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Role of STRO-1 sorting of porcine dental germ stem cells in dental stem cell-mediated bone tissue engineering

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ABSTRACT

Stem cells of dental origin emerged as a new source for the regeneration of tissues with advantages mainly including non-invasive collection procedures and lack of ethical contraversies with their harvest or use. In this study, porcine TGSCs (pTGSCs) were isolated from mandibular third molar tooth germs of 6-month-old domestic pigs. This is the first study that reports the isolation and characterization of TGSCs from porcine third molars and their differentiation depending on STRO-1 expression. PTGSCs were sorted according to their STRO-1 expression as STRO-1(+) and STRO-1(−). Sorted and unsorted heterogenous cells (US) were characterized by their osteogenic, chondrogenic and adipogenic differentiation capabilities. STRO-1(+) cells exhibited a higher proliferation rate owing to their clonogenic properties. All three groups of cells were found differentiated into osteogenic lineage as shown by ALP activity, calcium deposition assay, detection of osteogenic mRNAs and, proteins and mineralization staining. According to differentiation analysis, STRO-1(+) cells did not show a better performance for osteogenesis compared to STRO-1(−) and US cells. This might indicate that STRO-1(+) cells might require a heterogeneous population of cells including STRO-1(−) in their niche to perform their proposed role in osteogenesis.

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Mesenchymal stem cells; dental stem cells; porcine tooth germ stem cells; STRO-1; bone tissue engineering

Introduction

Critical size bone defects are major concern since they significantly decrease the quality of life of the patients and become an important socioeconomic burden to the healthcare system globally as the world population ages [1]. Bone tissue engineering has been introduced as a very promising alternative therapy to the conventional methods such as bone grafting and prostheses. It offers a lifelong solution and a better quality of patients' life reducing the need for repeated hospitalization and health care costs associated with pharmaceutical therapy.

Mesenchymal stem cells (MSCs) are the most common type of cells that are used for bone tissue engineering applications as the cell source. MSCs are plastic-adherent multipotent cells that are capable of differentiation into tissues of mesodermal origin such as bone, cartilage and adipose tissue [2]. They can be isolated from many adult tissue types and bone marrow stroma is used as the most common source. However, since bone marrow collection from patients is an invasive procedure, other sources with less invasive collection procedures are still being explored.

Dental tissues are one of those sources that appeared as a rich mesenchymal stem cell source since their first isolation from dental pulp in 2000. They gained attention because they are easily accessible and have multilineage differentiation capacity [3].

In humans, tooth germ tissues of third molars (wisdom teeth) contain progenitor cells that give rise to dental structures at around age 6 [4] unlike other teeth that begin their development prenatally. It means that until third molars completely develop, cells with stem cell characteritics remain in this tissue. In addition, due to tooth decay and orthodontic reasons, third molar tooth germs are routinely extracted and discarded as surgical waste. Therefore, stem cell harvesting from this source causes no ethical controversy [5].

Regardless of the tissue they are isolated from, MSCs are a heterogeneous mixture of cells with varying proliferation and differentiation potentials. Although it is a common practice to use this heterogenous group of cells for cell-based therapeutic applications, a better understanding of the MSC properties is required. On the other hand, depending on the source MSCs can be contaminated with cells of different types, such as hematopoietic cells, fibroblasts, endothelial cells during isolation, which is an undesired but unavoidable situation. Thus, attempts have been made to develop a cell-surface antigen profile for the better purification and identification of MSCs [2]. According to the International Society for Cellular Therapy

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Supplemental data for this article can be accessed here.

(ISCT), ≥95% of the MSC population must express CD105, CD73 and CD90, as measured by flow cytometry. Additionally, these cells must lack expression (≤ 2% positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19 and HLA class II [6].

STRO-1 is another useful MSC marker that distinguishes only a subpopulation of MSCs that show characteristics like high clonogenicity, multipotency, differentiation to fibroblasts, smooth muscle cells, adipocytes, osteoblasts and chondrocytes [7] and expression of telomerase [8]. STRO-1(+) populations consist of all detectable clonogenic CFU-Fs [8]. Also, it was reported that the level of STRO-1 expression in MSCs was strongly correlated with mRNA expression of transcription factors that are associated with immature stem cell phenotype and play roles in the regulation of proliferation and differentiation [9]. It was also reported that STRO-1(+) cells in human bone marrow stem cells are capable of differentiation into functional osteoblasts and that osteoprogenitors are present in the STRO-1(+) population [10]. In this study, TGSCs were isolated from porcine third molars and selected for their STRO-1 expression. Osteogenic potential of STRO-1-positive (STRO-1(+)) pTGSCs were compared with STRO-1-negative (STRO-1(−)) and unsorted (US) heterogeneous cells for the first time in the literature.

Materials and methods

Isolation of stem cells from porcine tooth germ (pTGSCs)

The study was approved by Local Animal Committee of the Government of Middle Franconia, Ansbach, Germany. Adequate measures were taken to minimize pain or discomfort. Mandibular third molar tooth germs were surgically removed from 6-month-old porcine jaws ($n = 5$) under I.V. anesthesia using Ketamine HCL (Ketavet, Ratiopharm, Germany). After elevation of a full-thickness flap, mandibular bone tissue over the tooth germ (Figure 1(a)) 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 (Figure 1(b)), including the dental mesenchyme residing in the developing crown and its surrounding follicle, was subjected to mechanical disruption. The small tissue pieces were transferred into six-well tissue culture plates (TCP) (Figure 1(c)). The culture medium was changed every other day. Approximately, after 5–7 days of incubation at 37 °C in a humidified atmosphere of 5% CO_2 in the incubator, the cells were subcultured using TrypLE™ Express trypsin replacement enzyme (Gibco, Carlsbad, CA).

Flow-cytometry analysis

The immunophenotypic antigens of pTGSCs (passage P1 and P5) were characterized by flow-cytometry analysis. Briefly, cells were trypsinized and incubated with monoclonal PE-conjugated CD105, FITC-conjugated CD90, CD45 (BD Pharmingen, Franklin Lakes, NJ), and CD44 (Abcam, England), PE-Cy5 conjugated CD34 (BD Pharmingen) antibodies for 1 h at 4 °C. Excess antibodies were discarded by washing and centrifugation. Cells were then resuspended in 400 µL PBS and the flow-cytometry analysis of 1×10^4 cells was carried out using the BD FACSCalibur flow cytometry system (BD Biosciences, Franklin Lakes, NJ). The data were analysed using Cell Quest software (Becton Dickinson).

Sorting of STRO-1(+) and STRO-1(−) cells

Passage 2 pTGSCs (1×10^7 cells) were incubated with unconjugated STRO-1 monoclonal antibody (R&D Systems) for 1 h at 4 °C. After washing and centrifugation steps, the samples were incubated with phycoerythrin (PE)-conjugated goat anti-mouse IgG antibody (R&D Systems, Minneapolis, MN) for 1 h at 4 °C. Cells were then washed for the removal of excess antibodies, resuspended in 5% FBS–PBS solution and kept on ice until sorting. Fluorescence emitting cells were sorted as STRO-1(+) and the cells that did not emit fluorescence were sorted as STRO-1(−) into 10% FBS and 100 u/mL PSA containing DMEM by FACS Aria III flow-cytometry system (BD Biosciences). STRO-1(+) and STRO-1(−) cells were collected in separate tubes and efficiency analysis of sorting experiment was carried out. STRO-1(+) cells were cultured in T75 flasks and expanded for approximately 10 days until they reached confluency. Unsorted (US) cells were used as controls. When they reached confluency, cells were trypsinized and seeded into TCP for further experiments. Remaining cells were labeled by STRO-1 antibody using the above protocol and checked for STRO-1 expression at the time of seeding.

Characterization of sorted and unsorted cells by osteogenic, chondrogenic and adipogenic differentiation

For osteogenic, adipogenic and chondrogenic differentiation, on the third day of the incubation after seeding the cells, medium was replaced with relevant differentiation medium. The cells were incubated for 21 days in relevant differentiation medium and the medium was changed twice a week. On days 7, 14 and 21, cells were fixed with 3.7%

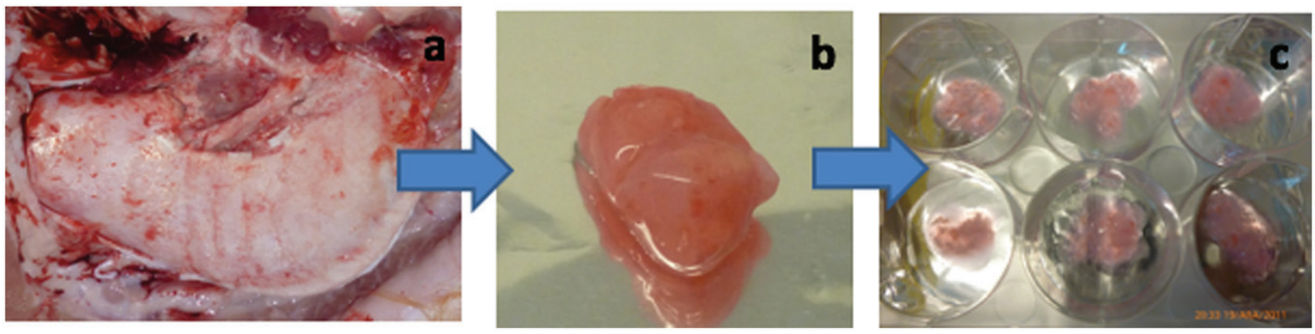

Figure 1. Isolation of porcine tooth germ stem cells. (a) mandibular third molars that tooth germs (b) are isolated from. (c) minced tooth germ tissue on TCP.

formaldehyde and stained with Alizarin Red to assess mineralization, Alcian Blue to assess the presence of acidic polysaccharides such as glycosaminoglycan and mucopolysaccharides in cartilage specific ECM, and Oil Red O to assess the presence of oil droplets accumulated by adipogenic cells (see Supplementary data for detailed procedure of differentiation medium and stainings).

Determination of clonogenic and osteogenic capacity of pTGSCs

P2 (after sorting) STRO-1(+), STRO-1(−) and US cells were counted and cultured in tissue culture plates at a concentration of 3000 cells/cm^2 in growth medium. For confocal microscopy studies, cells were cultured on positively charged microscope slides (KGW-Isotherm, Germany) with the same conditions. On the third day of the incubation, medium of the half of the plates was replaced with the osteogenic medium. This group was called as osteogenic medium containing (OM) group and other half of the plates were continued with the growth medium as controls and called growth medium containing (GM) group. Cells were incubated for 21 days and the medium was changed twice a week.

Determination of cell proliferation by MTS assay

On 1, 4, 7, 14 and 21 days of incubation, GM and OM groups of STRO-1(+), STRO-1(−) and unsorted pTGSCs were analysed for cell proliferation by CellTiter 96®AQueous One Solution Cell Proliferation Assay (MTS) assay. MTS reagent was added to the medium of each well (1:5) of the 12-well plate and incubated for 2 h at 37 °C in a CO_2 incubator. Absorbance was determined at 490 nm using an ELISA Plate Reader (BIO-TEK, Elx800, Winooski, VT).

Determination of alkaline phosphatase activity

pTGSCs were analysed for alkaline phosphatase (ALP) activity after 1, 4, 7, 14 and 21 days of incubation. Cells were first lysed with Tris buffer (0.1 M, pH 9.0) (Sigma-Aldrich, Germany) containing 0.01% Triton® X-100 (Sigma-Aldrich, Germany). Cell lysates were subjected to three successive freeze-thaw cycles and then sonicated for 10 min on ice. Each sample (100 μL) was mixed with 20 μL of p-nitrophenyl phosphate solution supplied by ALP kit (RANDOX Laboratories, Ireland). Absorbances of p-nitrophenol products were measured at 405 nm by ELISA Plate Reader (BIO-TEK, Elx800, Winooski, VT). Using a calibration curve of known concentrations of *p*-nitrophenol (Sigma-Aldrich, St. Louis, MO), ALP activity was calculated in μmol/min. Total protein concentration of the samples was determined using Smart Micro BCA Protein Assay kit (Intron Biotechnology, South Korea). ALP activity values were normalized according to calculated protein concentrations.

Determination of calcium deposition

Calcium content of the cultures was examined by QuantiChrom™ Calcium Assay Kit (Bioassay Systems, Hayward, CA) after 7, 14 and 21 days of incubation. Tissue culture wells were washed twice with PBS and incubated overnight with 200 μL 0.6 N HCl for decalcification. Following day, the cell layers were scraped from the well surfaces and collected in eppendorf tubes. For analysis, kit's instructions were followed, and then, absorbance was recorded at 612 nm in an ELISA plate reader. Ca concentrations of wells were calculated using a Ca calibration curve constructed with standards and then normalized using protein concentrations obtained from the same samples.

Von kossa staining of osteogenically induced pTGSCs

Mineralized nodules in cultures were assessed with von Kossa staining after fixation with 3.7% (w/v) formaldehyde for 45 min. Briefly, 5% silver nitrate solution was added on the cells after fixation, and the cells were exposed to UV for 30 min. Reaction was stopped by the addition of 5% sodium thiosulfate solution. Change in color in the wells was photographed and brightfield (BF) microscopy images of the mineralized bodies were obtained through inverted microscope (Nikon, Eclipse TC100, Japan).

Immunocytochemistry

At days 7, 14 and 21, cells were fixed and incubated in blocking solution. Rabbit anti-human collagen type I (Coll) (Abcam, United Kingdom) and mouse anti-human osteocalcin (OC) (Abcam, United Kingdom) antibodies were added in 1:500 ratio and incubated overnight at 4 °C. Next day, anti-rabbit and anti-mouse secondary antibodies were added in 1:500 ratio and incubated on the cells overnight at 4 °C. As a counter stain, DAPI was used (1:1000) and incubated for 15 min at RT. Antibody tagged cells were analyzed using Carl Zeiss SM 700 confocal microscopy under 20X objective.

q-PCR of osteogenic mRNAs

Total RNA content of the cells was isolated using Roche High Pure RNA isolation kit (Roche, Germany). mRNA was converted to single-strand cDNA using oligo(dT) primers with Sensiscript Reverse Transcription Kit (Qiagen, the Netherlands). Real-time PCR experiments were performed using Maxima SYBR Green Master Mix (Thermo Scientific, Waltham, MA) and reaction was carried out in CFX96 Touch™ Real Time PCR Detection System (Bio-Rad, Hercules, CA). Primer sequences for porcine mRNAs of housekeeping gene β-actin, and osteogenic genes Runx2, ALP, Coll, OC, Osteonectin (ON), Osteopontin (OP) are shown in Table 1.

Table 1. Sequence, product size and annealing temperature (Tm) of primers specific to osteogenic mRNAs.

	Primer sequence (5′->3′)	Product size (bp)	T_m (°C)
OC	F: CCTAGTGGTGCGGATTCTGG	241	58.0
	R: GCTGCGAGGTCTAGGCTATG		58.0
B-actin	F: GACTTCGAGCAGGAGATGG	232	58.0
	R: GCACCGTGTTGGCGTAGAG		58.0
ALP	F: CGACAACTACCAGGCACAGT	230	58.0
	R: GCCCTCAGAACAAGATGCCT		58.0
ON	F: GGATCTTCTTCCTCCTTTGCC	316	58.0
	R: CATACCTTCTCAAACTCGCCG		58.0
Runx2	F: ACTGAACCCCACGCTTGTTC	253	58.0
	R: AGTCACCTCCGCTTTCAAGG		58.0
OP	F: AGTCCAACGAAAGCCCTGAG	292	58.0
	R: GCTTCGGATCTGCGGAACTT		58.0
Coll	F: CCAGCCGCAAAGAGTCTACA	251	58.0
	R: AACACATTGCCGTTGTCGC		58.0

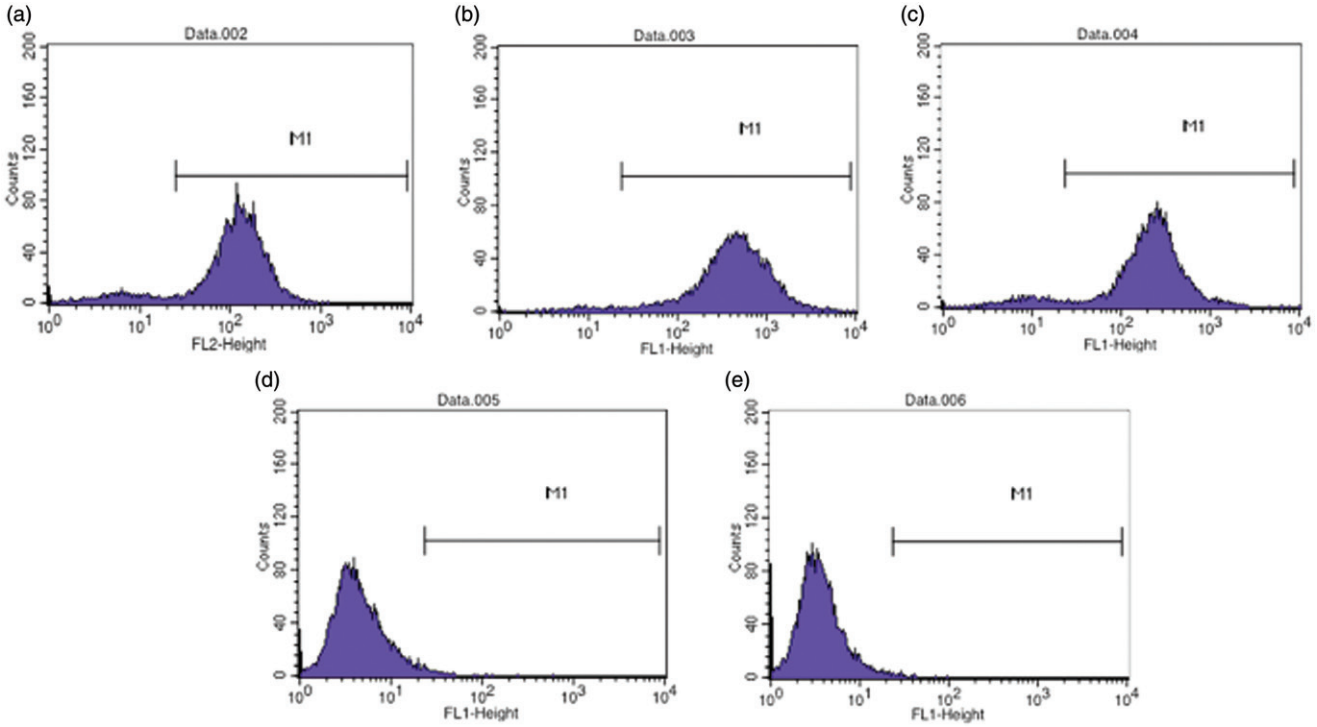

Figure 2. Flow cytometry histogram of (a) CD105, (b) CD90, (c) CD44, (d) CD45 and (e) CD34-labeled P1 pTGSCs (obtained by FACSCalibur).

ELISA assay of osteogenic proteins

Total protein content of the cells was isolated using RIPA lysis buffer (supplied with PMSF, sodium orthovanadate and protease inhibitor cocktail) (SantaCruz, Dallas, TX). Cell lysate was collected in microcentrifuge tubes, sonicated for 1 min on ice and centrifuged at 10,000 *g* for 10 min. Supernatant was used for the determination of the amount of osteogenic proteins Runx2, Coll and OC by ELISA assay (BlueGene Biotech, China) using manufacturer's instructions. Osteogenic protein amounts in samples were normalized using total protein concentrations.

Statistical analysis

Data were presented as the mean ± standard deviation resulting from independent experiments. The statistical data were analysed using software IBM SPSS Statistics 22 (IBM SPSS, Turkey). Kruskal–Wallis test was used to compare the parameters between test groups and the Mann–Whitney *U*-test was used to evaluate the discrepant group. Differences were considered significant when $p < .05$.

Results

Flow cytometry analysis of pTGSCs

The flow cytometry of pTGSCs indicated that the cells were positive for mesenchymal stem cell markers CD105, CD90 and CD 44 (Figure 2(a,b,c)) and negative for the hematopoietic stem cell markers CD45 and CD34 (Figure 2(d,e)), and even after passage 1, cross-contamination by other cell types due to explant culture did not occur. Throughout five passages, cells still expressed CD105, CD90 and CD44 and did not express CD34 and CD 45. CD105 expression decreased at passage 5 (Table 2).

Table 2. Percent positiveness of surface antigens after 1 and 5 passages of pTGSCs.

Surface markers	Passage 1	Passage 5
CD105	92.58	75.10
CD90	98.14	98.57
CD45	1.02	1.25
CD44	93.33	97.86
CD34	0.36	1.55

STRO-1 sorting

STRO-1(+) cell fraction from heterogenous mixture of pTGSCs was sorted by FACS Aria. Fraction of cells expressing STRO-1 was 40.9% (Q4 region in Figure 3(a)). Of those, 27.4% was sorted as STRO-1(+) (P2 region shown in green in Figure 3(a)) and 31.7% of the cells (P3 region shown in blue in Figure 3(a)) was sorted as STRO-1(−). pTGSCs (1×10^4) were run in FACS Aria to test the efficiency of sorting. It was >90% each time (data not shown). In order to reach the desired number of sorted cells, two more passages were performed and the effect of culture time and passaging to cells' STRO-1 characteristics was assessed by flow cytometry prior to cell seeding for experiments. It was shown that ~90% of cells that were sorted as STRO-1(+) were still STRO-1(+) (Figure 3(b)) and cells that were sorted as STRO-1(−) were still STRO-1(−) (Figure 3(c)).

Osteogenic, chondrogenic and adipogenic differentiation

Differentiation of pTGSCs towards osteoblastic lineages was assessed by alizarin red staining. Mineralization was observed even after 7 days of incubation with the positive red staining on the wells but significantly increased after 14 and 21 days of osteogenic induction (Supplementary Figure 1). ECM mineralization by osteoblastic cells was observed macroscopically

on the wells (Supplementary Figure 1) and microscopically under the light microscope (Figure 4(a)). According to the quantification of alizarin red-stained regions (Figure 4(b)), mineralization significantly increased in STRO-1(+), STRO-1(−) and US cell groups throughout 21 days of osteogenic induction, being significantly higher in STRO-1(+) cells after 21 days.

Differentiation of pTGSCs towards chondrogenic cells was assessed by alcian blue staining. After 21 days of incubation, positive staining of chondrogenic ECM was clearly visible in all cell groups by the cyan-colored staining in the culture (Supplementary Figure 2). Also, cell aggregation and condensation was observed during chondrogenesis (Figure 4(a)).

Differentiation of pTGSCs towards adipogenic cells was assessed by oil red staining. Lipid droplets appeared in the cells after 7 days of incubation and observed as bright red staining of circular lipid droplets located in the cells (Supplementary Figure 3). After 14 and 21 days of incubation,

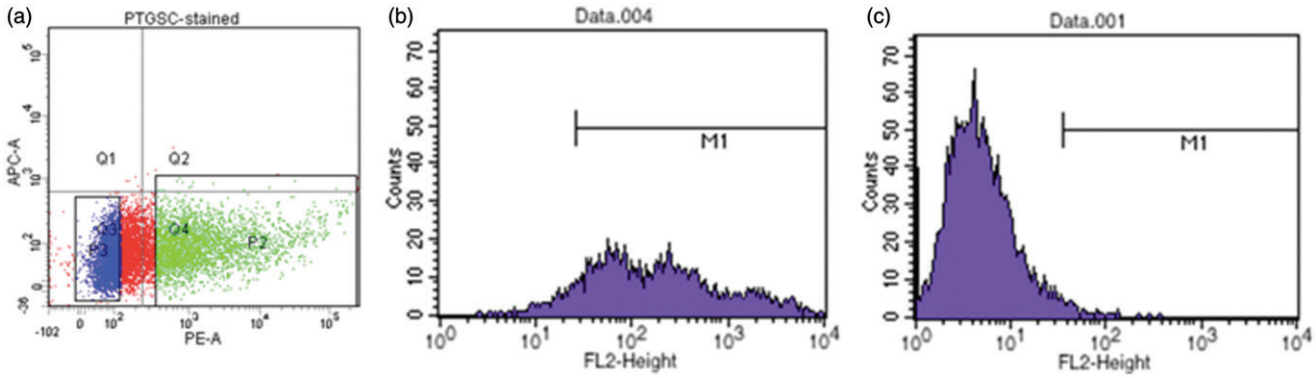

Figure 3. (a) Histogram of PE-STRO-1 antibody labeled cells obtained by FACS Aria: Dots in Q4 region represents STRO-1(+) cells, dots in Q3 region represents STRO-1(−) cells. Cells in P2 region were sorted as STRO-1(+) and cells in P3 region were sorted as STRO-1(−). Verification of STRO-1 phenotype of cells before seeding: b) STRO-1(+) cells, (c) STRO-1(−) cells.

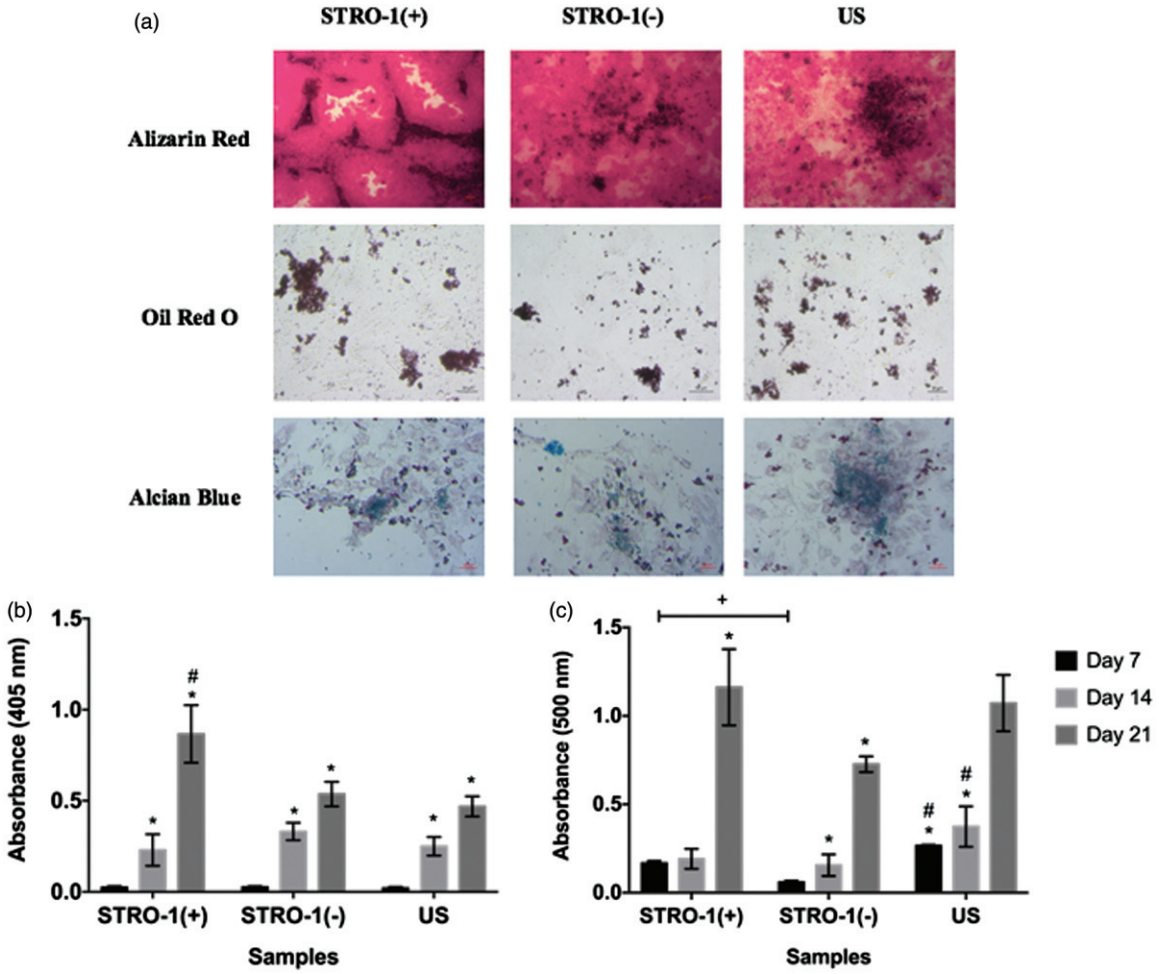

Figure 4. Osteogenic, adipogenic and chondrogenic differentiation of pTGSCs; (a) Microscopic observation of Alizarin Red (Scale bars show 100 μm), Oil Red O (Scale bars show 20 μm) and Alcian Blue (Scale bars show 50 μm) stained pTGSCs under BF) microscope after 21 days in differentiation medium, (b) Quantification of alizarin red staining, (c) Quantification of oil red O staining.

lipid droplets accumulated in and around adipogenic cells and appeared as dark red (Supplementary Figure 3). After 7 days of incubation in adipogenic medium, both STRO-1(+) and US cells accumulated significantly higher amount of lipid droplets than STRO-1(−) cells (Figure 4(c)). After 14 days, lipid accumulation by US cells was significantly higher than the other groups. However, after 21 days of incubation, oil red staining was significantly increased in all samples without any significant differences among the groups.

Cell proliferation

Increase in the cell number up to 14 days was observed in all groups (Figure 5). After 14 days, a decrease in the cell number of STRO-1(+) OM, STRO-1(−) GM and OM was observed possibly due to limited area available for cells on TCP. Proliferation of cells was the highest in STRO-1(+) cells after day 1, 4, 7 and 21 days of incubation in GM and also in OM 4, 7 and 21 days. Osteogenic medium addition did not significantly suppress the cell proliferation of STRO-1(+) and STRO-1(−) groups; however, cell proliferation was suppressed by OM addiditon to US groups. STRO-1(+) cells formed colonies and become confluent when those colonies merged, unlike STRO-1(−) and US pTGSCs which proliferated normally. Growth curve of cells also confirmed the results obtained by MTS assay (Supplementary Figure 4).

ALP activity

ALP activity was measured using *p*-nitrophenylphosphate which is the substrate of ALP enzyme and converted into *p*-nitrophenol in the presence of ALP. *p*-Nitrophenol is a yellow colored product of which absorbance at 405 nm is directly proportional to the amount of ALP enzyme produced by cells. All groups of pTGSCs synthesized high amounts of ALP (in μmol scale) and addition of OM did not increase ALP synthesis significantly. At day 4, STRO-1(+) GM had higher ALP activity compared to STRO-1(−) GM group. At days 4 and 14, ALP activity of STRO-1(+) OM group was significantly higher than STRO-1(−) OM group. At day 4, US GM group had the highest ALP activity compared to other GM groups. At days 4, 7 and 14, US OM group had the highest ALP activity compared to other OM groups. US OM group expressed the highest ALP activity at day 7 decreasing through day 14 and 21 (Figure 6).

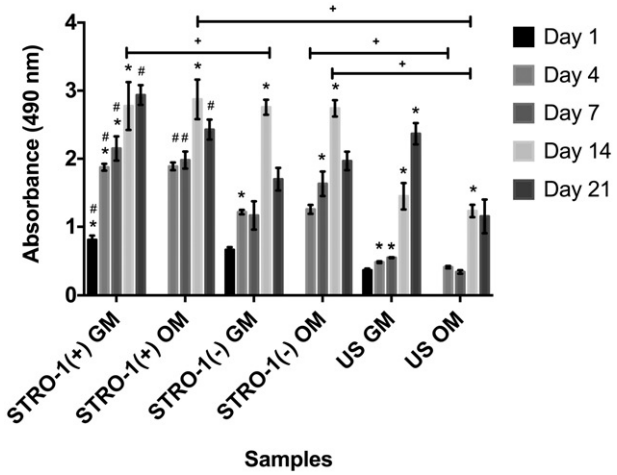

Figure 5. Cell proliferation of GM or OM added STRO-1(+), STRO-1(−) and US pTGSCs throughout 21 days of incubation. * shows the significant difference among days of the same group, # shows the significant difference among the same days of all cell types' GM or OM groups, + shows the significance among the indicated samples ($p < .05$).

Mineralization

Mineralization is a late marker and the final stage of osteogenesis. Osteoblasts deposit minerals in the form of calcium phosphate nodules. Deposited calcium (Ca) can be quantified or stained with von Kossa or alizarin red stains. A positive staining (brown) of mineralized nodules by von Kossa was observed in all OM groups beginning from 14 days of incubation (Figures 7(a) and 8). After 21 days, all groups mineralized their ECM as observed by dark black von Kossa staining of wells. Positive staining was not detected in GM groups both macroscopically (Figure 7(a)) and microscopically (Figure 8). Ca deposited by the cells was quantified by the formation of a stable blue-colored product (OD = 612 nm) when Ca reacts with phenolsulphonephthalein dye. It was also confirmed by Ca quantification that the cells could respond to OM by mineralizing their ECM especially after 21 days of incubation with a significant increase in Ca concentration by all OM groups compared to 7 and 14 days of incubation periods (Figure 7(b)). After 21 days, STRO-1(+) OM group had significantly the highest Ca concentration among other OM groups.

Immunocytochemistry

Coll was synthesized throughout 21 days of incubation in GM groups and amount of Coll increased due to the increase in cell number in this time interval (Figure 9). Coll synthesis also increased in OM groups due to both the increase in cell

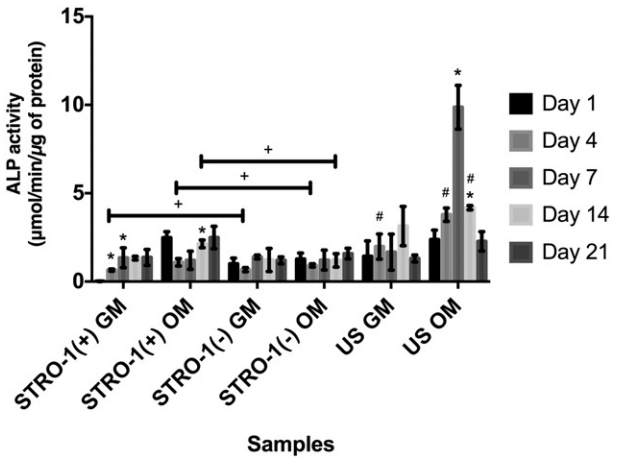

Figure 6. ALP activity of GM or OM added STRO-1(+), STRO-1(−) and US pTGSCs throughout 21 days of incubation. ALP activity was measured by OD measurements of *p*-nitrophenol at 405 nm and using a calibration curve of known *p*-nitrophenol concentrations. Values were then normalized by total protein concentrations. * shows the significant difference among days of the same group, # shows the significant difference among the same days of all cell types' GM or OM groups, + shows the significance among the indicated samples ($p < .05$).

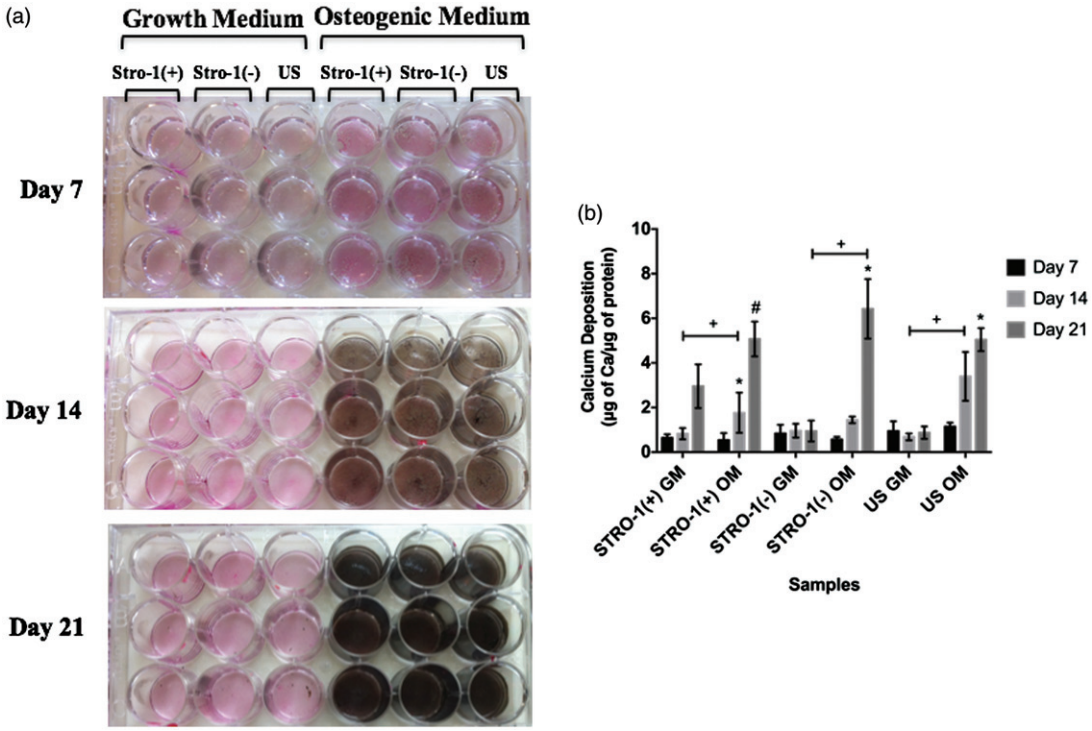

Figure 7. Mineralization of osteogenically induced GM or OM added STRO-1(+), STRO-1(−) and US pTGSCs throughout 21 days of incubation. (a) Macroscopic observation of von Kossa-stained wells, (b) Quantification of Ca deposited by cells. Ca concentration was measured by OD measurements at 612 nm and using a calibration curve of known Ca concentrations. Values were then normalized by total protein concentrations. * shows the significant difference among days of the same group, # shows the significant difference among the same days of all cell types' GM or OM groups, + shows the significance among the indicated samples ($p < .05$).

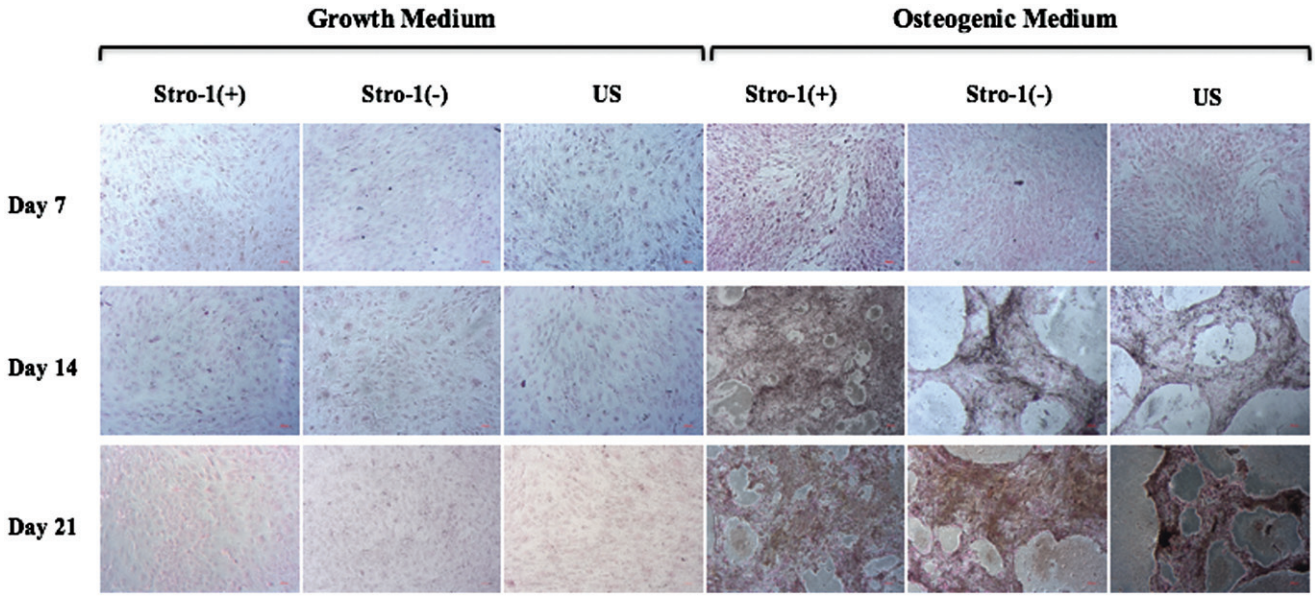

Figure 8. Microscopic observation of mineralized nodules in osteogenically induced GM or OM added STRO-1(+), STRO-1(−) and US pTGSCs cultures throughout 21 days of incubation under BF microscope. Scale bars show 100 µm.

number and differentiation of cells towards osteoblasts. OC increased in OM groups throughout 21 days due to their differentiation to osteoblasts. OC expression was higher in OM groups even after 7 days of incubation and seems to be higher in STRO-1(+) OM group after 21 days of incubation. Beginning from day 7 through days 14 and 21, OM groups exhibited a more organized morphology compared to monolayer appearance of GM groups. Besides, ECM of OM groups were more prominent and dense.

q-PCR of osteogenic mRNAs

Osteogenic differentiation was analysed at mRNA level by assessing expression levels of osteogenic genes. Expression levels were normalized using β-actin as housekeeping gene. Runx2 expression increased in OM groups and was significantly higher in STRO-1(+) OM group at 7 day samples (Figure 10(c)). Runx2 expression of STRO-1(+) pTGSCs decreased at 14 days of incubation and then increased again

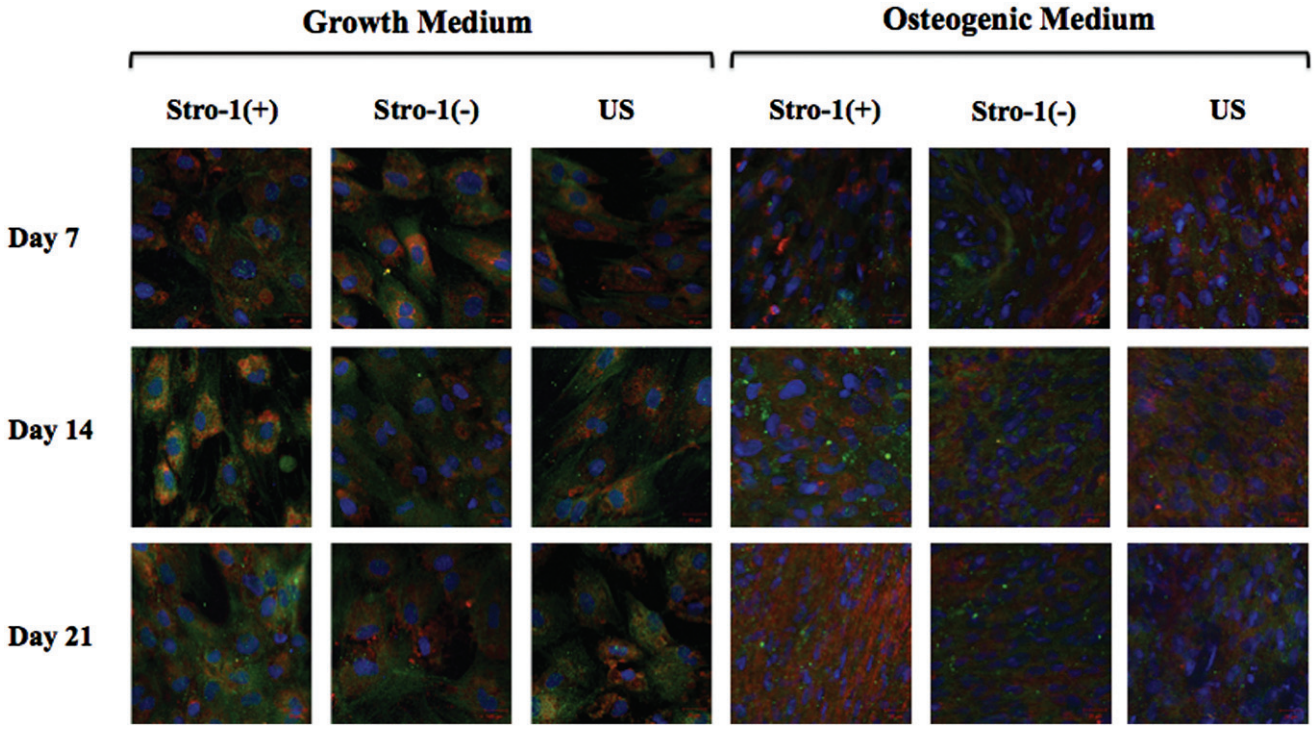

Figure 9. Confocal microscopy images of STRO-1(+), STRO-1(−) and US pTGSCs after 7, 14 and 21 days of incubation in GM or OM (20X objective). Scale bars show 20 μm. Osteocalcin (red), collagen type I (green).

at 21 days being significantly higher than STRO-1(−) and US pTGSCs. Cells responded to OM by increasing their OP expression and this expression peaked at 14 days. OP expression was significantly higher in STRO-1(−) OM group compared to STRO-1(+) OM group and significantly the highest in US OM group at 14 days compared to other groups (Figure 10(e)). Cells also responded OM by increasing OC expression and this expression was significantly higher in STRO-1(+) OM group at 7 and 21 days whereas at 14 days US OM expressed significantly the highest OC (Figure 10(d)). Coll (Figure 10(a)) and ON (Figure 10(b)) expressions were also detectable in all groups at every time point, whereas there was no significant difference among groups according to their STRO-1 expression or addition of GM or OM.

ELISA assay of osteogenic proteins

Osteogenic differentiation of pTGSCs was also analysed by assessing osteogenic protein levels by ELISA assay. Protein levels were normalized using total protein concentration of the samples. Collagen type I and OC was synthesized in all samples, but its synthesis decreased throughout 21 days of incubation in GM groups (Figure 11(a,c)). Coll and OC synthesis peaked at day 14 samples in all OM groups. Coll synthesis was significantly higher in STRO-1(+) OM group after 14 days of incubation compared to other groups. There was a decrease in synthesis of ECM proteins, Coll and OC, in all groups throughout 21 days of incubation since the cultures were confluent and cells slowed down their metabolism. Cells also responded to OM by increasing Runx2 protein synthesis throughout 21 days of incubation especially in OM groups. STRO-1(+) OM groups synthesized significantly higher Runx2 compared to other cell groups after 21 days of incubation (Figure 11(b)).

Discussion

Stem cells can be isolated from a variety of dental tissues including apical papilla of developing teeth (SCAP) and dental follicle (DFSCs). DFSCs and SCAP are generally cultured by dissecting dental follicle and apical papilla of immature tooth seperately. However, the use of whole tooth germ including dental follicle and its surrounding tissues is an alternative strategy which make use of reciprocal interactions between stem cells of ectoderm and mesoderm origin in order to preserve their stemness [11].

Domestic pig was chosen for the isolation of dental germ stem cells in this study for its anatomical, physiological and metabolical similarities with humans. Also, the similarities in dentition of the pig to that of humans make it a good candidate to study dental stem cell-mediated tissue engineering. Bone structure, mechanics and animal size are also important for the choice of pig to study bone tissue engineering.

Tooth germ stem cells isolated from porcine third molars and their characterization and differentiation depending on their STRO-1 expression were first reported in detail in this paper. pTGSCs, which were isolated for this study, were also used for bone tissue engineering on fibronection or laminin-modified poly(butylene succinate) (PBS) scaffolds simultaneously with this study [12].

Since MSCs are heterogenous in nature in terms of their proliferation and differentiation characteristics, researchers work on a cell-surface antigen profile to better identify and purify MSCs in a heterogeneous mixture. It was thought that a homogeneous source of stem cells might provide a better

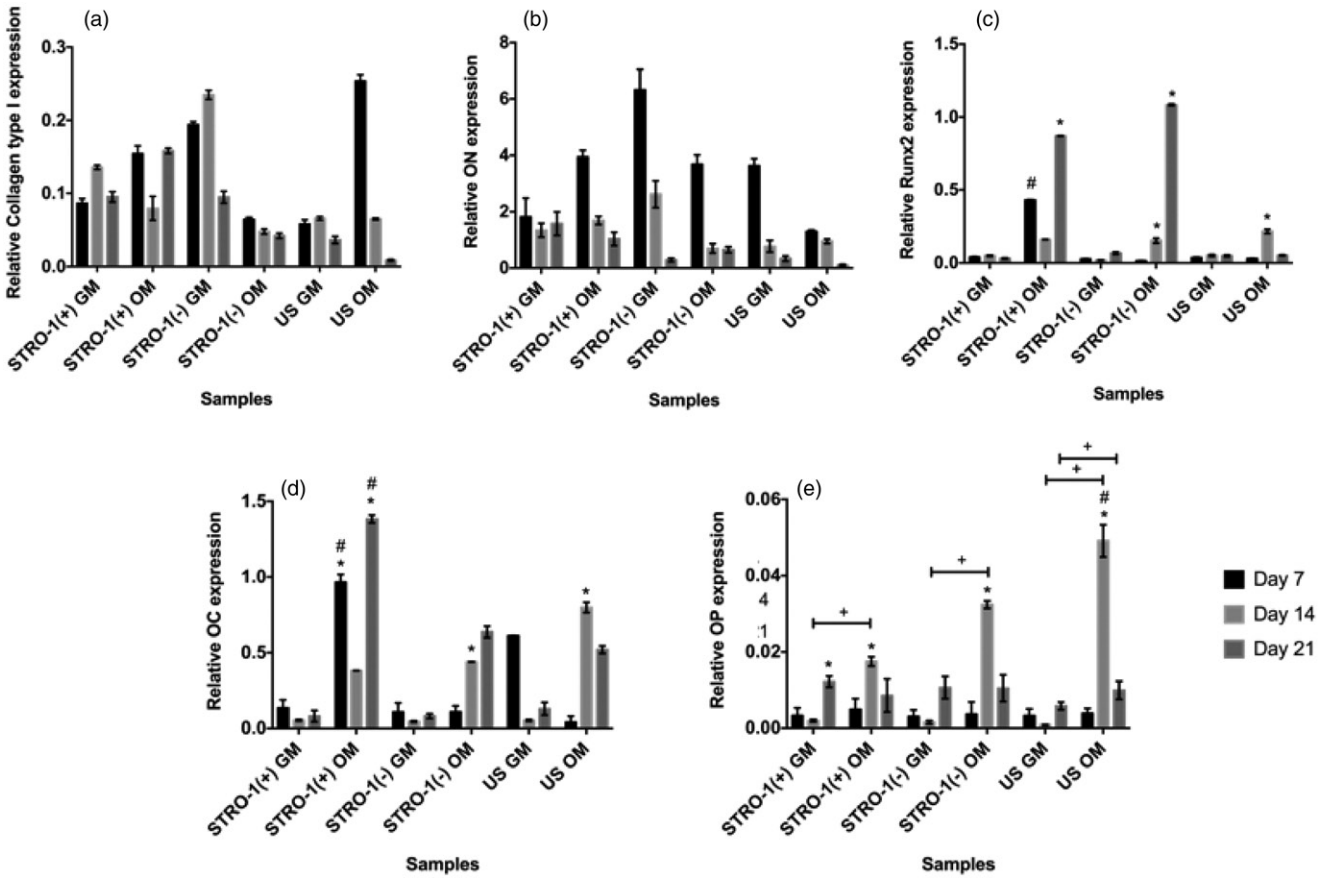

Figure 10. Analysis of osteogenic gene expression of STRO-1(+), STRO-1(−) and US pTGSCs by qPCR after 7, 14 and 21 days of incubation in GM or OM: (a) Collagen Type I (b) ON, (c) Runx2, (d) OC, (e) OP. * shows the significant difference among days of the same group, # shows the significant difference among the same days of all cell types' GM or OM groups, + shows the significance among the indicated samples ($p < .05$).

source for therapeutic purposes compared to unsorted heterogeneous cells. MSCs share a common surface marker expression profile, whereas STRO-1, a widely used MSC surface marker, distinguishes only a subpopulation of mesenchymal stem cells that are unique in terms of adherence, proliferation and multilineage differentiation potential [13]. pTGSCs exhibited the common surface marker profile of MSCs and kept their surface marker expression profile up to passage 5 without any significant differences in percentages of markers' expressions except CD105 whose expression decreased with passaging as previously reported for human MSCs elsewhere [14]. pTGSCs were found to contain ∼40% STRO-1(+) cells, although it was reported that the STRO-1 antigen is expressed on the surface of approximately 10–20% of adult human BM [9], 0.02–9.56% of human DPSCs [15], 6.7–20% of human PDLSCs [16], 9% of SHEDs [17], 10.9% of stem cells from miniature pig deciduous teeth (SPDs) [18], 18.2% of miniature pig PDLSCs [19].

It was shown in this study that STRO-1(+), STRO-1(−) and US pTGSCs could differentiate into osteoblasts, chondroblasts and adipocytes verifying their MSC characteristics besides their similar surface marker expression profile.

STRO-1(+) and STRO-1(−) cells require *in vitro* expansion after sorting to obtain adequate number of cells for the experiments. Thus, cells were cultured and passaged when they became confluent. However, it was previously reported that STRO-1 expression may gradually be lost during culture time and as the passage number of cells increases [15,20–23]. STRO-1 phenotype of expanded cells were verified for STRO-1(+) and STRO-1(−) cells by running through flow cytometry just before cell seeding for the experiments. STRO-1 phenotype was verified being ≥90% for STRO-1(+) pTGSCs and negative for STRO-1(−) pTGSCs.

From a practical point of view, although the possible necessity of STRO-1 sorting of MSCs for bone tissue engineering applications was previously reported [7,10,24], STRO-1 selection and expansion procedure after the selection are time- and money-consuming. Thus, it is useful to compare STRO-1(+), STRO-1(−) and unsorted cells in terms of their proliferative and osteogenic differentiation characteristics in order to decide whether it is worth to select for bone tissue engineering applications [22].

According to cell proliferation and growth curve analysis, STRO-1(+) cells were more clonogenic and had higher proliferation rates and colony-forming abilities compared to STRO-1(−) cells or parental unsorted cells as several other studies previously reported [8,9]. Proliferation rate of STRO-1(+) pTGSCs were higher at almost all time points and was not affected by OM addition which normally suppresses proliferation of stem cells due to differentiation. The reason for this could be the presence of osteoprogenitors in STRO-1(+) pTGSCs.

Differentiation of MSCs to osteoblast lineages can be analysed by the expression of bone-specific biomarkers. These

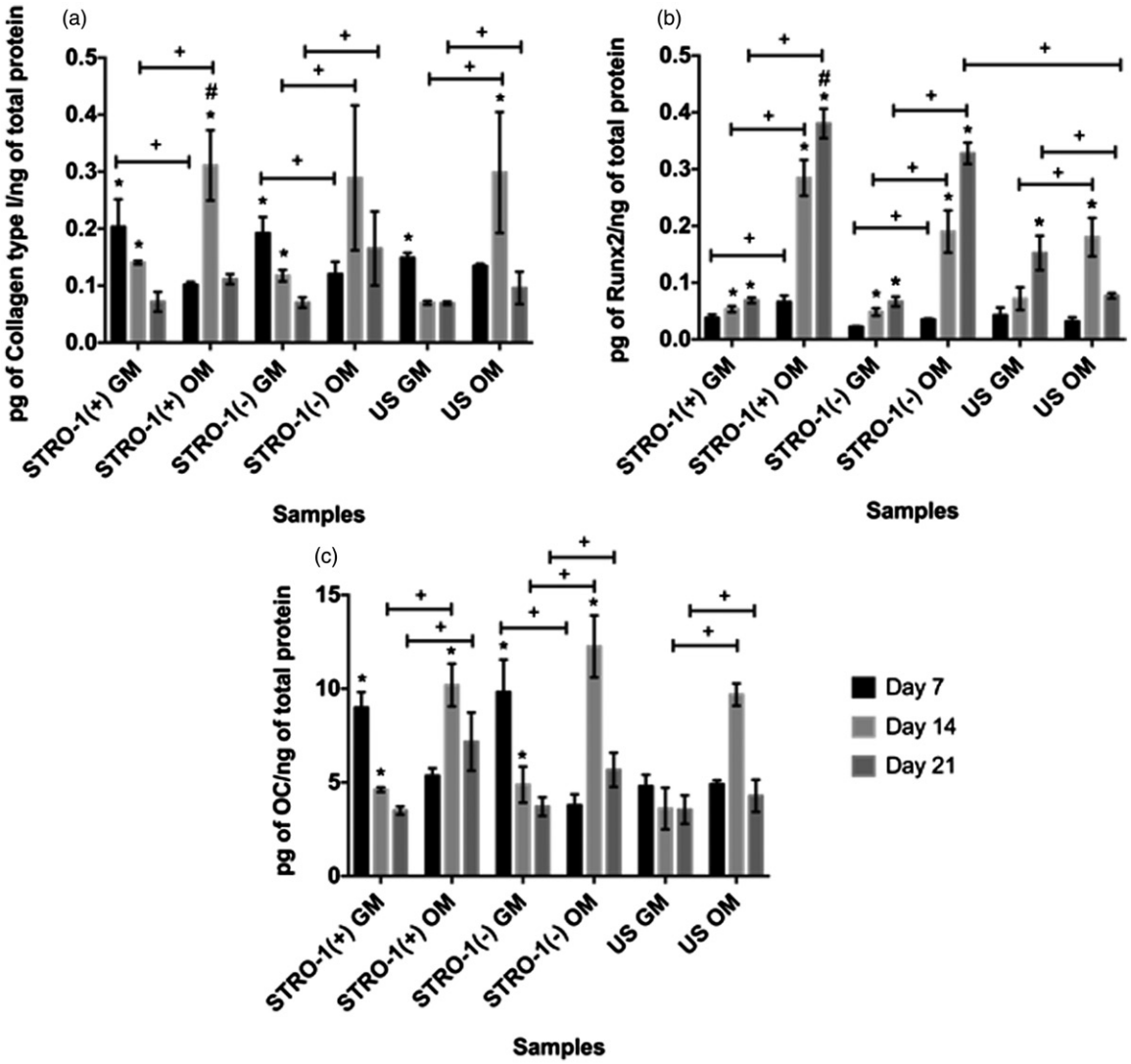

Figure 11. Analysis of osteogenic protein synthesis of STRO-1(+), STRO-1(−) and US pTGSCs by Elisa Assay after 7, 14 and 21 days of incubation in GM or OM: (a) Collagen type I, (b) Runx2, (c) OC. * shows the significant difference among days of the same group, # shows the significant difference among the same days of all cell types' GM or OM groups, + shows the significance among the indicated samples ($p < .05$).

biomarkers include transcription factors, enzymes and ECM components. Runx2, a key transcription factor in skeletal development, is expressed early in cells which are destined to be osteoblasts. Runx2 mRNA level at day 7 was significantly higher in STRO-1(+) OM group indicating that STRO-1(+) cells were more readily committed to osteogenic differentiation compared to STRO-1(−) and US pTGSCs. Runx2 protein expression was significantly upregulated by all cell types with the addition of OM, being significantly higher in STRO-1(+) OM after 21 days.

Bone ECM is important for bone since organic and inorganic matrix components are responsible from its unique compression and tensile characteristics. Collagen type I (Coll), the major constituent of bone organic matrix, was expressed in all groups of cells in both GM and OM as confirmed by mRNA and protein expression.

Maturation of bone matrix is achieved by mineralization through deposition of $Ca_3(PO4)_2$ nodules and this process is regulated by ALP and non-collagenous ECM proteins such as OC, OP and ON [25]. OC, ON and OP have multiple Ca^{+2}-binding sites and they are the nucleator of bone minerals. ALP is an important early osteoblastic differentiation marker, showing the commitment of stem cells to become an osteoblast. It regulates mineralization process by producing free phosphate by the reduction of phosphate-containing substances for bone mineralization and hydrolyzing pyrophosphate (PPi) which is an inhibitor of hydroxyapatite formation [26]. All types of pTGSCs exhibited high ALP activity, in both GM and OM, showing the commitment of cells to become osteoblasts. ALP activity of US OM group decreased through 7 and 14 days of incubation after it peaked at 7 days of incubation since it is an early osteoblastic marker and it decreases when mineralization is initiated as also previously noticed by our group [27]. In STRO-1(+) group, pTGSCs could accumulate Ca^{2+} in detectable amounts at 21 days of incubation even with the addition of GM indicating the possible presence of osteoprogenitors in this group of cells. STRO-1(+) OM group accumulated significantly more amount of Ca^{2+} after 21 days of incubation.

Having roles in bone matrix mineralization as previously discussed earlier, ON, OC and OP are typical biomarkers used for the mature osteoblast phenotypic behavior [28]. OC

mRNA expression significantly upregulated by all groups as a response to OM supplements, whereas ON was only upregulated at early stage in STRO-1(+) and US OM groups and OP in STRO-1(+) OM group. OC protein synthesis, according to ELISA assay, peaked at 14 days of incubation with the addition of OM and was significantly higher at 14 and 21 days of incubation in all OM groups compared to GM groups.

Overall, in this study, STRO-1(+) cells were found clonogenic but did not perform obviously better in osteogenic conditions. In accordance with our studies, STRO-1(+) rat dental stem cells were compared with unsorted cells for their odontogenic capacity by Yang *et al.* (2007) [29] and STRO-1(+) cells did not exhibit a better performance compared to unsorted cells. Yan *et al.* (2014) [22] also compared STRO-1(+) and unsorted periodontal ligament stem cells and did not observe any superiority of STRO-1(+) fraction in terms of osteogenic differentiation. Besides, STRO-1(−) pTGSCs also differentiated towards osteogenic lineage as shown in this study by mineralization and expression of osteogenic proteins. Since STRO-1 surface marker distinguishes the cells with the immature stem cell phenotype, the remaining cells present in STRO-1(−) group might contain cells committed to osteogenic lineages. Thus, this situation may account for the comparable osteogenic differentiation of STRO-1(−) and US pTGSCS with STRO-1(+) pTGSCs. On the other hand, several studies showed the superior performance of STRO-1(+) fraction in osteogenic differentiation [10,23,30]. The reason for the differences observed by different researchers can be explained with differences in isolation methods, batch-to-batch variation, and the impurity of cell populations [23].

STRO-1 sorting of pTGSCs is not a very effective strategy to enhance their osteogenic capacity. It has been proposed earlier that cell to cell interactions can provide cells the cues required for stem cell state or differentiation [27]. Therefore, STRO-1(+) cells might require a heterogeous niche to perform their role in osteogenesis.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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References

[1] Montoro DT, Wan DC, Longaker MT. Skeletal tissue engineering. In: Lanza R, Langer R, Vacanti J, editors. Principles of tissue engineering. 4th ed. Amsterdam (The Netherlands): Elsevier; 2013. pp. 1289–1302.

[2] Kolf CM, Cho E, Tuan RS. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. Arthritis Res Ther. 2007;9:204.

[3] Lei M, Li K, Li B, et al. Mesenchymal stem cell characteristics of dental pulp and periodontal ligament stem cells after *in vivo* transplantation. Biomaterials. 2014;35:6332–6348.

[4] d'Aquino R, Papaccio G, Laino G, et al. Dental pulp stem cells: a promising tool for bone regeneration. Stem Cell Rev. 2008;4:21–26.

[5] Yalvaç ME, Ramazanoglu M, Tekguc M, et al. Human tooth germ stem cells preserve neuro-protective effects after long-term cryopreservation. CNR. 2010;7:49–58.

[6] Dominici M, Le Blanc K, Mueller I, et al. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8:315–317.

[7] Dennis JE, Carbillet JP, Caplan ACP. The STRO-1+ marrow cell population is multipotential. Cells Tissues Organs (Print). 2002;170:73–82.

[8] Gronthos S, Zannettino ACW, Hay SJ, et al. Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. J Cell Sci. 2003;116:1827–1835.

[9] Psaltis PJ, Paton S, See F, et al. Enrichment for STRO-1 expression enhances the cardiovascular paracrine activity of human bone marrow-derived mesenchymal cell populations. J Cell Physiol. 2010;223:530–540.

[10] Gronthos S, Graves SE, Ohta S, et al. The STRO-1+ fraction of adult human bone marrow contains the osteogenic precursors. Blood. 1994;84:4164–4173.

[11] Ramazanoglu M, Schlegel KA, Kose GT. Potential use of dental stem cells for craniofacial tissue regeneration. In: Turksen K, editor. Stem cells: current challenges and new directions. New York, NY: Springer New York; 2013. p. 105–124.

[12] Abay N, Pekozer GG, Ramazanoglu M, et al. Bone formation from porcine dental germ stem cells on surface modified polybutylene succinate scaffolds. 2016;2016. doi:10.1155/2016/8792191.

[13] Wang X, Wang Y, Dai X, et al. Effects of intermittent administration of parathyroid hormone (1-34) on bone differentiation in stromal precursor antigen-1 positive human periodontal ligament stem cells. Stem Cells Int. 2016;2016:1–9. Available from: http://www.hindawi.com/journals/sci/2016/4027542/

[14] Pogozhykh O, Pogozhykh D, Neehus AL, et al. Molecular and cellular characteristics of human and non-human primate multipotent stromal cells from the amnion and bone marrow during long term culture. Stem Cell Res Ther. Stem Ther. 2015;6:150.

[15] Werle SB, Chagastelles P, Pranke P, et al. The effects of hypoxia on in vitro culture of dental-derived stem cells. Arch Oral Biol. 2016;68:13–20.

[16] Iwata T, Yamato M, Zhang Z, et al. Validation of human periodontal ligament-derived cells as a reliable source for cytotherapeutic use. J Clin Periodontol. 2010;37:1088–1099.

[17] Miura M, Gronthos S, Zhao M, et al. SHED: stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci USA. 2003;100:5807–5812.

[18] Zheng Y, Liu Y, Zhang CM, et al. Stem cells from deciduous tooth repair mandibular defect in swine. J Dent Res. 2009;88:249–254.

[19] Ding G, Liu Y, Wang W, et al. Allogeneic periodontal ligament stem cell therapy for periodontitis in swine. Stem Cells. 2010;10:1829–1838.

[20] Matsubara T, Suardita K, Ishii M, et al. Alveolar bone marrow as a cell source for regenerative medicine: differences between alveolar and iliac bone marrow stromal cells. J Bone Miner Res. 2005;20:399–409.

[21] Itaya T, Kagami H, Okada K, et al. Characteristic changes of periodontal ligament-derived cells during passage. J Periodontal Res. 2009;44:425–433.

[22] Yan XZ, Both SK, Yang PS, et al. Human periodontal ligament derived progenitor cells: Effect of STRO-1 cell sorting and Wnt3a treatment on cell behavior. Biomed Res Int. 2014;2014. doi: 10.1155/2014/145423.

[23] El-Sayed KMF, Paris S, Graetz C, et al. Isolation and characterisation of human gingival margin-derived STRO-1/MACS(+) and MACS(-) cell populations. Int J Oral Sci. 2014;7:80–88.

[24] Gronthos S, Zannettino AC, Graves SE, et al. Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells. J Bone Miner Res. 1999;14:47–56.

[25] Clarke B. Normal bone anatomy and physiology. Clin J Am Soc Nephrol. 2008;3(Suppl 3):131–139.

[26] Sun L, Wu L, Bao C, et al. Gene expressions of collagen type I, ALP and BMP-4 in osteo-inductive BCP implants show similar pattern to that of natural healing bones. Mater Sci Eng C. 2009;29:1829–1834.

[27] Pekozer GG, Kose GT, Hasirci V. Influence of co-culture on osteogenesis and angiogenesis of bone marrow mesenchymal stem cells and aortic endothelial cells. Microvasc Res. 2016;108:1–9.

[28] Luz GM, Mano JF. Mineralized structures in nature: Examples and inspirations for the design of new composite materials and biomaterials. Compos Sci Technol. 2010;70:1777–1788.

[29] Yang X, van den Dolder J, Walboomers XF, et al. The odontogenic potential of STRO-1 sorted rat dental pulp stem cells in vitro. J Tissue Eng Regen Med. 2007;1:66–73.

[30] Yang X, Zhang W, van den Dolder J, et al. Multilineage potential of STRO-1+ rat dental pulp cells in vitro. J Tissue Eng Regen Med. 2007;1:128–135.