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# Influence of STRO-1 selection on osteogenic potential of human tooth germ derived mesenchymal stem cells

Pinar Ercal[a,*], Gorke G. Pekozer[b,c], Osman Z. Gumru[d], Gamze T. Kose[b,c], Mustafa Ramazanoglu[d]

[a] Department of Oral Surgery, Faculty of Dentistry, Istanbul Kemerburgaz University, 34147 Bakirkoy, Istanbul, Turkey
[b] Department of Genetics and Bioengineering, Faculty of Engineering and Architecture, Yeditepe University, 34755 Kayisdagi, Istanbul, Turkey
[c] Center of Excellence in Biomaterials and Tissue Engineering, BIOMATEN, METU, 06800 Ankara, Turkey
[d] Department of Oral Surgery, Faculty of Dentistry, Istanbul University, 34390 Fatih, Istanbul, Turkey

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ABSTRACT

Mesenchymal stem cells derived from the human tooth germ (hTGSCs) are a heterogeneous cell population that can differentiate into osteogenic, neurogenic, and adipogenic lineages. The aim of this study was to compare the osteogenic differentiation capacity of STRO-1 positive (STRO-1 + ) hTGSCs and unsorted heterogeneous hTGSCs and to establish if STRO-1 + cells are more committed to osteogenic differentiation. HTGSCs were isolated from impacted third molar tooth germ tissues of adolescents, and a subpopulation of STRO-1 + hTGSCs was obtained by fluorescence-activated cell sorting. STRO-1 +, STRO-1 negative (STRO-1 −), and unsorted cells were cultured in osteogenic and standard culture media to compare their capacity to differentiate towards osteoblastic lineage. Cells were tested for proliferation rates, alkaline phosphatase activity, and amounts of accumulated calcium. Gene expression levels of the *RUNX2*, osteocalcin, and osteonectin genes were analyzed with real time PCR. Mineralization and osteogenic protein expression were examined by using von Kossa staining and confocal microscopy. Our results indicated that osteogenically induced cell populations showed greater mineralization capacity than non-induced cells. However, expression levels of early and late osteogenic markers were not significantly different between STRO-1 + and unsorted cells. In conclusion, the selection by STRO-1 expression does not yield cells with osteogenic capacity higher than that of the heterogeneous hTGSC population. Cell sorting using osteogenic markers other than STRO-1 might be beneficial in obtaining a more sensitive osteogenic sub-population from unsorted heterogenous hTGSCs.

## 1. Introduction

Multipotent human stem cells are found in various tissues of the body, such as the bone, cartilage, muscle, and the nervous system (Gazit, 2013). Bone marrow was the first tissue from which plastic-adherent, fibroblast-like cells were obtained that developed into colony-forming unit fibroblasts (Friedenstein, Latzinik, Grosheva, & Gorskaya, 1982; Friedenstein, Piatetzky-Shapiro, & Petrakova, 1966). That discovery paved the way for the continuing search of an ideal cell population for tissue regeneration. Mesenchymal stem cells (MSCs) have been identified as a plastic-adherent cell population that has the ability to differentiate into osteogenic, adipogenic, chondrogenic, myogenic, and other lineages (Aubin, Liu, Malaval, & Gupta, 1995; Mackay et al., 1998; Pittenger et al., 1999; Shake et al., 2002; Wakitani et al., 2002). Recently, MSCs have been found in tissues of dental origin and were classified as dental pulp stem cells, stem cells from apical papilla, stem cells from exfoliated deciduous teeth, periodontal ligament stem cells, dental follicle precursor cells, and tooth germ stem cells (Gronthos, Mankani, Brahim, Robey, & Shi, 2000; Gronthos et al., 2002; Miura et al., 2003; Morsczeck et al., 2005; Seo et al., 2008; Sonoyama et al., 2006; Yalvac et al., 2010).

Tooth germ, which includes dental follicle and the surrounding tissues, is a source of dental stem cells that can be harvested during routine orthodontic treatments. Human third molar tooth germs are exceptional in permanent dentition, because their calcification begins around the age of 8, but tooth development becomes complete with apex closure only at around 21 years of age (Jung & Cho, 2014). Due to late tooth formation of third molars in adolescence, developmentally young progenitor cells are present in dental germ tissues. Therefore, stem cells from third molars can be harvested from patients later in their lives during dental treatments and without ethical controversies or further morbidity.

HTGSCs can be differentiated into cells of osteogenic, adipogenic, neurogenic, odontogenic, and chondrogenic lineages (Calikoglu, Pekozer, Ramazanoglu, Kose, & Hasirci, 2015; Taşlı, Aydın,

* Corresponding author.
*E-mail addresses:* pinarercal@gmail.com, pinar.ercal@kemerburgaz.edu.tr (P. Ercal).

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Yalvaç, & Şahin, 2014; Taşlı, Yalvaç, Sofiev, & Şahin, 2013; Yalvac et al., 2010). HTGSCs have been shown to express significantly higher levels of *SOX2*, *MYC,* and *KLF4* mRNAs than human embryonic stem cells. Expression of such important developmental transcription factors indicates that hTGSCs can be a reliable source of stem cells that can differentiate into various lineages (Yalvac et al., 2010). However, MSC-like cells isolated as a heterogeneous population usually exhibit varying proliferation and differentiation properties. In order to sort MSC populations and to obtain a homogenous cell subpopulation more committed towards desired lineage differentiation, expression of specific cell surface markers is often examined (Dominici et al., 2006). STRO-1 is a surface marker that was initially identified in bone marrow stromal cells. It distinguishes clonogenic cell fraction that can differentiate into osteoblasts, chondrocytes, adipocytes, or smooth muscle cells (Dennis, Carbillet, Caplan, & Charbord, 2002; Gronthos, Graves, Ohta, & Simmons, 1994; Gronthos & Simmons, 1995; Simmons & Torok-Storb, 1991; Zannettino et al., 2007). It has been shown that osteoprogenitors in human bone marrow cells are represented only by STRO-1+ cells (Gronthos et al., 1994). Hence, it is possible that STRO-1+ hTGSCs have a greater propensity for osteogenesis. Recent studies revealed that MSCs derived from dental tissues express STRO-1 (Gronthos et al., 2003; El-Sayed et al., 2014; Miura et al., 2003; Xu, Wang, Kapila, Lotz, & Kapila, 2009; Yan et al., 2014; Yang, Zhang et al., 2007; Yu et al., 2010). STRO-1+ sorted dental stem cells displayed higher propensity to undergo odontogenesis and showed a more defined multilineage potential than unsorted cells (Bakopoulou, Leyhausen, Volk, Koidis, & Geurtsen, 2013; Yang, Van den Dolder et al., 2007). Therefore, selection for STRO-1 expression could help establishing a reproducible and defined *in vitro* culture protocol for hTGSCs that would have a high osteogenic differentiation potential.

The aim of this study was to investigate whether STRO-1+ subpopulation of hTGSCs had a higher potential for osteogenic differentiation than unsorted and STRO-1− cell populations. Thus, we explored in detail growth and mineralization of these groups of cells. Our hypothesis was that STRO-1+ hTGSCs would have superior osteogenic differentiation capacity as a homogenous subpopulation and thereby, be a better tool for regenerative purposes.

## 2. Materials and methods

### 2.1. Isolation of hTGSCs

This study was approved by the Human Ethics Committee of Istanbul University (Ethics Committee Approval No. 2013/647). HTGSCs were isolated from impacted third molars of consenting patients (13–20 years of age) undergoing orthodontic treatment and cultured according to the method described by Yalvac et al. (2010). The cells were grown in low glucose (1 g/L) Dulbecco's Modified Eagle medium (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin/amphotericin (PSA) at 37 °C, in a $CO_2$ incubator (Forma Series II 3110 Water Jacketed $CO_2$ Incubator, Thermo Scientific, USA) in a humidified (90%) atmosphere of 95% air and 5% $CO_2$. Cells were passaged at 80% confluence, and the medium was changed every other day.

### 2.2. Flow cytometry analysis

Surface antigen profiles of the established cells were analyzed by flow cytometry. Passage 3 (p3) hTGSCs were detached from culture plates with trypsin (TrypLE Express 1 × ,Gibco, USA) and fixed with 4% paraformaldehyde for 40 min at room temperature to achieve cell preservation and light scatter stabilization. Cells were washed with Dulbecco's phosphate-buffered saline (pH 7.4) and incubated with primary antibodies against CD34 (BD Pharmingen, USA), CD 45 (BD Pharmingen, USA), CD 105 (Abcam, UK), CD 90 (BD Pharmingen, USA), CD 44 (Abcam, UK), CD14 (Genway, USA), and CD117 (eBioscience, USA) for 45 min at 4 °C and then for 15 min at room temperature. Flow cytometry analysis of the cells was performed with FACS Calibur (Becton Dickinson, USA).

### 2.3. Fluorescence-activated cell sorting of STRO-1 positive cells

For fluorescence-activated cell sorting (FACS) experiments, p3 hTGSCs (80% confluence) were detached from culture plates using trypsin and then suspended in phosphate-buffered saline (PBS). Samples were incubated with an unconjugated primary monoclonal antibody against STRO-1 (R & D Systems, USA) for 1 h at 4 °C. After centrifugation and washes, cells were incubated in PBS with a phycoerythrin-conjugated secondary goat anti-mouse IgG antibody (R & D Systems, USA) at 4 °C for 1 h. Cell were then washed to remove excess antibodies, suspended in PBS supplemented with 5% FBS, and maintained on ice until cell sorting. Cells were sorted with a FACS Aria III flow-cytometry system (BD Biosciences, USA) into DMEM supplemented with 10% FBS and 100 units/mL PSA. STRO-1+ and STRO-1− cells were collected and cultured in T25 flasks. After sorting, STRO-1+ population was run through flow cytometry in order to test sorting efficiency. Sorted cells were expanded for 10–15 days until they reached confluence.

### 2.4. Osteogenic differentiation

For osteogenic differentiation of STRO-1+, STRO-1−, and unsorted (US) cell groups, we used osteogenic culture medium that contained DMEM supplemented with 10% FBS, 1% PSA, 10 nM dexamethasone (AppliChem, Germany), 10 nM ß-glycerophosphate (AppliChem, Germany), and 50 μM ascorbic acid (Sigma, USA). P5 hTGSCs were cultured in 12-well plates ($2 \times 10^5$ per well) in standard growth medium and on the 3rd day of incubation, osteogenic medium was added to test groups. Standard growth medium was used for control experiments in each cell group. All cell groups were cultured for 21 days. The medium was changed twice a week.

### 2.5. Determination of cell proliferation by MTS assay

After 1, 4, 7, 14, and 21 days of incubation, proliferation of hTGSCs was analyzed by CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS, Promega, USA). MTS/DMEM (1:6) solution was prepared and added to each well of the 12-well plates and incubated for 2 h at 37 °C in a $CO_2$ incubator. Absorbance was measured at 490 nm with an ELISA Plate Reader (Bio-Tek, Elx800, USA).

### 2.6. Determination of alkaline phosphatase activity

On days 4, 7, 14, and 21, alkaline phosphatase (ALP) activity was analyzed using an ALP kit (RANDOX Laboratories, Ireland). Cell lysis was done in 0.1% Triton® X-100 (Sigma-Aldrich, Germany) dissolved in 0.1 M Tris buffer solution (pH 9.0) (Sigma-Aldrich, Germany). Cell lysates underwent three consecutive freeze-thaw cycles by freezing for 10 min at −20 °C and thawing for 10 min at 37 °C. Samples were subjected to sonication for 10 min on ice with 30-s intervals every minute. P-nitrophenyl phosphate (20 μL) solution provided by the ALP kit was added to 100 μL of each sample. Absorbance was measured at 405 nm for 14 min at 2-min intervals by an ELISA Plate Reader (BIO-TEK, Elx800, USA). ALP activity was expressed in nmol/min units determined by using a calibration curve (Millipore, USA). Total protein concentrations of the samples were measured with a Smart Micro BCA Protein Assay kit (Intron Biotechnology, South Korea) following manufacturer's protocols. Cells were lysed according to the manufacturer's protocol, and 100 μL of cell lysate was transferred to 96-well plates. For constructing the calibration curve, serial dilutions of bovine serum albumin (BSA) were prepared, and 100 μL of those solutions were also transferred to 96-well plates. Working solution (100 μL) prepared from