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Stemness genes expression in naïve *vs.* osteodifferentiated human dental-derived stem cells

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Abstract. – OBJECTIVE: Mesenchymal stem cells (MSCs) have been deeply investigated in regenerative medicine because of their crucial role in tissue healing, such as tissue regeneration. Dental-derived stem cells (d-DSCs) are easily available from dental tissues, which can be isolated from all age patients with minimal discomfort.

PATIENTS AND METHODS: Normal unerupted third molars tooth buds were collected from adolescents' patients underwent to extractions for orthodontic reasons. The expression of the genes Kruppel-like factor 4 (Klf-4), octamer-binding transcription factor 4 (Oct-4), homeobox transcription factor Nanog (NANOG) was investigated in d-DSCs obtained from dental bud (DBSCs), differentiated toward osteoblastic phenotype and not.

RESULTS: Our results showed that DBSCs expressed Oct-4, Nanog, and Klf-4 in undifferentiated conditions and interestingly the expression of such genes increased when the cells were kept in osteogenic medium.

CONCLUSIONS: These attractive stemness properties, together with the effortlessly isolation, during common oral and maxillofacial surgical procedures, from undifferentiated tissues such as dental bud, make this kind of d-DSCs a promising tool in regenerative medicine, having the potential for clinical applications, and reinforcing the present challenge to develop new preventive and healing strategies in tissue regeneration.

Key Words:
Mesenchymal stem cells, Stemness genes, Dental bud, Tissue regeneration, Oral and maxillofacial surgery.

Introduction

A breakthrough in tissue engineering was the discovering of progenitor/stem cells in several tissues: this new research topic opened the door to new therapies for several disorders[1]. In the last years, stem cell research has improved the techniques associated to stem cell characterization at molecular levels and to their isolation from animal and human tissues[2].

In vitro proliferation and differentiation capabilities of embryonic and postnatal stem cells in-

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clined the researchers towards the idea of using such cells in diseased and impaired tissues and organs for their regeneration[3]. Mesenchymal stem cells (MSCs) have the ability to self-renewing and differentiate into multiple mesenchymal lineages such as osteoblasts, adipocytes, and chondrocytes among others, thus representing optimal candidates for regenerative medicine. In addition, compared to embryonic stem cells, MSCs have the advantage of being free from ethical controversies and they can be isolated postnatally from patients and used for autologous therapies.

The current studies on bone tissue engineering concerning dental-derived stem cells (d-DSCs) and adipose-derived stromal/stem cells (ASCs) have noticeably established that such cells can play a key role in the treatment of cranio-maxillofacial defects, given their strong commitment towards the osteogenic phenotype[4-8].

The maintenance of self-renewal and differentiation potential is critical for the use of these cells in the regenerative therapies, but *in vitro* expansion can lead to the progressive loss of stem features[9].

Stemness connect the capability of a cell to maintain its lineage, to produce differentiated cells, and to interrelate with its environment to uphold a balance among quiescence, proliferation, and regeneration. Although adult stem cells exhibit these properties when taking part in tissue homeostasis, cancer stem cells (CSCs) act as their malignant equal using their stemness properties to maintain their lineage and survive stress and chemotherapy, while, in this context, electrochemotherapy acts as an effective treatment for cancer along with the expression change in stemness genes[10-14].

The expression of stemness genes, as the transcription factors Kruppel-like factor 4 (Klf-4), octamer-binding transcription factor 4 (Oct-4), homeobox transcription factor Nanog (Nanog), is associated with maintenance of self-renewal and multi-differentiation capacity and was reported in MSCs of different origin[15-21], confirming the great potential of these cells in tissue regeneration. We have previously shown that MSCs from dental tissues express high levels of mesenchymal stem markers and have great osteogenic potentials, becoming a promising tool after tissue injury[22-27].

In this study, we investigated the expression of the stemness-associated genes *Oct-4, Nanog* and *Klf-4* in differentiated *vs* undifferentiated dental bud derived MSCs (DBSCs), which represent a good mesenchymal stem cell model with optimal osteogenic differentiation capacity.

Patients and Methods

Subjects and Cell culture

Experimental procedures were conducted following our previous experience in the field, and according to manufacture specifications[19-21].

After a detailed medical and dental history was obtained, patients were selected following inclusion/exclusion criteria. Inclusion criteria were: patients between-12 years old requiring extractions of unerupted third molars for orthodontics reasons, following oral and maxillofacial piezo-surgery procedures. Exclusion criteria were: systemic diseases (ASA III or higher) that contraindicate surgery or impair wound healing, periodontal disease, patients under any pharmacological treatment or patients with contraindications to the extraction under local anesthesia[23-27]. Parents or guardians of each patient gave informed consent to tooth extraction obtained with piezo-surgery technology, in accordance with the Declaration of Helsinki, for the re-use of biospecimens in research applications. With the purpose to preserve dental tissues for consequent cell isolation and expansion, piezo-surgery technology enables selective tissue cutting, and consequently, tooth buds or embedded third molars can effortlessly be removed from bones with a slight wound to periodontal fibers or bud follicles.

In addition, tooth extraction, especially through piezosurgery technique, can be considered less invasive in comparison to bone marrow or other tissues biopsy[28].

All patients were operated by the same surgeon (S.C.) under local anesthesia with 2% mepivacaine, after a one-stage full-mouth disinfection and quadrant scaling[28,29]. Morphologic analysis of third-molar maturity, to determine bud stage on the basis of root development, was performed by digital radiographic images[30,31], a full-thickness flap was elevated and a piezosurgical device (Silfradent, Surgybone, S. Sofia, Italy) was used to cut a precisely defined surgical area. Then, the tooth socket was exposed, and the dental bud was delivered in the mesio vestibular direction by inserting a straight elevator (Figure 1 A-C). The cutting was continuously accompanied by abundant irrigation with refrigerated saline solution. The extraction socket was debrided and closed by interrupted 4/0 absorbable silk (SKD, Italy) sutures. The central part of the bud was cut in small pieces and digested in presence of 3 mg/mL collagenase I, 4 mg/mL dispase (Gibco Ltd., Uxbridge, UK) for 1 h at 37°C by shacking. The suspension

Table I. Primer sequences used for qPCR.

Gene	Sequence (5′-3′)	NCBI Accession No.
Klf-4	Forward: CCATCTTTCTCCACGTTCG Reverse: AGTCGCTTCATGTGGGAG	NM_004235.4
Oct-4	Forward: GTATTCAGCCAAACGACCATC Reverse: CTGGTTCGCTTTCTCTTTCG	NM_002701.5
Nanog	Forward: ATTCAGGACAGCCCTGATTCTTC Reverse: TTTTTGCGACACTCTTCTCTGC	NM_024865.3
HPRT	Forward: TGACACTGGCAAAACAATGCA Reverse: GGTCCTTTTCACCAGCAAGCT	NM_000194.2

Klf-4, kruppel-like factor 4; Oct-4, octamer-binding transcription factor 4; Nanog, homeobox transcription factor Nanog; HPRT, hypoxanthine phosphoribosyltransferase.

was filtered, and the cells were seeded in α-MEM supplemented with 5% fetal bovine serum (FBS), 100 U/ml penicillin-G, 100 µg/ml streptomycin (Gibco Ltd., Uxbridge, United Kingdom) at 5 × 103 cells/cm^2. Flasks were incubated in a Thermo Scientific Heracell CO_2 (5%) at 37°C (Thermo Fisher Scientific, Waltham, MA, USA), and the medium was replaced every 3 days. For induction of osteogenic differentiation, the cells were seeded 3000/cm^2 in α-MEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 2% FBS, 10^{-8} M dexamethasone and 50 µg/ml ascorbic acid (Sigma-Aldrich, St. Louis, MO, USA)[19-21].

Total RNA Extraction and qPCR

Total RNA was extracted from DBSCs using the Purelink™ RNA mini kit (Applied Biosystems, Monza, Italy) and RNA was (were) reverse-transcribed using M-MuLV reverse transcriptase (Applied Biosystems, Monza, Italy). The cDNA samples were amplified by real-time PCR (qPCR) using primers sequences (Table I) specific for the Klf-4, Oct-4, Nanog and hypoxanthine phosphoribosyltransferase (HPRT). The qPCR reactions were performed using a Pikoreal 96 system (Thermo Fisher Scientific, Carlsbad, CA, USA).

The qPCR conditions were: an initial denaturation step at 95°C for 10 min; 40 cycles of 10 s at 95°C and 1 min 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 through the $2^{-\Delta\Delta Ct}$ method with the levels

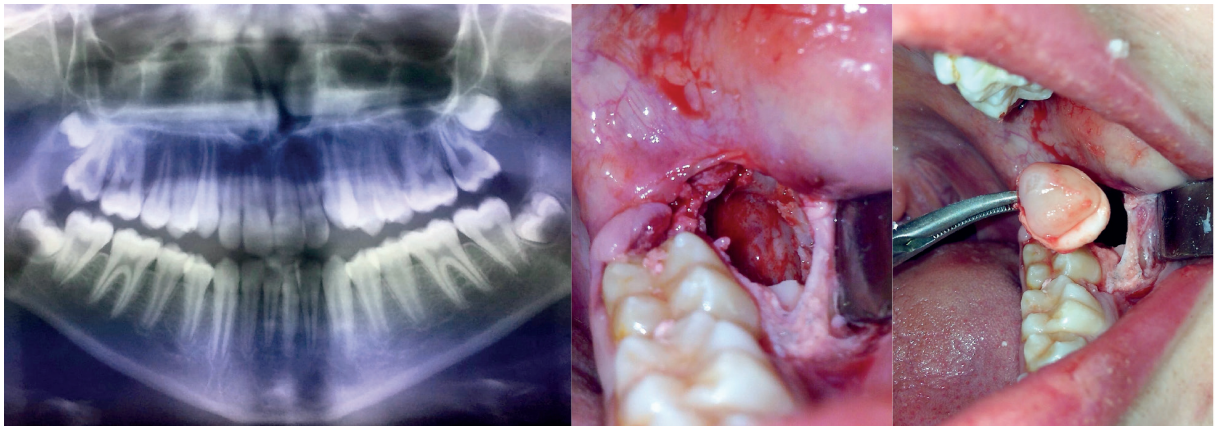

Figure 1. *A-C*. Clinical Images. (**A**) Orthopantomogram of the site before treatment. ***(B)*** After the alveolar bone was removed by a periosteal detacher, the tooth socket was exposed and ***(C)*** delivered in mesiolvestibular direction by inserting a straight elevator.

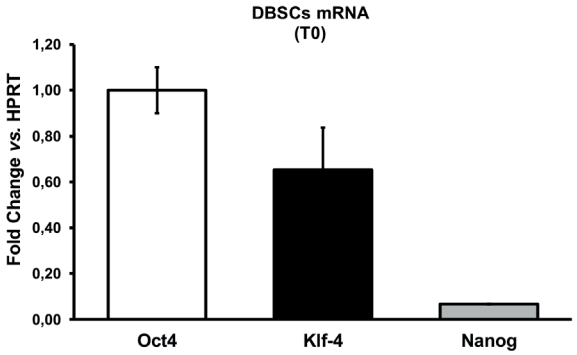

Figure 2. Quantification of Expression of Stemness Associated Genes *Oct-4, Klf-4* and *Nanog* in Undifferentiated DBSCs. The graph in the Figure 1 represents the qPCR performed on undifferentiated DBSCs showing the expression of the three stemness genes *Oct-4, Klf-4* and *Nanog.* Expression was normalized to HPRT. Each graph represents means ± SD of 3 independent donors. Statistics: unpaired Student's *t*-test.

of gene expression normalized to the housekeeping gene HRPT[21].

Statistical Analysis

Experiments were performed in triplicate. Data are shown as means ± standard deviation (SD). Data were evaluated by unpaired two-tailed Student's *t*-test, in all comparisons, and a *p*-value <0.05 with 95% confidence intervals was considered statistically significant (GraphPad Prism software package, La Jolla, CA, USA).

Results

DBSCs Express the Stemness-Associated Genes Oct-4, Klf-4 and Nanog

In this study, we analyzed, by qPCR, the expression of three pluripotent genes (Oct-4, Klf-4, and Nanog) in DBSCs in basal, undifferentiated conditions. The cells at low passage were maintained in culture with mesenchymal stem cells medium for 48 h and lysed for RNA collection. As shown in the Figure 2 gene expression levels showed that DBSCs cultured in basal medium express the three pluripotent genes Oct-4, Klf-4 and Nanog, although showing different levels. These results strengthened our previous findings of DBSCs stemness features and confirmed the expression of pluripotent genes in mesenchymal stem cells of dental origin as showed in MSCs from other sources[15-18].

DBSCs Differentiation, Under Osteogenic Conditions, Increases the Expression of the Stemness-Associated Genes Oct-4, Klf-4 and Nanog.

The previous results have led us to investigate whether the expression of such genes could change in DBSCs differentiated in osteogenic conditions. For this purpose, the cells were cultured for one week in osteogenic medium (DIFF) and lysed for RNA collection. Furthermore, qPCR analyses were performed to determine if the osteogenic medium influenced the expression of the genes compared to basal undifferentiated conditions (T0).

Interestingly, as reported in Figure 3, our results showed that DBSCs controlled differentiation, with osteogenic medium (DIFF), significantly increased the expression levels of Oct-4, Klf-4 and Nanog if compared with (T0). These results indicated that targeted osteogenic differentiation conditions did not promote genes suppression but increased their expression. This finding is not surprising, because it is in agreement with other literature data indicating that Oct-4, Klf-4 and Nanog are involved in the self-renewal and multi differentiation potential of MSCs[15-18].

Discussion

MSCs were first discovered as clonogenic and adherent colony forming fibroblastic cells present in bone marrow stroma[32], but subsequent studies demonstrated that these cells can reside in a variety of postnatal tissues[9], including dental tissues[7]. MSCs harvesting from bone marrow is often considered invasive for patients, thus many scientists have been motivated to deepen the study of other sources, such as the dental tissues. Indeed, despite to other sources of MSCs like bone marrow, adipose tissue, skin, and liver, the dental tissues from permanent teeth, in particular wisdom tooth ones, are formed at a later stage of development and are a source of large amount of stem cells, due to the late completion of odontogenesis process and tooth eruption. We have previously demonstrated[19] that dental bud derived MSCs expressed ≥95% of common mesenchymal stem markers (CD73, CD90, CD105) and, under osteogenic conditions, they expressed the typical osteoblastic markers producing mineralized matrix nodules, representing an optimal model of cell differentiation toward the osteoblastic/odontoblastic lineage. In addition, in the present study, we demonstrated, for

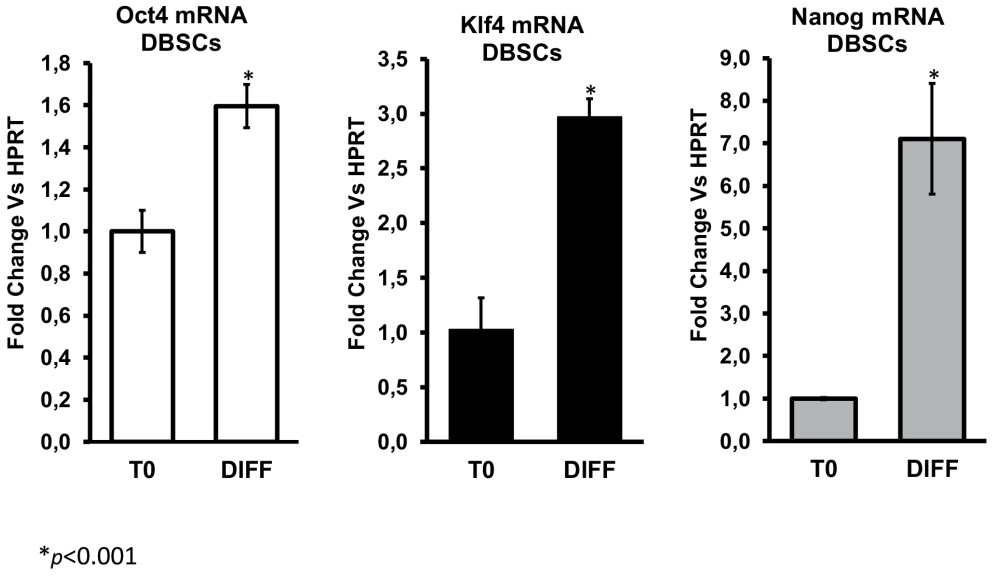

Figure 3. Effect of Osteogenic Differentiation on Stemness Associated Genes. The qPCR performed on DBSCs differentiated for 1 week or T0 showed that osteogenic differentiation significantly increased the expression of the three stemness associated markers *Oct-4*, *Klf-4* and *Nanog*. Expression was normalized to HPRT. Each graph represents means ± SD of 3 independent donors. Statistics: unpaired Student's *t*-test. *$p < 0.001$ compared to T0.

the first time, that DBSCs express the stemness associated genes Oct-4, Klf-4, and Nanog in undifferentiated conditions, as issued by qPCR data (Figure 2). Such genes were first recognized as essential for maintenance of pluripotency in embryonic stem cells[33-35] and thus identified as stemness-associated genes. More recently it has emerged that these genes play an important role in maintaining self-renewal and multi-differentiation potential of MSCs[17,18,36-42] but they are also involved in the inhibition of spontaneous differentiation[35]. In this study, we demonstrated the expression of these genes by DBSCs, which is in agreement with literature data concerning MSCs[15-18] and further strengthens the issue that these cells are endowed with stemness features and can be good candidates for tissue regeneration therapies[43-50]. The study continued exploring, again by qPCR, the expression of the same genes in DBSCs differentiated in osteogenic conditions (DIFF) compared with undifferentiated (T0) (Figure 3). Interestingly, we noticed that the addressed commitment of these cells toward the osteogenic lineage was associated with a significant increase of all analyzed genes. These results are consistent with the findings recently emerged and reported in the literature, showing that overexpression of Oct-4 and Nanog in MSCs from human bone marrow enhanced the proliferation rate and differentiation potential in the osteogenic, adipogenic and chondrogenic lineage. Moreover, spontaneous differentiation was inhibited[36]. In addition, Oct-4 overexpression, also in association with another stemness gene Sox-2, in human adipose tissue MSCs, was associated with upregulated osteogenic and adipogenic differentiation markers[37]. Furthermore, the expression of Klf-4 in association with Sox-2 and c-MYC, for 6 days was sufficient to convert murine fibroblasts into SOX9+/RUNX2+ cellular aggregates able to differentiate in osteoblasts and chondrocytes[51]. Notably, our results indicated that Nanog was the most upregulated gene (6-fold change) in differentiated cells, suggesting a major role for this transcription factor in osteogenic differentiation. Intriguingly a previous work showed that constitutive expression of Nanog alone in C3H10T1/2, a mouse mesenchymal cell line, strongly induced osteogenic differentiation of C3H10T1/2 cells by modulating BMP signaling[52].

Conclusions

Conversely to what is described for the normal stem cell aspects, we showed that this source of MSCs during the osteogenic differentiation increases the stemness genes.

All these features, together with the transcription factors Oct-4, Klf-4 and Nanog, shape an overall integrated system that modulates the stemness gene of DBSCs as well as the differentiation processes. We extend the data of stemness during osteogenic differentiation and adjunct understanding about MSCs, derived from a human dental bud.

We suggested that the examined genes can play an important role in maintaining stemness properties and differentiation capacity of DBSCs, making necessary further clarification studies in this regard. In addition, they encourage to take into consideration the use of DBSCs in a different branch of regenerative medicine, such as the bone tissue regeneration which is a promising alternative cell source for MSC-based therapeutic applications.

Author Contributions

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

Conflict of Interest

The Authors declare that they have no conflict of interest.

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