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A comparative study on different stemness gene expression between dental pulp stem cells *vs.* dental bud stem cells

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Abstract. – OBJECTIVE: The clinical use of mesenchymal stem cells (MSCs) in regenerative medicine either in tissue repair or tissue reconstruction has given highly interesting results thanks to their particular nature. Sources that have attracted the attention of medical scientists from where stem cells (SCs) in adults could be obtained are different and, dental tissues have certainly become an optimal source of MSCs. Dental tissue is a main reservoir of two types of MSCs dental bud (DBSCs) that constitute the immature precursor of the tooth and dental pulp (DPSCs) that are derived from dental inner pulp and partly from dental follicle tissue and can differentiate into several cell phenotypes as osteoblast, chondrocyte, hepatocytes, cardiomyocytes, neuron and β cells.

PATIENTS AND METHODS: Normal impacted third molars and tooth buds were collected from adults and adolescents underwent to extractions for orthodontic reasons. The expression of the five stemness genes Nanog, OCT4, Sox2, c-Myc and Klf4 were investigated by qRT-PCR in two different dental stem/progenitor cells: dental pulp stem cells (DPSCs) and stem cells from dental bud (DBSCs), differentiated toward osteoblastic phenotype and not.

RESULTS: Both DPSCs and DBSCs are easy to access and we found their expression of the typical mesenchymal stemness makers and osteogenic capacity due to the effective presence of embryonic gene regulators like Nanog, OCT4, Sox2, c-Myc and Klf4. Both DBSCs and DPSCs could represent a valid tool in regenerative medicine and translational applications.

CONCLUSIONS: The results depicted here provide, for the first time to our knowledge, a comparative outcome about the stemness properties generated from accessible tissues such as DPSCs and DBSCs. These two types of SCs showed few different distinctive genetic traits supposedly in relation to their origin, location and stage of maturation. Certainly these SCs reserve solid potential for human clinical application in autologous procedure for bone, hard tissue and soft tissue regeneration, easy to isolate, ready availability, high-biocompatibility and safety and no ethical restrictions.

Key Words:
Dental Bud Stem Cells (DBSCs), Dental Pulp Stem Cells (DPSCs), Mesenchymal Stem Cells (MSCs), Stemness genes, Osteogenic differentiation.

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Introduction

Currently different types of stem cells (SCs) have been isolated from dental and surrounding tissue. Their involvement in tissue repair and regeneration, local bone and teeth homeostasis and maintenance has been proved[1]. It is difficult to characterize exclusive dental stem cells using just surface protein markers by Flow-cytometry or gene expression by Real-time PCR (qRT-PCR) due to their ubiquitous characteristics, since these markers are equally expressed by all different typology of stem cells[2]. Gene expression of multipotent and pluripotent markers such as Kruppel-like factor 4 (Klf4), octamer-binding transcription factor 4 (OCT4), homeobox transcription factor Nanog (Nanog), v-myc avian myelocytomatosis viral oncogene homolog (c-Myc), SRY (sex determining region Y)-box 2 (Sox2), osteocalcin (OCN), dentin matrix protein-1 (DMP-1) and protein markers such as CD44, CD73, CD90, CD133, CD34, CD45, CD14, Nestin, Stage-specific embryonic antigen-3 (SSEA-3) and Transcription-associated protein 1 (Tra1) are in fact evidence of stemness which is specific feature of stem cells from the bone marrow (BM), umbilical cord blood (UCB), placenta, peripheral blood (PB) as well from teeth tissue [2-4]. Dental tissue and more specifically dental bud and dental pulp are prominent source of these types of multipotent and pluripotent stem cells, known as dental-derived stem cells (d-DSCs). The focus on these cells has attracted highly interest not only within dentists and orthopedics but also in the Regenerative Medicine community. Both dental bud and dental pulp stem cells (DBSCs-DPSCs) have shown great potential in dental tissue repair and regeneration and confirmed the expression of transcription factors like Nanog, OCT4, Sox2, c-Myc, and Klf4 that play a key regulatory activity in the stem cell self-renewal process in replenishing mature cells that constantly die due to normal and constant tissue turnover reprogramming. The DPSCs and DBSCs both displayed a similar plasticity and typical features of pluripotency/multipotency typical of other sub-set of mesenchymal stem cells (MSCs)[5-7]. The human dental pulp cells from deciduous and permanent teeth can undergo reprogramming to establish pluripotent stem cell lines without c-Myc. These surgical residues, usually regarded as medical waste, can be used as an alternative source of pluripotent stem cells for personalized medicine[5-7]. The role of OCT4, Nanog and Sox2 in keeping the pluripotency status of stem cells has been well confirmed either in embryonic stem cells (ESCs) or in induced pluripotent stem cells (iPS). Takahashi and Yamanaka[9] showed in 2006 that the introduction of these specific genes encoding transcription factors could convert adult cells into pluripotent SCs[8-15]. The OCT4, Sox2 and Nanog are mutually involved in the regulation of embryo growth, modulating the cellular differentiation at the very early passages; OCT4, Sox2 and Nanog directly work on ESCs keeping them in a stemness state preventing their differentiation, and sustaining their self-renewal[16-20]. The active participation of Klf4 and c-Myc in this stemness activity has been confirmed though the way they interact has not been fully understood yet[21-26]. The data about c-Myc are sometimes discordant probably due to the multilevel expression of this gene. In normal cell c-Myc participate in proliferation and growth mechanism, an interesting feature is that c-Myc is generally low in quiescent cells as could be the case of MSCs in BM niches or in PB circulation; conversely, c-Myc switch on quick and become highly active once induced by growth factors[10]. Intriguingly, outcomes from c-Myc knockout mouse lead to embryonic death and can act the same as Klf4 either as an oncogene or tumor suppressor. This redundancy was described by Chang et al[26] who reprogrammed human dental pulp cells from deciduous and permanent teeth into pluripotent stem cells without inserting c-Myc. Similarly, Klf4 participate in cell proliferation and growth expressed in different tissues regulating the final commitment of stem cell differentiation as well apoptosis[21]. These results suggest that both Klf4 and c-Myc may exert differently depending on general micro-molecular condition, nevertheless they both are involved in the important modulatory function ESCs stemness, self-renewal and pluripotency as it was shown in Yamanaka's iPS project[9,21-26]. The aim of this study was to investigate the natural stemness feature of both DPSCs and DBSCs in the view to considered they as non-conventional, valid alternative source of MSC like cells that might be used for autologous cell therapy and drug screening applications, in particular in oral and maxillofacial practice.

Patients and Methods

Culture of d-DSCs

Dental bud stem cells (DBSCs) and dental pulp stem cells (DPSCs) were cultured as previously reported[5,7]. In brief, unerupted third molars and dental buds were obtained from healthy paediatric male donors (8-12 years) underwent to surgical extractions for orthodontics reasons. Written informed consent to tooth extraction by

Table I. Primer sequences used for quantitative Real-Time PCR.

Gene	Sequence (5′-3′)	NCBI Accession Number
Klf4	Forward: CCATCTTTCTCCACGTTCG Reverse: AGTCGCTTCATGTGGGAG	NM_004235.4
OCT4	Forward: GTATTCAGCCAAACGACCATC Reverse: CTGGTTCGCTTTCTCTTTCG	NM_002701.5
Sox2	Forward: GACTTCACATGTCCCAGCACTA Reverse: CTCTTTTGCACCCCTCCCATT	NM_003106.3
Nanog	Forward: ATTCAGGACAGCCCTGATTCTTC Reverse: TTTTTGCGACACTCTTCTCTGC	NM_024865.3
c-Myc	Forward: GCTGCTTAGACGCTGGATTT Reverse: TAACGTTGAGGGGCATCG	NM_002467.4
HPRT	Reverse: TGAAACTCAACCTTCCCTTGGT Forward: TGACACTGGCAAAACAATGCA Reverse: GGTCCTTTTCACCAGCAAGCT	NM_000194.2

Klf4, Kruppel-like factor 4; OCT4, octamer-binding transcription factor 4; Sox2, SRY-related HMG-box 2; Nanog, homeobox transcription factor Nanog; c-Myc, v-myc avian myelocytomatosis viral oncogene homolog; HPRT, hypoxanthine phosphoribosyltransferase.

piezo-surgery equipment (Silfradent Surgybone, S. Sofia, Italy) was obtained from all patient's guardians and parents.

Culture of MSCs

Dental bud stem cells (DBSCs) and dental pulp stem cells (DPSCs) were cultured for 7 days in a basal medium containing 10% fetal bovine serum (FBS) (Gibco Ltd., Uxbridge, UK), 100 μM L-ascorbic acid-2-phosphate, 100 U/ml penicillin, 2 mM glutamine and 100 μg/ml streptomycin (Gibco Limited, Uxbridge, United Kingdom), incubated in a Thermo Scientific Heracell CO_2 (5%) at 37°C (Thermo Fisher Scientific, Waltham, MA, USA).

For osteogenic experiments, DBSCs and DPSCs cells were cultured for 7 days with osteogenic medium containing α-MEM (Sigma-Aldrich, St. Louis, MO, USA), 20% fetal bovine serum (FBS (Invitrogen, Carlsbad, CA, USA) with 10 mM β-glycerophosphate (Sigma-Aldrich, St. Louis, MO, USA), 100 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, USA), 0.2 mM L-ascorbic acid-2-phosphate (Sigma-Aldrich, St. Louis, MO, USA), 0.1 mg/mL streptomycin, 0.25 mg/mL amphotericin B and 100 U/mL penicillin (Gibco Limited, Uxbridge, United Kingdom).

Total RNA Extraction and qRT-PCR

Total RNA was extracted from DBSCs and DPSCs using the Purelink™ RNA mini kit (Applied Biosystems, Monza, Italy) and RNA were reverse-transcribed using M-MuLV reverse transcriptase (Applied Biosystems, Monza, Italy). The cDNA samples were amplified by Real-time PCR (qRT-PCR) using primers sequences (Table I) specific for the kruppel-like factor 4 (Klf4), octamer-binding transcription factor 4 (OCT4), homeobox transcription factor Nanog (Nanog), v-myc avian myelocytomatosis viral oncogene homolog (c-Myc), SRY (sex determining region Y)-box 2 (Sox2) and hypoxanthine phosphoribosyltransferase (HPRT). The qPCR reactions were performed using a Pikoreal 96 system (Thermo Fisher Scientific, Waltham, MA, USA). The qRT-PCR conditions were: an initial denaturation step at 95°C for 10 min; 40 cycles of 10 s at 95°C and 1 minute at 60°C. Melting curve analyses were performed at the end of each PCR assay to verify the specificity of the PCR products. mRNA expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method with the levels of gene expression normalized to the housekeeping gene hypoxanthine phosphoribosyltransferase (HRPT).

Statistical Analysis

All experiments were performed in triplicate. Data is shown as means ± standard deviations (SD). Data was evaluated by unpaired two-tailed Student's *t*-test (GraphPad Prism software package, La Jolla, CA, USA). The differences between groups were considered statistically significant when *p*-values were less than 0.05.

Results

In this study, we compared the expression of five stemness genes (OCT4, Sox2, c-Myc, Nanog and Klf4) DBSCs *vs.* DPSCs in basal medium and during

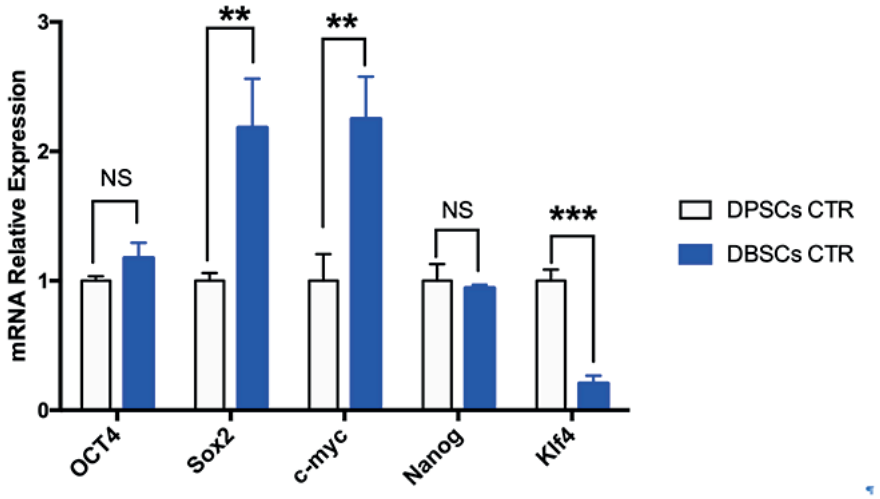

Figure 1. Expression of the stemness molecular markers in human DBSCs compared to DPSCs in basal medium. mRNA expression of the stemness genes OCT4, Sox2, c-Myc, Nanog and Klf4 in DBSCs compared to DPSCs cultured in basal medium for 1 week. Results are represented as fold increase compared to the level expressed in DPSCs cultured in basal medium. *($p < 0.05$); **($p < 0.01$); ***($p < 0.001$); (NS.= not significant).

osteogenic differentiation process. Gene expression levels showed that DBSCs cultured in basal medium express significantly higher mRNA levels of Sox2 (2.2-fold) and c-Myc (2.2-fold) than DPSCs.

On the contrary, DBSCs express significantly lower levels of Klf4 mRNA (0.21-fold) when compared to DPSCs at the same conditions (Figure 1). Furthermore, Real-time PCR (qPCR) analyses were performed to determine if osteogenic medium influenced the expression of the major stemness-associated genes OCT4, Sox2, c-Myc, Nanog and Klf4 in DBSCs vs. DPSCs. Gene expression levels showed that DBSCs cultured in osteogenic medium express significantly higher mRNA levels of OCT4 (1.8-fold), Sox2 (1.4-fold), c-Myc (1.68-fold) and Nanog (3.77-fold), whereas they express significantly lower levels of KLf4 mRNA (0.55-fold) when compared to DPSCs at the same

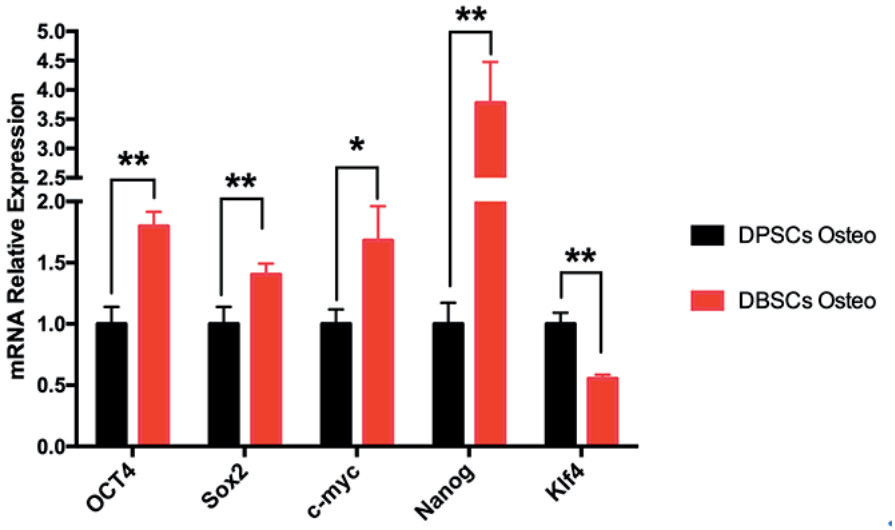

Figure 2. Expression of the stemness molecular markers in human DBSCs compared to DPSCs cultured in osteogenic medium. mRNA expression of the stemness genes OCT4, Sox2, c-Myc, Nanog and Klf4 in DBSCs compared to DPSCs cultured in osteogenic medium for 1 week. Results are represented as fold increase compared to the level expressed in DPSCs cultured in basal medium *($p < 0.05$); **($p < 0.01$).

conditions (Figure 2). Comparative image of total stemness expression pattern for both d-DSCs is reported in Figure 3.

Discussion

Mesenchymal stem cells were first discovered as fast growing, high plasticity and adherent colony forming like-fibroblastic cells present in bone marrow stroma; however, they also present in fat tissue, umbilical cord blood, placenta and peripheral blood. Mesenchymal stem cells sub-set was also obtained from different typology of oral cavity tissues. These MSCs have been studied and used with different typology of scaffolds showing a very interactive activity with the internal scaffold microenvironment[27-34].

The co-expression of gene like OCT4, Nanog, Sox2, c-Myc and Klf4 certainly proved the stemness attribute of these cells. Of note, in order to appreciate entirely the interactivity of these genes it is crucial to understand that they dynamically intersect each other. For instance, mouse and human cells studies showed that OCT4 as transcription factors (TF), also includes the homeobox protein of Nanog and homebox transcription factors of Sox2 that maintain pluripotency in ESCs.

Another research performed on blastocytes revealed that Sox2 is arranged into a heterodimeric complex with OCT4 with a spacer of 3bp that is in charge of regulating the expression of fibroblast growth factor-4 (Fgf4) gene. This spacer is exactly located in between the Sox and OCT4, CATTGTCATGCAAAT, important for Fgf4 expression[35-37].

Detection of OCT4, Sox2 and Nanog expression in human MSCs from BM, UCB, adipose tissue, PB and many other tissues is consistent with earlier reported data by many other authors[6,38-42]. Although currently these genes are better understood we still need to know more clear their functional role in different sub-sets of adult stem cells as these genes ubiquitously may refer either to adult stem cells or embryonic like stem cells that are kept in quiescent state in special niches, in circulation or organ tissues.

In fact, our data indicated that both DBSCs and DPSCs cultured *in vitro* expressed a hetero-geneous assortment of markers associated with embryonic stemness, showing a well consolidated self-renewal capability and multilineage differentiation potential to chondrocytes, adipocytes, odontoblasts, and neural-like cells under appropriate induction conditions[32-34].

Despite other sources of MSCs like BM, UCB, PB, skin, brain, liver in the adult body the dental tissues are formed at a later stage and are a sour-

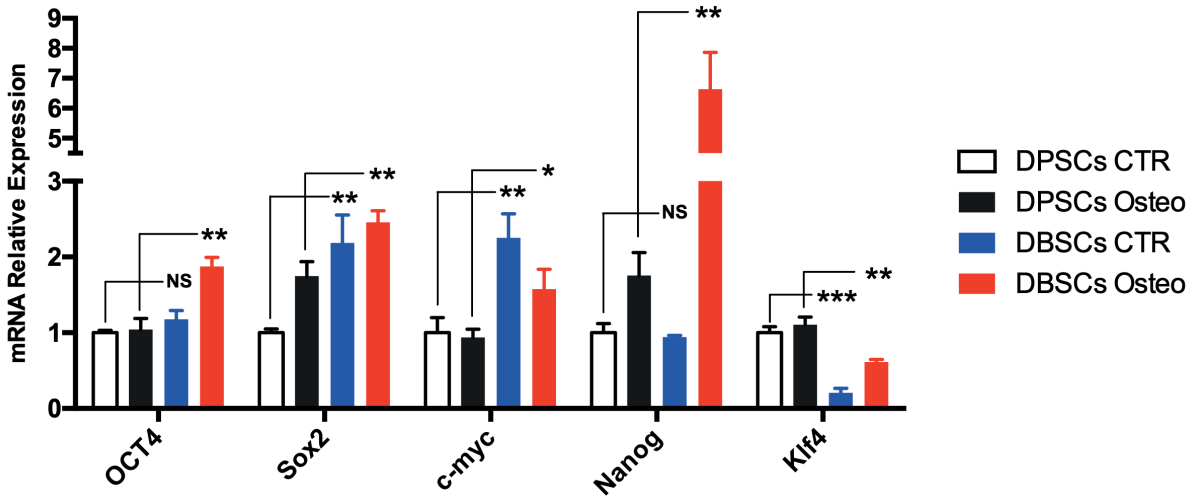

Figure 3. Gene expression of OCT4, Sox2, c-Myc, Nanog and Klf4 in DBSCs compared to DPSCs in both basal medium and osteogenic medium. mRNA expression obtained from two lines of cells at day 7 cultured in either basal medium or osteogenic medium (without the adjunct of other osteo-inducers such as vitamin D, Dexamethasone and BMP2) were compared. The DBSCs eventually at day 7 expressed in osteogenic medium environment higher level of OCT4, Nanog and Sox2 with a remarkable decrease of Klf4 and no valuable changes in c-Myc. Conversely, the DPSCs did not show as much fluctuance in gene expression in comparing; however, in the osteogenic medium it has been noted a decrease in c-Myc whilst OCT4 and Kfl4 showed no valuable changes. The *c-Myc* down expression in both samples might be related to the fact that this gene need a presence of different inducers such as vitamin D, in addition the expression of c-Myc, OCT4 and Klf4 indicate cells osteo-differentiation in later period which is between 21 day for mature osteoblasts and SCs and as early as 14 days for emerging undifferentiated SCs.

ce of large amount of stem cells, caused by late completion of odontogenesis process and tooth eruption.

Additionally wisdom tooth that are mature and at the stage of germ, can be a source of DPSCs and DBSCs. Wisdom teeth are not essential for human masticatory function and frequently extracted for orthodontic reasons or dysodontiasis. The tooth extraction, by piezo-surgery technique, is less invasive when compared to bone marrow or other tissue biopsy.

The challenge of new bone formation and graft integration is strictly dependent on recruitment and adhesion of stem cells on the scaffolds, to attain a successful cell differentiation and interaction with the microenvironment.

In this study, the expression of five stemness genes namely OCT4, Sox2, c-Myc, Nanog and Klf4 were compared during expansion for DBSCs and DPSCs (Figures 1, 2, 3). The results concluded that DBSCs cultured in basal medium significantly expressed higher mRNA levels of both Sox2 and c-Myc almost twice than DPSCs. This was revealed in the course of gene expression analysis. In contrast, when associated to DPSCs in similar conditions, DBSCs showed significant lower levels of Klf4 mRNA. Similarly, real-time PCR (qPCR) analyses was performed to determine if osteogenic medium had any influence on the expression of the major stemness-associated genes OCT4, Sox2, c-Myc, Nanog and Klf4 in DBSCs with respect to DPSCs.

Gene expression levels showed significantly higher mRNA levels of OCT4 (1.8-fold), Sox2 (1.4-fold), c-Myc (1.68-fold) and Nanog (3.77-fold), however they express significantly lower levels of Klf4 mRNA (0.55-fold) when compared to DPSCs under same culture condition. So, we can speculate that d-DSCs are fitting for stemness potential related clinical applications since their effortless isolation, easiness of culture conditions and reprogramming aptitude. It may be suitable to apply these cells to achieve the regeneration of tissues/organs, in particular for head and neck tissues regeneration such as the gingiva, the tooth and salivary gland, which are formed through the interaction of epithelial and mesenchymal tissues during organogenesis[43-50]. This study evaluated and confirmed at least *in vitro* the stemness state, the multipotency and pluripotency of both line of cells obtained from oral tissues, the DPSCs and DBSCs. We are, therefore, highly confident that these SCs could be an alternative and a real valid solution to be used in Regenerative Medicine for soft tissue and bone regeneration due to their distinctive biological features and safety. However, any gene variation that has been showed in this study may be the results of few variabilities that, in part, indicate the substantial differences that contradistinguish the two lines of cells, one from the pulp the others from Bud. The differences might be related to the medium used *in vitro* culture and the intrinsic natural final commitment towards different types of tissues and cell phenotypes of both DPSCs and DBSCs; the lack of different stimulators in osteogenic medium that has been directly connected with OCT4, c-Myc and Klf4 gene over expression such as vitamin has probably to be considered as an adjunctive cause; eventually we should also consider the period of culture that in our case was 1 week, which is probably too short to see a fully expression of those genes as showed by different data in which either 14 or 21 days is the right time to either identify iPS formation and osteoblast maturity confirmed by the additional characterization of alkaline phosphatase (AP)[51]. Last but not least, we should highlight the structural limitations of the *in vitro* microenvironment and the lack of molecular and bio-chemical influences that eventually contribute to cell behavior and maturity.

Conclusions

We showed for the first time *in vitro* the genetic expression existing between DPSCs and DBSCs under different media induction and their stemness pertinence that put them at the same level of other sub-set of adult SCs and MSCs.

Conflict of Interest

The Authors declare that they have no conflict of interest.

Author Contributions

A.B. made substantial contributions to the conception and design of the study, experiments and coordination, and supervised the manuscript. C.I.G. contributed to the data analysis and interpretation, and manuscript revision. S.C. was responsible for cell culture and manuscript first draft and contributed to data analysis. J.S. contributed to molecular biology analyses. S.K.A. contributed to statistical analysis. M.C. and S.S. contributed to the isolation and expansion of mesenchymal stem cells and helped to draft the manuscript. L.S., G.D and K.CD.N. contributed in bibliographic research. L.P., A.S., and F.I. participated in the design of the study, collected the biological material, performed data analysis and helped to revise the manuscript. All authors read and approved the final manuscript.

References

1) Mitrano TI, Grob MS, Carrión F, Nova-Lamperti E, Luz PA, Fierro FS, Quintero A, Chaparro A, Sanz A. Culture and characterization of mesenchymal stem cells from human gingival tissue. J Periodontol 2010; 81: 917-925.
2) Ballini A, Cantore S, Scacco S, Coletti D, Tatullo M. Mesenchymal stem cells as promoters, enhancers, and playmakers of the translational regenerative medicine 2018. Stem Cells Int 2018; 2018: 6927401.
3) Allison MR, Islam S. Attributes of adult stem cells. J Pathol 2009; 217: 144-160.
4) Mimeault M, Hauke R, Batra SK. Stem cells: a revolution in therapeutics-recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. Clin Pharmacol Ther 2007; 82: 252-264.
5) Di Benedetto A, Posa F, De Maria S, Ravagnan G, Ballini A, Porro C, Trotta T, Grano M, Lo Muzio L, Mori G. Polydatin, natural precursor of resveratrol, promotes osteogenic differentiation of mesenchymal stem cells. Int J Med Sci 2018; 13: 944-952.
6) Labusca L, Herea DD, Mashayekhi K. Stem cells as delivery vehicles for regenerative medicine-challenges and perspectives. World J Stem Cells 2018 26; 10: 43-56.
7) Ballini A, Mastrangelo F, Gastaldi G, Tettamanti L, Bukvic N, Cantore S, Cocco T, Saini R, Desiate A, Gherlone E, Scacco S. Osteogenic differentiation and gene expression of dental pulp stem cells under low-level laser irradiation: a good promise for tissue engineering. J Biol Regul Homeost Agents 2015; 29: 813-822.
8) Daltoé FP, Mendonça PP, Mantesso A, Deboni MC. Can SHED or DPSCs be used to repair/regenerate non-dental tissues? A systematic review of in vivo studies. Braz Oral Res 2014; 28: pii: S1806-83242014000100401.
9) Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 2006; 126: 663-676.
10) Takeda-Kawaguchi T, Sugiyama K, Chikusa S, Iida K, Aoki H, Tamaoki N, Hatakeyama D, Kunisada T, Shibata T, Fusaki N, Tezuka K. Derivation of iPSCs after culture of human dental pulp cells under defined conditions. PLoS One 2014; 9: e115392.
11) Rodda DJ, Chew JL, Lim LH, Loh YH, Wang B, Ng HH, Robson P. Transcriptional regulation of nanog by OCT4 and SOX2. J Biol Chem 2005; 280: 24731-24737.
12) Boyer LA, Lee T, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R, Young RA. Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 2005; 122: 947-956.
13) Beltrami AP, Cesselli D, Bergamin N, Marcon P, Rigo S, Puppato E, D'Aurizio F, Verardo R, Piazza S, Pignatelli A, Poz A, Baccarani U, Damiani D, Fanin R, Mariuzzi L, Finato N, Masolini P, Burelli S, Belluzzi O, Schneider C, Beltrami CA. Multipotent cells can be generated in vitro from several adult human organs (heart, liver, and bone marrow). Blood 2007; 110: 3438-3446.
14) Raposio E, Bertozzi N. How to isolate a ready-to-use adipose-derived stem cells pellet for clinical application. Eur Rev Med Pharmacol Sci 2017; 21: 4252-4260.
15) Riekstina U, Cakstina I, Parfejevs V, Hoogduijn M, Jankovskis G, Muiznieks I, Muceniece R, Ancans J. Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis. Stem Cell Rev 2009; 5: 378-386.
16) Kuang WB, Deng Q, Deng CT, Li WS, Zhang YG, Shu SW, Zhou MR. MiRNA regulates OCT4 expression in breast cancer cells. Eur Rev Med Pharmacol Sci 2018; 22: 1351-1357.
17) Chen HY, Han XL, Wang RG, Song XF, Zhang HZ. The expression of OCT4 and its clinical significance in laryngeal squamous carcinoma tissues. Eur Rev Med Pharmacol Sci 2017; 21: 4591-4594.
18) Ambrosetti DC, Basilico C, Dailey L. Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites. Mol Cell Biol 1997; 17: 6321-6329.
19) Zhou L, Zhao LC, Jiang N, Wang XL, Zhou XN, Luo XL, Ren J. MicroRNA miR-590-5p inhibits breast cancer cell stemness and metastasis by targeting SOX2. Eur Rev Med Pharmacol Sci 2017; 21: 87-94.
20) Silva J, Nichols J, Theunissen TW, Guo G, van Oosten AL, Barrandon O, Wray J, Yamanaka S, Chambers I, Smith A. Nanog is the gateway to the pluripotent ground state. Cell 2009; 138: 722-737.
21) Zhang P, Andrianakos R, Yang Y, Liu C, Lu W. Kruppel-like factor 4 (Klf4) prevents embryonic stem (ES) cell differentiation by regulating Nanog gene expression. J Biol Chem 2010; 285: 9180-9189.
22) Rowland BD, Bernards R, Peeper DS. The KLF4 tumour suppressor is a transcriptional repressor of p53 that acts as a context-dependent oncogene. Nat Cell Biol 2005; 7: 1074-1082.
23) Rowland BD, Peeper DS. KLF4, p21 and context-dependent opposing forces in cancer. Nat Rev Cancer 2006; 6: 11-23.
24) Chen X, Johns DC, Geiman DE, Marban E, Dang DT, Hamlin G, Sun R, Yang VW. Krüppel-like factor 4 (gut-enriched Krüppel-like factor) inhibits cell proliferation by blocking G1/S progression of the cell cycle. J Biol Chem 2001; 276: 30423-30428.
25) Nakagawa M, Koyanagi M, Tanabe K, Takahashi K, Ichisaka T, Aoi T, Okita K, Mochiduki Y, Takizawa N, Yamanaka S. Generation of induced pluripotent stem cells without Myc from mouse and human fibroblasts. Nat Biotechnol 2009; 26: 101-106.
26) Chang YC, Li WC, Twu NF, Li HY, Lo WL, Chang YL, Lee YY, Lin CF, Shih YH, Chen MT. Induction of dental pulp-derived induced pluripotent stem cells in the absence of c-Myc for differentiation into neuron-like cells. J Chin Med Assoc 2014; 77: 618-625.

27) Zippel N, Schulze M, Tobiasch E. Biomaterials and mesenchymal stem cells for regenerative medicine. Recent Pat Biotechnol 2010; 4: 1-22.

28) Guillot PV, Cui W, Fisk NM, Polak DJ. Stem cell differentiation and expansion for clinical applications of tissue engineering. J Cell Mol Med 2007; 11: 935-944.

29) Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. J Cell Physiol 2007; 213: 341-347.

30) Santiago JA, Pogemiller R, Ogle BM. Heterogeneous differentiation of human mesenchymal stem cells in response to extended culture in extracellular matrices. Tissue Eng Part A 2009; 15: 3911-3922.

31) Tormin A, Brune JC, Olsson E, Valcich J, Neuman U, Olofsson T, Jacobsen SE, Scheding S. Characterization of bone marrow-derived mesenchymal stromal cells (MSC) based on gene expression profiling of functionally defined MSC subsets. Cytotherapy 2009; 11: 114-128.

32) Ballini A, Boccaccio A, Saini R, Van Pham P, Tatullo M. Dental-derived stem cells, their secretome and interactions with bioscaffolds/biomaterials in regenerative medicine: from the in-vitro research to translational applications. Stem Cells Int 2017; 2017: 6975251.

33) Ballini A, Scacco S, Coletti D, Pluchino S, Tatullo M. Mesenchymal stem cells as promoters, enhancers, and playmakers of the translational regenerative medicine. Stem Cells Int 2017; 2017: 3292810.

34) Di Benedetto A, Posa F, Carbone C, Cantore S, Brunetti G, Centonze M, Grano M, Lo Muzio L, Cavalcanti-Adam EA, Mori G. NURR1 downregulation favors osteoblastic differentiation of MSCs. Stem Cells Int 2017; 2017: 7617048.

35) Yuan H, Corbi N, Basilico C, Dailey L. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. Genes Dev 1995; 9: 2635-2645.

36) Ambrosetti DC, Basilico C, Dailey L. Synergistic activation of the fibroblast growth factor 4 enhancer by Sox2 and Oct-3 depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites. Mol Cell Biol 1997; 17: 6321-6329.

37) Wei CX, Wong H, Xu F, Liu Z, Ran L, Jiang RD. IRF4-induced upregulation of lncRNA SOX2-OT promotes cell proliferation and metastasis in cholangiocarcinoma by regulating SOX2 and PI3K/AKT signaling. Eur Rev Med Pharmacol Sci 2018; 22: 8169-8178.

38) Dai WW, Liu S, Liu XJ, Peng ZP. Stemness-related changes of CD133- cells in nasopharyngeal carcinoma after x-ray radiation at the median lethal dose. Eur Rev Med Pharmacol Sci 2018; 22: 2334-2342.

39) Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop DJ, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The international society for cellular therapy position statement. Cytotherapy 2006; 8: 315-317.

40) Bao CS, Li XL, Liu L, Wang B, Yang FB, Chen LG. Transplantation of Human umbilical cord mesenchymal stem cells promotes functional recovery after spinal cord injury by blocking the expression of IL-7. Eur Rev Med Pharmacol Sci 2018; 22: 6436-6447.

41) Xue ZL, Meng YL, Ge JH. Upregulation of miR-132 attenuates osteoblast differentiation of UC-MSCs. Eur Rev Med Pharmacol Sci 2018; 22: 1580-1587.

42) Wei CX, Wong H, Xu F, Liu Z, Ran L, Jiang RD. IRF4-induced upregulation of lncRNA SOX2-OT promotes cell proliferation and metastasis in cholangiocarcinoma by regulating SOX2 and PI3K/AKT signaling. Eur Rev Med Pharmacol Sci 2018; 22: 8169-8178.

43) Cantore S, Ballini A, De Vito D, Martelli FS, Georgakopoulos I, Almasri M, Dibello V, Altini V, Farronato G, Dipalma G, Farronato D, Inchingolo F. Characterization of human apical papilla-derived stem cells. J Biol Regul Homeost Agents 2017; 31: 901-910.

44) Miura Y, Gronthos S, Allen MR, Cao C, Uveges TE, Bi Y, Ehirchiou D, Kortesidis A, Shi S, Zhang L. Defective osteogenesis of the stromal stem cells predisposes CD18-null mice to osteoporosis. Proc Natl Acad Sci U S A 2005; 102: 14022-14027.

45) Ballini A, Scattarella A, Crincoli V, Carlaio RG, Papa F, Perillo L, Romanazzo T, Bux MV, Nardi GM, Dituri A, Cantore S, Pettini F, Grassi FR. Surgical treatment of gingival overgrowth with 10 years of follow-up. Head Face Med 2010 12; 6: 19.

46) Brunetti G, Di Benedetto A, Posa F, Colaianni G, Faienza MF, Ballini A, Colucci S, Passeri G, Lo Muzio L, Grano M, Mori G. High expression of TRAIL by osteoblastic differentiated dental pulp stem cells affects myeloma cell viability. Oncol Rep 2018; 39: 2031-2039.

47) Cantore S, Crincoli V, Boccaccio A, Uva AE, Fiorentino M, Monno G, Bollero P, Derla C, Fabiano F, Ballini A, Santacroce L. Recent advances in endocrine, metabolic and immune disorders: mesenchymal stem cells (MSCs) and engineered scaffolds. Endocr Metab Immune Disord Drug Targets 2018; 18: 466-469.

48) Li C, Wei GJ, Xu L, Rong JS, Tao SQ, Wang YS. The involvement of senescence induced by the telomere shortness in the decline of osteogenic differentiation in BMSCs. Eur Rev Med Pharmacol Sci 2017; 21: 1117-1124.

49) Mori G, Brunetti G, Ballini A, Di Benedetto A, Tarantino U, Colucci S, Grano M. Biological characteristics of dental stem cells for tissue engineering. Key Engineering Materials 2013; 541: 51-59.

50) Chen B, Meng J, Zeng YT, Du YX, Zhang J, Si YM, Yuan X. MicroRNA-7-5p regulates osteogenic differentiation of hMSCs via targeting CMKLR1. Eur Rev Med Pharmacol Sci 2018; 22: 7826-7831.

51) Singh U, Quintanilla RH, Grecian S, Gee KR, Rao MS, Lakshmipathy U. Novel live alkaline phosphatase substrate for identification of pluripotent stem cells. Stem Cell Rev 2012; 8: 1021-1029.