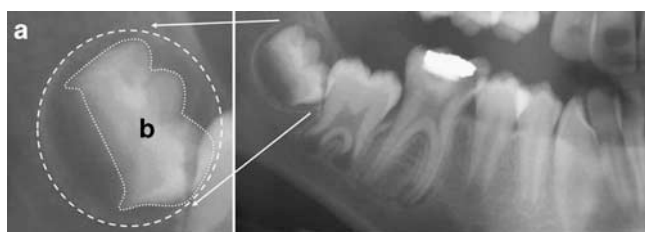


Figure 1 Isolation of human tooth germ stem cells from an impacted third molar tooth germ. (a) Whole tooth germ was extracted (dashed line) and (b) the crown part (dotted line) was removed. Afterward, all remaining tissues, including the dental mesenchyme residing in the crown and its surrounding follicle, were used for stem cell isolation.

were minced into small pieces (approximately 0.1 mm in diameter) with a sterile scalpel in growth medium containing Dulbecco's modified essential medium (Sigma Chemical Co, St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Biological Industries, Beit Haemek, Israel), 2 mM L-glutamine (Biological Industries) and 1% of penicillin, streptomycin and amphotericin solution (Biological Industries). Upon mincing, the small tissue pieces were then transferred into six-well plates (5–6 pieces per well) containing growth medium. After 10–15 days of incubation at 37 °C in a humidified atmosphere of 5% CO₂ in the incubator, the cells reached 80% confluency. The culture medium was changed every other day. Established hTGSC lines were sub-cultured using trypsin-EDTA solution (1 ×, Sigma Chemical Co., St Louis, MO, USA).

Flow-cytometry analysis

The immuno-phenotypic antigens of hTGSCs (passage 3–4) were characterized by flow-cytometry analysis as described earlier.²⁴ Cells were trypsinized and incubated with CD34, CD45, CD73, CD90, CD105, CD133 and CD166 primary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 45 min. After washing the excess primary antibodies, cells were incubated with fluorescein-iso-thio-cyanate-conjugated secondary antibodies at 4 °C for 45 min (Santa Cruz Biotechnology Inc.). The flow-cytometry analysis of the cells was carried out using the Becton Dickinson FACSCalibur flow-cytometry system (Becton Dickinson, San Jose, CA, USA).

Differentiation of human tooth germ stem cells

Human tooth germ stem cells were differentiated into osteogenic, neurogenic and adipogenic cells based on protocols obtained by slightly modifying earlier established dental stem cell differentiation procedures.^{25–26} hTGSCs with low passage number (3–4) were used for differentiation experiments.

For osteogenic differentiation, cells were counted and cultured in six-well plates at a concentration of 3000 cells cm⁻² in growth medium. After 48 h, it was replaced with osteogenic medium (Dulbecco's modified essential medium supplemented with 10% FBS, 0.1 mmol l⁻¹ dexamethasone, 10 mmol l⁻¹ β-glycerol-phosphate, 50 mmol l⁻¹

ascorbate) (Sigma Chemical Co.). The cells were incubated in osteogenic medium for 10 days, and the solution was changed every other day. On day 10, vonKossa staining, immuno-cytochemistry and RNA isolation were conducted to confirm osteogenic differentiation.

For neurogenic differentiation, cells at first step were seeded at a concentration of 3000–5000 cells cm⁻² in six-well plates on cover-slips coated with 100 μg ml⁻¹ poly-D-lys (Sigma Chemical Co.) and incubated in Dulbecco's modified essential medium supplemented with 20% FBS, 1 mmol l⁻¹ β-mercapto-ethanol (AppliChem, Darmstadt, Germany) and 5 ng ml⁻¹ basic fibroblast growth factor (Promega, Madison, WI, USA) for 24 h. At the second step, cells were incubated in NeuroBasal medium (Invitrogen, Carlsbad, CA, USA) complemented with B-27 stem cell culture supplement (Invitrogen), 50 ng ml⁻¹ basic fibroblast growth factor and 50 ng ml⁻¹ nerve growth factor (Promega) without FBS for 12 days. Neuron-specific β₃-tubulin (TuJ1, Promega) was used to assess the potential for neuronal differentiation.

For adipogenic differentiation, hTGSCs were cultured in six-well plates at a concentration of 3000 cells cm⁻² in growth medium. After 24 h, the solution was replaced with Dulbecco's modified essential medium supplemented with 10% FBS, 1 mmol l⁻¹ dexamethasone, 5 mg ml⁻¹ insulin, 0.5 mmol l⁻¹ iso-butyl-methyl-xanthine and 60 mmol l⁻¹ indomethacin (Sigma Chemical Co.) for 2 weeks. Intracellular lipid vesicles were observed under a light microscope (Nikon TS100, Minnesota, MN, USA).

Real-time PCR analysis

Total RNA from hTGSCs was isolated using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. ESC messenger RNA (mRNA) from human embryonic stem cell line Moscow 01 (hESM01) was kindly provided by Dr Sergey L Kiselev (Institute of Gene Biology, Russian Academy of Sciences, Moscow).³² Complementary DNA synthesis was conducted using random hexamer primers and moloney murine leukemia virus reverse transcriptase (MMLV-RT, Promega) at 37 °C for 1 h. For real-time PCR, primer sets of each gene and PCR conditions were chosen and applied as reported in the literature (Table 1). In addition, TaqMan primers and probes (Table 2) were designed using PrimerExpress (Applied Biosystems, Foster City, CA, USA) software. Real-time PCR primers and TaqMan probes were synthesized by Syntol (Moscow, Russia). The Premix (2.5 ×) for TaqMan real-time PCR was purchased from Syntol and used according to the manufacturer's instructions. The amount of RNA was normalized by using β-actin. Serial dilution of ESC complementary DNA was used for relative quantitation of the expression of *oct4*, *klf4*, *sox2*, *c-myc* and *nanog* genes. Relative expression represents the level of expression of target mRNA compared with that seen with control ESCs.

Immuno-cytochemical analysis

Human tooth germ stem cells grown on glass cover-slips were fixed with 2% of para-formaldehyde and permeabilized

Table 1 Primers for RT-PCR

Marker	Sequence	Length (bp)	Ref.
oct4	Forward: 5'-CGACCATCTGCCGCTTTGAG-3' Reverse: 5'-CCCCCTGTCCCCATTCTA-3'	572	McLaughlin <i>et al.</i> ³⁸
NS	Forward: 5'-GGAGTCCTGGATTCCTTCC-3' Reverse: 5'-GCCCTGACCACTCCAGTTTA-3'	98	Kafienah <i>et al.</i> ³⁴
β ₃ -Tubulin	Forward: 5'-CTCAGGGGCTTTGGACATC-3' Reverse: 5'-CAGGCAGTCGCAGTTTTTAC-3'	159	Xiao <i>et al.</i> ³⁹
Nestin	Forward: 5'-GGAGTCCTGGATTCCTTCC-3' Reverse: 5'-GCCCTGACCACTCCAGTTTA-3'	200	McLaughlin <i>et al.</i> ³⁸
β-Actin	Forward: 5'-GACAGGATGCAGAAGGAGATTACT-3' Reverse: 5'-TGATCCACATCTGCTGGAAGGT-3'	141	Kafienah <i>et al.</i> ³⁴
Osteo-calcin	Forward: 5'-GGTGCAAAGCCCAGCGACTCT-3' Reverse: 5'-GGAAGCCAATGTGGTCCGCTA-3'	190	Yoon <i>et al.</i> ⁴⁰
NFL	Forward: 5'-ACCTCTCCGCCGCTCTCAAG-3' Reverse: 5'-TCTCCTCCACCTCTGACTGCTCC-3'	612	Somogyi <i>et al.</i> ⁴¹
hTERT	Forward: 5'-AGAGTGTCTGGAGCAAGTTGC-3' Reverse: 5'-CGTAGTCCATGTTTACAATCG-3'	183	Saji <i>et al.</i> ⁴²
ALP	Forward: 5'-GACATCGCCTACCAGCTCAT-3' Reverse: 5'-TCACGTTGTTCTGTTCAGC-3'	306	Tare <i>et al.</i> ⁴³
Vimentin	Forward: 5'-TGAGGCTGCCAACCAGGAACA-3' Reverse: 5'-TTGGCCGCTGCAGGATGAG-3'	208	Kameda <i>et al.</i> ⁴⁴

Abbreviations: ALP, alkaline-phosphatase; hTERT, human telomerase reverse-transcriptase; NFL, neuro-filament; NS, nucleo-stemin; RT, real time.

Table 2 Primers and probes for real-time PCR

Oligo-nucleotide	Sequence	Genebank no.
β-Actin-TMprobe(human) ^a	5'-CCAGCCATGTACGTTGCTATCCAGGC-3'	NM_001101
β-Actin-TM-F(human)	5'-GCGAGAAGATGACCCAGGATC-3'	
β-Actin-TM-R(human)	5'-CCAGTGGTACGGCCAGAGG-3'	
h-oct4-TMprobe621 ^a	5'-TCTGCAGCTTAGCTTCAAGAACATGT-3'	NM_002701
h-oct4-TM499F	5'-CGACCATCTGCCGCTTTG-3'	
h-oct4-TM664R	5'-GCAAGGGCCCGCAGCTTA-3'	
h-c-myc-TMprobe1494 ^a	5'-TACGCAGCGCCTCCCTCCACTC-3'	NM_002467
h-c-myc-TM1472F	5'-CGTCTCCACACATCAGCACAA-3'	
h-c-myc-TM1539R	5'-TCTTGGCAGCAGGATAGTCCTT-3'	
h-klf4-TMprobe1414 ^a	5'-CCGGTTCCTGCATGCCAGAGGA-3'	NM_004235
h-klf4-TM1387F	5'-CGTCCATTACCAAGAGCTCAT-3'	
h-klf4-TM1463R	5'-CGATCGTCTTCCCCTTTG-3'	
h-nanog-TMprobe453 ^a	5'-TGAGAGAAGAGTGTGCGAAAAAAGG-3'	NM_024865
h-nanog-TM431F	5'-CCAAAGGCAAACAACCCACTT-3'	
h-nanog-TM499R	5'-TCTTGACCGGGACCTTGTCT-3'	
h-sox2-TMprobe763 ^a	5'-CCGGCGGAAAACCAAGACGCT-3'	NM_003106
h-sox2-TM717F	5'-TGCGAGCGCTGCACAT-3'	
h-sox2-TM809R	5'-GCAGCGTGACTTATCCTTCTTCA-3'	

^aTaqMan probes contain 5' FAM fluorescent dye and 3' RTQ-1 quencher.

by incubating with 0.1% Triton-X100/phosphate buffered saline for 5 min. Nonspecific binding of antibodies was blocked by incubating with 2% goat serum (diluted in phosphate buffered saline) for 20 min. Samples were incubated with primary antibodies overnight at 4 °C. Each sample was washed twice for 5 min with phosphate buffered saline to remove un-bound primary antibodies. After washing, secondary antibodies (goat poly-clonal anti-rabbit

IgG-Alexa 488, goat poly-clonal anti-mouse IgG-Alexa 488 conjugate, goat poly-clonal anti-mouse IgG-Alexa 647: Invitrogen) were added and incubated for 1 h. Stained cover-slips were mounted on clean glass slides using Mowiol mounting medium (Calbiochem, La Jolla, CA, USA). Prepared slides were observed under a confocal microscope (Leica TCS SP2 SE, Leica, Bensheim, Germany) immediately after preparation.

vonKossa staining

After 10 days of incubation with osteogenic medium in six-well plates, cells were fixed with 2% of para-formaldehyde at 4 °C for 30 min. After fixation, cells were stained using the vonKossa method (Bio-optica, Milan, Italy) and calcium depositions were observed with a light microscope (Nikon TS100). Accountability of mineralization by vonKossa stain and calcium estimation can provide evidence of osteogenic type of cells.

In vitro tube formation assay

Induction of tube formation was carried out using Matrigel: an *in vitro* assay that shows the angiogenic potential of cells. Matrigel was thawed on ice and 50 μ l of that was plated on each well of a pre-chilled 96-well plate (TPP TechnoPlastic-Products, Trasadingen, Switzerland) by using chilled pipette tips. The plate was placed in an incubator at 37 °C for 30 min, which allowed Matrigel to polymerize. Passage 3 hTGSCs were removed from a 70% confluent plate by trypsinization. Cells were re-suspended in serum-containing medium to 10⁶ cells mL⁻¹ and 100 μ l of cell suspension was added into the Matrigel-coated wells. The tube-like structure formation was monitored for 7 h.

Statistical analysis

Standard error and *t*-test values were calculated using Microsoft Excel statistics.

Results

A total of eight hTGSC lines were established from the extracted tooth germs (Figures 1 and 2). Cells expanded *in vitro* showed fibroblast-like morphology. All cell lines were cultured between passages 20 and 35 without any changes in their morphology (Figure 2). hTGSCs were expanded extensively without feeder cells and doubled approximately every 24 h, which increased up to 36 h as the cell lines reached approximately passage 24. All established hTGSC lines were positive for CD73, CD90, CD105 and CD166, but negative for CD34, CD45 and CD133 (Figure 3). On the other hand, dental pulp stem cell ($n=3$) lines established from patients of the same age group (11–17 years) expressed these markers at statistically lower levels than hTGSCs (data not shown).

Real-time PCR analysis showed that primary hTGSCs express *oct4*, nucleostemin, nestin, alkaline-phosphatase, vimentin, β_3 -tubulin and human telomerase reverse-transcriptase, but not osteocalcin and neurofilament mRNAs, which are specific markers for osteocytes and neurons, respectively (Figure 4). In addition, real-time PCR analysis showed that hTGSCs express developmentally important transcription factors, *sox2* and *c-myc* mRNA, and high levels of *klf4* mRNA compared with ESCs, while expressing relatively lower levels of *oct4* and *nanog* mRNAs (Figure 5). It was also shown that expression levels of *oct4* decreased as the passage number increased from 1 to 27. When hTGSCs differentiated into osteogenic and neurogenic cells, expression levels of *oct4* decreased significantly (Figure 6).

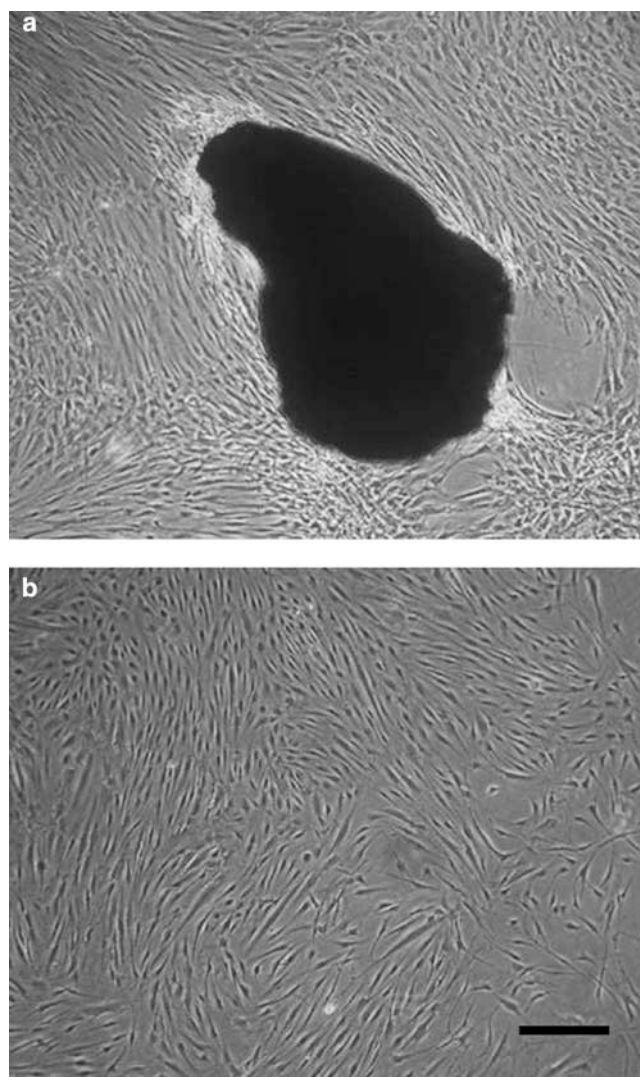


Figure 2 Morphology of human tooth germ stem cells (hTGSCs). Each human tooth germ tissue was dissected into smaller pieces in a plastic culture dish containing growth media, which were seeded on six-well plates (5–6 pieces per well). In a few days, hTGSCs started to grow around the tooth material ((a), day 5) and proliferated as a mono-layer, reaching 80% confluence by 10–15 days ((b), day 10). Scale bar: 400 μ m.

Immuno-cytochemistry analysis revealed that hTGSC cells stained positively for *klf4*, *c-myc*, *oct4*, nestin, nucleostemin and *sox2* (Figure 7). After osteogenic induction, differentiated hTGSCs were positive for bone-specific collagen type I and formed calcium depositions that were visualized using the vonKossa method (Figure 8a, b). After neurogenic induction, hTGSCs gained neuron-like morphology and were positive for β_3 -tubulin (Figure 8c). Adipogenic differentiation of hTGSCs was confirmed by the presence of small cytoplasmic lipid vesicles formed upon induction with adipogenic medium (Figure 8d). hTGSCs formed tube-like structures within 4–7 h incubation on Matrigel (Figure 9).

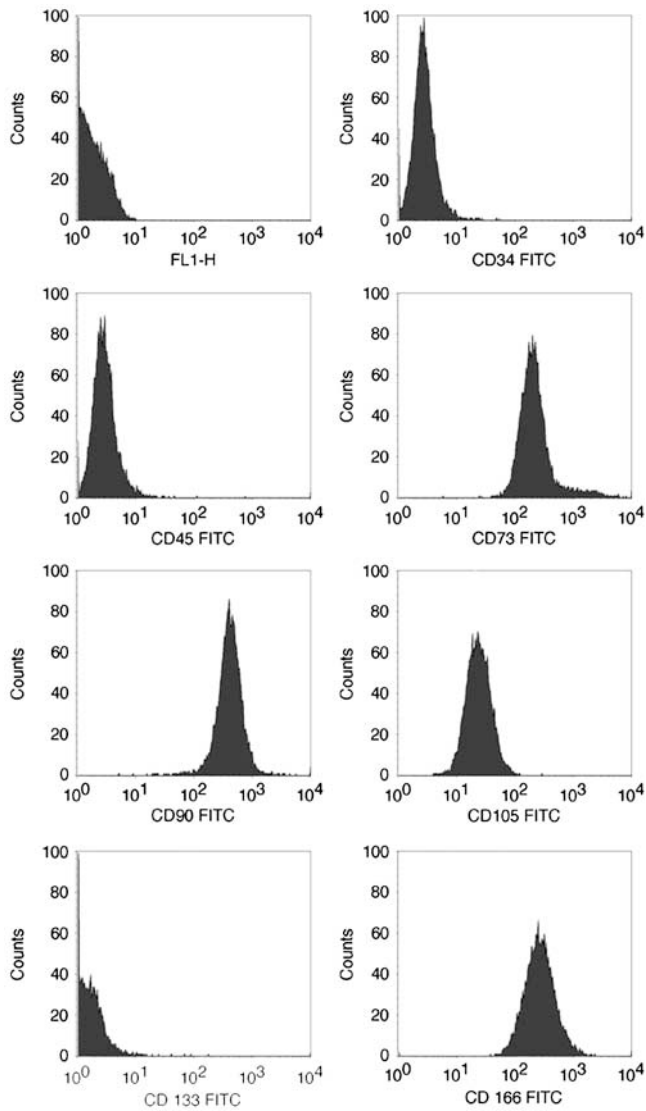


Figure 3 Immuno-phenotypic characteristics of human tooth germ stem cells (hTGSCs). Flow-cytometry analyses revealed that hTGSCs expressed cell surface antigens CD90, CD73, CD106 and CD105 (typical for mesenchymal stem cells (MSCs)), and were negative for CD34, CD45 and CD133.

Discussion

Bone marrow is one of the main sources of the mesenchymal stem cells (MSCs) and it is currently the preferred source to obtain large quantities of these cells. Besides the bone marrow, adult stem cells have been isolated from a variety of tissues, such as the liver, nerve, muscle, skin, synovium and cartilage.^{33,34} Recent studies have shown that extracted non-decayed impacted third molar tooth germ tissues could serve as a very valuable source of stem cells. As these tissues are removed during standard prophylactic interventions and do not require additional surgical procedures, utilization of such resources to isolate stem cells offers an attractive alternative. Isolation of multipotent MSC-like

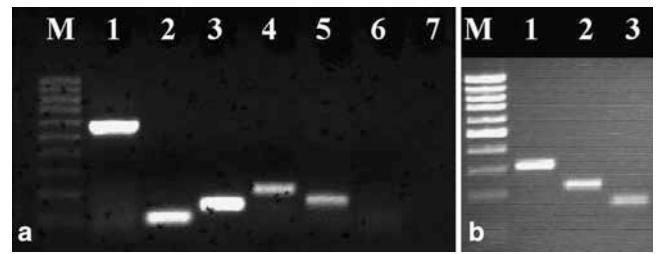


Figure 4 Real-time PCR analysis of hTGSCs. Human tooth germ stem cells (hTGSCs) express messenger RNAs of stem cell markers such as *oct4*, nucleo-stemin (*NS*), vimentin, alkaline-phosphatase (ALP), nestin, human telomerase reverse-transcriptase (hTERT), but not that of mature cell markers such as neuro-filament (NFL) and osteo-calcin. (a) 100-bp ladder (M), *oct4* (1), nucleo-stemin (2), β -actin (3), nestin (4), β_3 -tubulin (5), NFL (6) and osteo-calcin (7). (b) 100-bp ladder (M), ALP (1), vimentin (2) and hTERT (3).

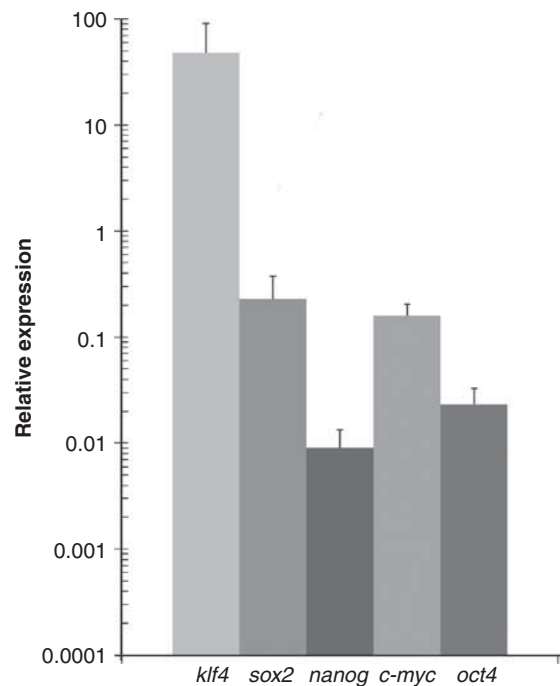


Figure 5 Real-time PCR comparison of *klf4*, *sox2*, *nanog*, *c-myc* and *oct4* gene-expression levels in human tooth germ stem cells and human embryonic stem cells. Messenger RNA (mRNA) expression levels are represented relative to the expression of the corresponding mRNA in embryonic stem cells.

cells from dental follicles, dental pulp, dental papilla and peri-odontal ligaments, all of which originate from the tooth germ, has been successfully achieved by various groups, including our laboratory.^{17-20,23,24,26-28} Unlike earlier reports, in this study we isolated stem cells for the first time from the entire tooth germ, which includes aforementioned tissues. Although we did not compare side-by-side, this approach yielded higher numbers of cells, showing a higher proliferation rate compared with the dental follicle cells we isolated earlier.²⁴

We have also shown that hTGSCs express MSC-specific cell surface antigens, CD73, CD90, CD105, CD166, and do not express CD34, CD45 and CD133, which are mostly related to the hematopoietic stem cells. Real-time PCR analysis revealed that hTGSCs present undifferentiated cell markers such as nucleo-stemin and human telomerase reverse-transcriptase, indicating that these cells are in an undifferentiated, proliferative state.³⁴ Although hTGSCs were proliferative, the expression of *oct4* decreased gradually as the passage number increased. As *oct4* is crucial for the maintenance of the pluri-potency,³⁵ decrease in *oct4* in

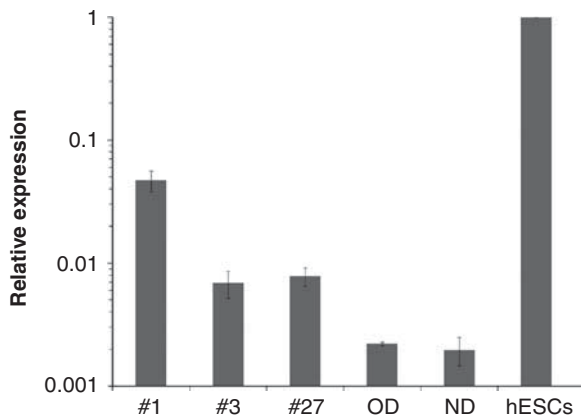


Figure 6 Real-time PCR analysis of *oct4* messenger RNA (mRNA) expression levels in human tooth germ stem cells (hTGSCs) in response to increased number of passages (1, 3, 27) and osteogenic and neurogenic differentiation. The level of *oct4* messenger RNA (mRNA) was over 10-times less in hTGSCs when compared with embryonic stem cells, and its expression further decreased significantly after the first passage. As expected, *oct4* mRNA abundance decreased in differentiated cells.

subsequent passages might indicate the loss of differentiation capacity of hTGSCs and potential senescence. It might also be an indication of the loss of a specific cell type, present originally in a heterogeneous cell population.

Our results from differentiation studies are in agreement with earlier reports that showed the ability of tooth germ-derived stem cells to differentiate into osteogenic, adipogenic and neurogenic cells. In this study, nestin and β_3 -tubulin markers were used to analyze the neurogenic potential of hTGSCs. The results showed that hTGSCs express nestin and β_3 -tubulin mRNAs, but not neurofilament mRNA, indicating that differentiated hTGSCs contain some neuro-progenitor cells but not mature neurons. After neurogenic induction, hTGSCs gained neuron-like morphology and stained positive for β_3 -tubulin. It has been shown that bone MSCs are able to trans-differentiate into endothelial-like cells *in vivo* and *in vitro*, which is especially important for providing responses to the angiogenic factors released by tumors.³⁶ An *in vitro* tube formation assay was used to understand the role of MSCs in neurovascularization by various groups.³⁷ In this study, we showed that hTGSCs form tube-like structures, which might be an indication of the possible contribution of these cells to vascularization. It might be valuable to compare the responses of bone marrow-derived MSCs and hTGSCs to angiogenic factors such as the vascular endothelial growth factor. Taken together, hTGSCs might be used as an efficient model for vascularization and neurogenesis experiments.

Until age 6, human third molar tooth germs contain undifferentiated embryonic tissues that give rise to the entire tooth.¹³ With regard to this, isolation of tooth germ cells from individuals as young as possible might enable derivation of less differentiated stem cells. It is important to analyze the expression levels of transcription factors such as

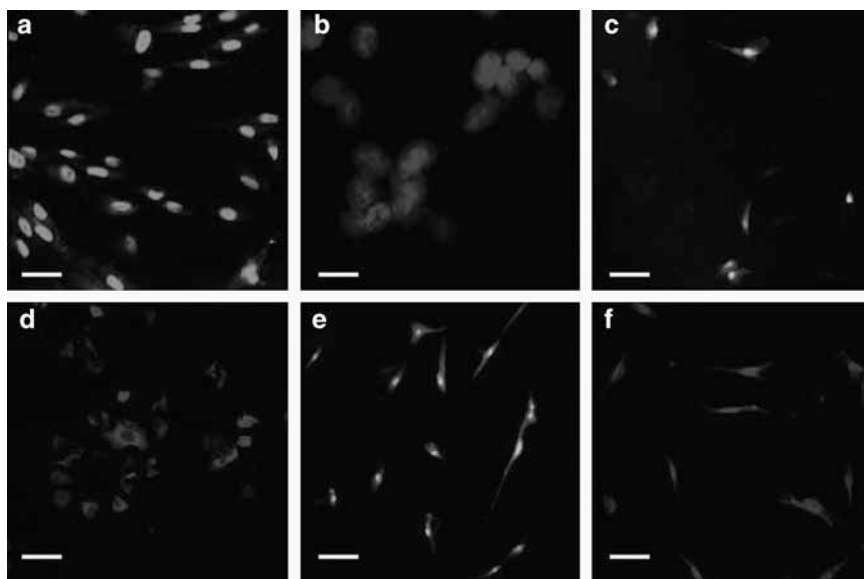


Figure 7 Immuno-cytochemical analysis of human tooth germ stem cells (hTGSCs) using specific antibodies for *klf4* (a), *c-myc* (b), *oct4* (c), nestin (d), nucleo-stemin (e) and *sox2* (f) proved that all markers showed correct localization inside the cell. Scale bars: (a) 20 μ m, (b) 10 μ m, (c) 100 μ m, (d) 40 μ m, (e) 100 μ m and (f) 100 μ m.

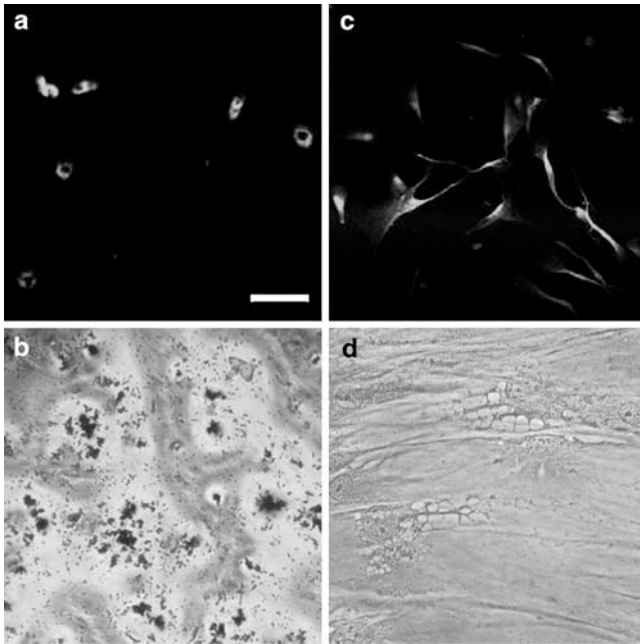


Figure 8 Immuno-cytochemical and light microscopy analysis of the multilineage differentiation potential of human tooth germ stem cells (hTGSCs). hTGSCs present collagen type I protein upon osteogenic induction (a) Osteogenic differentiation was also confirmed by visualization of calcium depositions by using the vonKossa staining technique (b) hTGSCs were positive for β_3 -tubulin (TuJ1) after induction of neuronal differentiation (c) hTGSCs accumulated intracellular lipid vesicles after adipogenic differentiation (d) Scale bar: 50 μm .

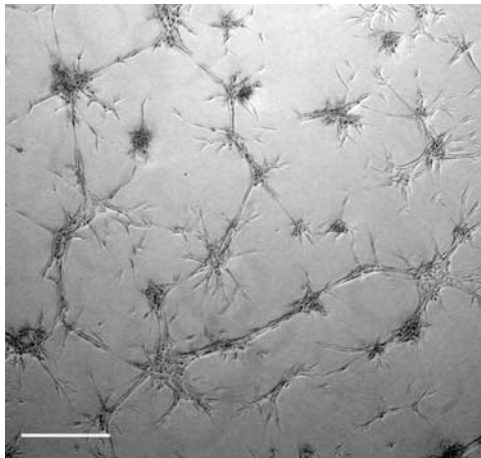


Figure 9 *In vitro* Matrigel tube formation assay. Human tooth germ stem cells were plated onto polymerized Matrigel layer in a 96-well plate. Tube-like structures were observed after 7 h of incubation. Scale bar: 100 μm .

oct4, *sox2*, *c-myc*, *nanog* and *klf4* in hTGSCs to compare their stem cell state with that of ESCs. Analyzing the endogenous expression levels of some of these genes in cell culture can help determine their capacity to undergo re-programming into the pluri-potent state. Although the first re-programming

studies used four transcription factors,^{29–30} recent studies suggest that fewer factors might be sufficient to re-write specific cell types. For example, neural stem cells were re-programmed back to the pluri-potent state by inducing them with only two transcription factors (*oct4* and *klf4*) instead of the original 4.³¹ This finding is significant because these factors are transferred to the cell via viral vectors and remain integrated in host genome. Reducing the number of factors required to induce cells to a pluri-potent state will facilitate the discovery of alternative approaches to re-program these types of cells that endogenously express these factors. In this study, we have shown that hTGSCs express significant levels of transcription factors *klf4*, *sox2* and *c-myc*, which are required for the generation of induced pluri-potent stem cells. We found it remarkable that *klf4* expression was significantly higher in hTGSCs compared with that seen with human embryonic stem cells. These results suggest that hTGSCs might be an attractive source for various cell-based biomedical applications, particularly for generation of induced pluri-potent stem cells.

In conclusion, we showed that the entire human tooth germ tissues of young adults contain stem cells that can be isolated and expanded efficiently. These multipotent stem cells may provide a potential source of autologous transplantation for treatment of various diseases. hTGSCs can also be used as an alternative cell source to create human embryonic stem cell-like cells that are immortal and pluri-potent, as they may require less transgenic manipulations to induce.

Conflict of interest

The authors declare no conflict of interest.

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