

Stem cells derived from human exfoliated deciduous teeth: a narrative synthesis of literature

S. ANNIBALI, M.P. CRISTALLI, F. TONOLI, A. POLIMENI

Department of Oral and Maxillofacial Sciences, "Sapienza" University of Rome, Rome, Italy

Abstract. – BACKGROUND: Stem Cells from Human Exfoliated Deciduous Teeth (SHEDs) represent an immature stem cell population, easily accessible without ethical concerns, able to induce pluripotent stem cells and to differentiate in osteoblasts, hepatocytes, adipocytes, neural cells, chondrocytes, myocytes, skin cells and odontoblasts.

AIM: The purpose of this review is to present a comprehensive synthesis of current knowledge of SHEDs, through the description of their tissue sources, properties, differentiation potential, and comparative assessment of their advantages for tissue engineering.

MATERIALS AND METHODS: Studies were identified by searching electronic databases (MEDLINE via PubMed, Medscape and Web of Science) from 2003 to 30 September 2013 and scanning references lists of the included publications and of the reviews. No publication date or publication status restrictions were imposed. Only evidence available in English language was reviewed.

RESULTS: A total of 72 studies were identified for inclusion in the review. Clinical heterogeneity didn't allow for meta-analysis but only for a narrative synthesis. The outcomes of the present narrative synthesis are presented separately for methods of isolation and culture, characterization of SHEDs, differentiation in vitro and in vivo, use in animal model, and stem cell banking.

CONCLUSIONS: SHEDs display multifactorial potential such as strong and high proliferative capacity, easy accessibility, high viability and multilineage differentiation capacity. Their retrieval is relatively simple and non-invasive, no risks for developing immune reactions or rejection following transplantation exist and no immunosuppressive therapy is needed.

Key Words:

Tooth, "Deciduous" [MeSH Terms], "Stem cells" [Mesh].

Introduction

Tissue engineering was defined by Langer and Vacanti as "an interdisciplinary field that applies the principles of engineering and life sciences to-

ward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ"¹. Tissue engineering aims to stimulate the body either to regenerate tissue on its own or to grow tissue outside the body which can then be implanted as natural tissue.

Several stem cells, have been extensively used in tissue engineering to develop a biologically functional tissue that can be used to restore malfunctioned or damaged tissues. Conventional tissue transplantation solutions are limited by factors, such as the lack of accessibility, insufficient donor tissue and graft rejection and failure. By contrast, stem cells, instead, are capable of renewing themselves through cell division and are able to regenerate new tissue and to restore function, without any limiting factors. With these features stem cells are considered as a new source of seed cells in tissue engineering in a wide range of applications.

To date the most common source of mesenchymal stem cells (MSCs) to use in clinical applications were bone marrow, adipose tissues, skin, umbilical cord and placenta. Recently, the presence of multi-potent stem cells in dental tissues, such as dental pulp of permanent (DPSCs) or deciduous teeth (SHEDs), dental follicle (DFSCs), dental papilla (SCAPs), periodontal ligament (PDLSCs), was reported. More specifically dental pulp from deciduous teeth provides a better source of stem cells when compared with dental pulp isolated from permanent teeth, because a significant number of stem cells decrease with aging and stem cell quality within niches could be affected by genetic and/or environmental factors².

SHEDs, identified for the first time in 2003 by Miura as a population of heterogeneous cells, represent a more immature stem cell population, easily accessible without ethical concerns, that shows some advantages. The pulp can easily be dissected and stored long-term in liquid nitrogen in stem cell banks, to be used in future for trans-

plantation without risk of immunologic reaction^{3,4}. The proliferation rate and the cell population doublings are higher when compared with stem cells from permanent teeth (DPSCs) and bone marrow derived mesenchymal stem cells (BMSCs)⁵⁻⁷. The expansion *in vitro* is easy, and the multilineage differentiation capacity is similar to other MSCs. Therefore, SHEDs are characterized by the ability to induce pluripotent stem cells and to differentiate in osteoblasts, hepatocytes, adipocytes, neural cells, chondrocytes, myocytes, skin cells and odontoblasts.

To our knowledge no systematic literature review exists on SHEDs.

The purpose of this review is to present a comprehensive synthesis of current knowledge of SHEDs, through the description of their tissue sources, properties, differentiation potential, and comparative assessment of their advantages for tissue engineering.

Materials and Methods

Studies were identified by searching electronic databases and scanning references lists of the included publications and of reviews to search for additional studies that could have been missed. The search on electronic databases was applied to MEDLINE via PubMed, Medscape and Web of Science. The literature search was performed from 2003 (when the SHEDs were identified for the first time) to 30th September 2013.

Eligibility Criteria

Case Reports and Comparative Studies were included. Reviews were not included. No Case Series, Clinical Trial, Controlled Clinical Trials (CCT), Randomized Clinical Trials (RCT) or Systematic Review have been found. No publication date or publication status restrictions were imposed. Only evidence available in the English language was reviewed.

Search Method

Full text articles published in English languages were found with an electronic search using the key words: “tooth, deciduous” [MeSH Terms] AND “stem cells” [Mesh].

Study Selection and Data Collection

Eligibility assessment was performed independently in a standardized method by two reviewers. Disagreements between reviewers were re-

solved by discussion. The first review Author extracted the following data from included studies and the second author checked the extracted data. Any disputes were sent to a third Author for arbitration. To determine the validity of the narrative synthesis the reviewers analyzed each study selected independently.

A total of 72 studies were identified for inclusion in the review (Tables Ia and Ib). The electronic search provided a total of 251 studies and the hand search provided a total of 13 papers. Of these, 131 were excluded based on their title, 45 were excluded based on the abstract (31 reviews and 24 did not meet the inclusion criteria) and 16 were excluded based on the text. The excluded full text papers are listed in Table II with the reasons for exclusion (Figure 1).

Abstracts with an unclear analysis were included in full text analysis.

Data Items

Data obtained was extracted from each study included and recorded on flow chart. The list of papers included are reported in Table I divided into (a) first Author; (b) year; (c) study design; (d) target cells; (e) journal; (f) impact factor.

Research Synthesis

Outcomes were recorded in evidence tables.

The marked heterogeneity allowed just a narrative synthesis.

Papers in which SHEDs were compared with stem cells from other sources were included.

The importance of the papers was evaluated considering the Impact Factor (IF) of the publications because no limits were applied for types of studies and no Systematic Review, Randomized Clinical Trials (RCT) or Controlled Clinical Trials (CCT) had been found, and so it was impossible to classify according to the type of study.

Clinical heterogeneity didn't allow for meta-analysis but only for a narrative synthesis.

Results

The outcomes of the present narrative synthesis are presented separately for each of the five issues:

1. Methods of isolation and culture
2. Characterization of SHEDs
3. Differentiation *in vitro* and *in vivo*
4. Use in animal model
5. Stem cell banking

Table 1a. List of papers included.

Author	Year	Study design	Target cells	Journal	IF
Abedini F	2007	Case report <i>In vitro</i> study	Embryonic origin	Pak J Biol Sci	–
Alipour R	2013	Case report <i>In vitro</i> study	Culture	Iran J Allergy Asthma Immunol	0.968
Alipour R	2010	Case report <i>In vitro</i> study	Adipocytes	Int J Prev Dent	–
Bakopoulou A	2011	Case report <i>In vitro</i> study	Osteoblasts, odontoblasts	Calcif Tissue Int	2.376
Bento LW	2013	Comparative study <i>In vitro/vivo</i> study (mice)	Endothelial cells	J Dent Res	3.486
Casagrande L	2010	Case report <i>In vitro/vivo</i> study (mice)	Odontoblasts	J Dent Res	3.486
Chadipiralla K	2010	Case report <i>In vitro</i> study	Osteoblasts	Cell Tissue Rev	3.114
Cordeiro MM	2008	Case report <i>In vivo</i> study (mice)	Endothelial cells, odontoblasts	J Endod	2.880
Coyac BR	2013	Case report <i>In vitro</i> study	Culture	J Dent Res	3.486
De Mendonca Costa A	2008	Case report <i>In vivo</i> study (mice)	Osteoblasts	J Craniofac Surg	0.822
Eslaminejad MB	2010	Case report <i>In vitro</i> study	Osteoblasts, odontoblasts, adipocytic, chondrocytes	J Dent (Tehran)	–
Felthaus O	2012	Case report <i>In vitro/vivo</i> study (mice)	Osteoblasts	Differentiation	3.316
Galler KM	2008	Case report <i>In vitro</i> study	Osteoblasts	Tissue Eng Part A	4.022
Gioventù S	2012	Case report <i>In vitro</i> study	Stem cells, Storage cells	Transfus Apher Sci	1.321
Gosau M	2013	Case report <i>In vitro</i> study	Odontoblasts	Arch Oral Biol	1.603
Gotlieb EL	2008	Case report <i>In vitro</i> study	Odontoblasts	J Am Dent Assoc	1.773
Govindasamy V	2010	Comparative study <i>In vitro</i> study	Osteoblasts, adipocytes, chondrocytes	J Endod	2.880
Govitvattana N	2013	Case report <i>In vitro</i> study	Culture	Oral Dis	2.495
Hara K	2011	Comparative study <i>In vitro</i> study	Osteoblasts	J Endod	2.880
Hirata TM	2010	Case report <i>In vitro</i> study	Culture	J Endod	2.88
Inoue T	2012	Case report <i>In vivo</i> study (rats)	Cerebral stroke	Tissue Eng Part A	4.022
Ishkitiev N	2013	Case report <i>In vitro</i> study	Hepatocytes	Regen Med	3.873
Ishkitiev N	2012	Case report <i>In vitro</i> study	Hepatocytes	J Endod	2.880
Ishkitiev N	2012	Case report <i>In vitro</i> study	Hepatocytes	J Breath Res	2.541
Ishkitiev N	2010	Case report <i>In vitro</i> study	Hepatocytes	J Endod	2.880
Jarmalaviciute A	2013	Case report <i>In vitro</i> study	Neural cells	J Mol Neurosci	2.891
Kanafi MM	2013	Case report <i>In vitro</i> study	Culture	Cell Biol Int	1.64
Kanafi MM	2013	Case report <i>In vivo</i> study (mice)	Systemic disease (diabetes)	Cytherapy	3.275

Table continued

Table 1a (Continued). List of papers included.

Author	Year	Study design	Target cells	Journal	IF
Kerkis I	2006	Case report <i>In vitro/vivo</i> study (mice)	Osteoblasts, neural cells, myocytes, chondrocytes, storage cells	Cells Tissues Organs	2.2
Kerkis I	2008	Case report <i>In vivo</i> study (dogs)	Systemic disease (muscular dystrophy)	J Transl Med	3.47
Kim JK	2011	Case report <i>In vitro</i> study	Odontoblasts	J Endod	2.880
Koyama N	2009	Case report <i>In vitro</i> study	Osteoblasts, adipocytes, chondrocytic	J Oral Maxillofac Surg	1.64
Laino G	2006	Case report <i>In vitro/vivo</i> study (rats)	Osteoblasts, adipocytes, myocytes	J Cell Physiol	2.827
Li B	2012	Case report <i>In vivo</i> study (mice)	Osteoblasts	Oral Dis	2.495
Lizier NF	2012	Case report <i>In vitro</i> study	Stem cells	PLoS One	4.411
Ma L	2012	Case report <i>In vitro/vivo</i> study (mice)	Osteoblasts, odontoblasts, adipocytic, chondrocytic, endothelial cells, hepatocytes, neural cells	PLoS One	4.411
Mikami Y	2011	Case report <i>In vitro</i> study	Osteoblasts, adipocytes	Stem Cells Dev	4.459
Miura M	2003	Case report <i>In vitro/vivo</i> study (mice)	Osteoblasts, odontoblasts, neural cells	Proc Natl Acad Sci USA	9.681
Morsczeck C	2010	Comparative study <i>In vitro</i> study	Neural cells	Clin Oral Investig	2.364
Nakamura S	2009	Comparative study <i>In vitro</i> study	Stem cells	J Endod	2.880
Nam H	2009	Case report <i>In vitro</i> study	Epithelial cell	Biochem Biophys Res Commun	2.548
Nikolic N	2011	Case report <i>In vitro</i> study	Osteoblasts, myocytes, chondrocytic, adipocytes	Arch Biol Sci	0.36
Nishino Y	2011	Case report <i>In vivo</i> study (mice)	Epithelial cells	J Craniofac Surgery	0.822
Nishino Y	2011	Case report <i>In vivo</i> study (mice)	Osteoblasts	Cytotherapy	3.550
Nourbakhsh N	2011	Case report <i>In vitro</i> study	Neural cells	Int J Dev Biol	2.820
Osathanon T	2013	Case report <i>In vitro</i> study	Osteoblasts	J Cell Biochem	3.062
Pivoriunas A	2010	Case report <i>In vitro</i> study	Embryonic origin	Stem Cells Dev	4.459
Saha R	2011	Case report <i>In vitro</i> study	Osteoblasts, odontoblasts, adipocytes, chondrocytes	J Clin Pediatr Dent	0.444
Sakai K	2012	Case report <i>In vivo</i> study (rats)	Neural cells	J Clin Invest	12.812
Sakai VT	2010	Case report <i>In vivo</i> study (mice)	Odontoblasts, endothelial cells	J Dent Res	3.486
Seo BM	2008	Comparative study <i>In vivo</i> study (mice)	Osteoblasts	Oral Dis	2.495
Shekar R	2012	Case report <i>In vitro</i> study	Skin cells	Indian J Dent Res	0.665
Suchanek J	2010	Case report <i>In vitro</i> study	Culture	Acta Medica (Hradec Kralove)	–
Sukarawan W	2013	Case report <i>In vitro</i> study	Culture	Odontology	1.222
Taghipour Z	2012	Case report <i>In vivo</i> study (rats)	Neural cells	Stem Cells Dev	4.459

Table continued

Table 1a (Continued). List of papers included.

Author	Year	Study design	Target cells	Journal	IF
Takahashi N	2012	Case report <i>In vitro</i> study	Osteoblasts, adipocytes	Arch Oral Biol	1.603
Tarle SA	2011	Case report <i>In vitro</i> study	Culture	J Cell Physiol	3.874
Ueda M	2010	Case report <i>In vivo</i> study (mice)	Systemic disease (Dermatoheliosis)	J Craniofac Surgery	0.822
Vakhrushev IV	2012	Case report <i>In vivo</i> study (mice)	Osteoblasts	Bull Exp Biol Med	0.305
Vakhrushev IV	2010	Case report <i>In vitro</i> study	Osteoblasts, adipocytes	Bull Exp Biol Med	0.822
Viale-Bouroncle S	2012	Case report <i>In vitro</i> study	Osteoblasts	Differentiation	3.316
Wang J	2010	Case report <i>In vitro</i> study	Stem cells	Biochem Biophys Res Commun	3.583
Wang J	2010	Case report <i>In vitro/vivo</i> study (rats)	Systemic disease (Parkinson)	Stem Cells Dev	4.459
Wang X	2012	Comparative study <i>In vitro/vivo</i> study (mice)	Osteoblasts	Arch Oral Biol	1.603
Yamagata M	2013	Case report <i>In vivo</i> study (mice)	Hypoxic-Ischemic Brain Injury	Stroke	6.158
Yamaza T	2010	Case report <i>In vivo</i> study (mice)	Systemic disease (SLE)	Stem Cell Res Ther	3.21
Yan X	2010	Case report <i>In vitro</i> study	Stem cells	Stem Cells Dev	4.459

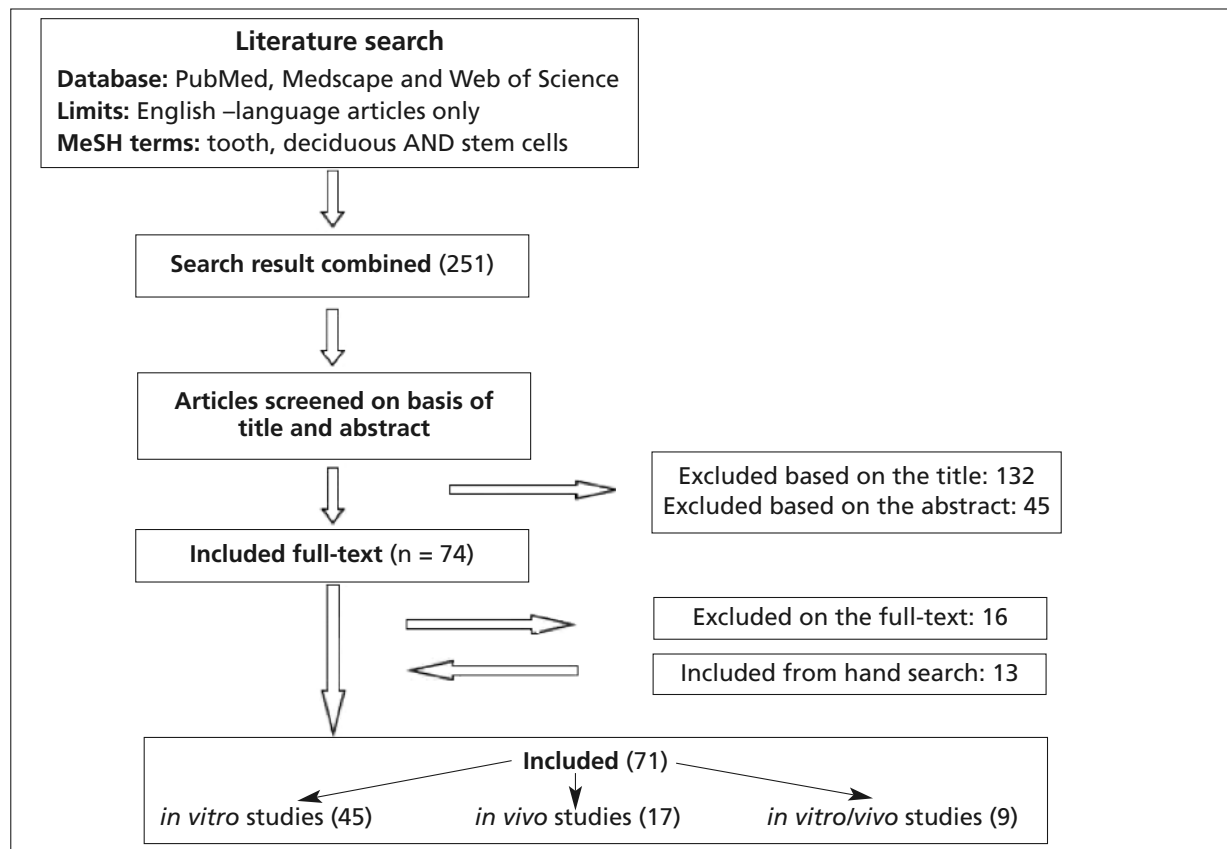

Figure 1. Flow chart of the screening process.

Table Ib. List of papers included from hand search.

Author	Year	Study design	Target cells	Journal	IF
Beltrao-Braga P	2011	Case report In vitro study	Stem cells	Cell Transplant	5.13
De Souza LM	2010	Comparative study In vitro study	Culture	Braz J Oral	–
Feitosa MLT	2010	Case report In vivo study (sheep)	Osteoblasts	Acta Cirurgica Brasileira	0.58
Govindasamy V	2011	Case report In vitro study	Pancreatic, Osteoblasts, chondrocytes, adipocytes	J Dent Res	3.486

Methods of Isolation and Culture

Dental pulp tissues were retrieved from exfoliated deciduous teeth, only in one study stem cells were retrieved from a discarded tissue waste⁸. Cell cultures were established using two different isolation methods, isolating two different populations: immature dental pulp stem cell (IDPSC) and SHED³.

For IDPSC isolation the minced pulp tissue was placed on culture dish with Dulbecco modified Eagle medium (D-MEM) supplemented with 15-20% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin and incubated at 37°C in 10% CO₂ using the direct cell outgrowth method. The outgrown cells at confluence were transferred to a large 75 cm² flask and then continuously passed for further experiments^{3,9-12}.

For SHED isolation, the dental pulp was minced and digested in a solution of 3 mg/ml collagenase type I and 4 mg/ml dispase for 1h at 37°C, using the enzymatic dissociation described for the first time in 2003^{2,7,12-36}.

After dental tissue explants and stem cells isolation, single-cell suspensions were obtained by passing the cell mass through a 40-70 µm pore size strainer, and cultured in growth medium. Two different media are commonly used in the literature:

1. α Modification of Eagle medium (α -MEM) supplemented with 20% fetal calf serum (FCS), 100 µmol/L l-ascorbic acid 2-phosphate, 2 mmol/L l-glutamine, 100 units/mL penicillin, 100 µg/ml streptomycin, at 37°C in 5% CO₂^{3,5,7,21,24,26,29,32-35,37-41}.

Table II. List of excluded full text papers and reasons for exclusion (SHEDs: Human Exfoliated Deciduous Teeth; BMSSCs: Bone Marrow Stromal Stem Cells; PDLSCs: Periodontal Ligament Stem Cells).

First author (year of publication)	Journal	Reason for exclusion
Coppe C (2009)	Pediatr Dent	Primary teeth extracted for caries
D'Aquino R (2007)	Cell Death Differ	Stem cells from permanent teeth
Iohara K (2008)	Stem Cells	Stem cells from animals teeth
Karaoz E (2010)	Histochem Cell Biol	Stem cells from neonatal tooth
Karaoz E (2011)	Histochem Cell Biol	Stem cells from permanent teeth
Lee JY (2011)	In Vitro Cell Dev Biol Animal	Effect of PRP on osteogenic differentiation of SHED
Nakahara (2006)	Dent Clin North Am	Stem cells from BMSSCs and PDLSCs
Oktar PA (2011)	Stem Cell Rev	Expression of nucleostamina during SHED proliferation
Pierdomenico L (2005)	Transplantation	Stem cells from permanent teeth
Siqueira da F. S (2009)	Cell Prolif	Stem cells from permanent teeth
Stevens A (2008)	Stem Cells Dev	Stem cells from permanent teeth
Suchanek J (2007)	Acta Medica Kralove	Stem cells from permanent teeth
Yamada Y (2010)	Tissue Eng Part A	Stem cells from animals teeth
Yamada Y (2011)	Cell Transplant	Stem cells from animals teeth
Yuk Kwan C (2010)	J Oral Pathol Med	Stem cells from permanent teeth
Zheng Y (2012)	J Dent Res	Stem cells from animals teeth

2. Dulbecco modified Eagle medium (D-MEM) supplemented with 10-20% fetal bovine serum (FBS), 100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin at 37°C in 10% CO₂^{2,4,8-10,13-15,17,19,20,22,23,25,28,31,35,36,42-46}.

In one study the knockout Dulbecco's modified Eagle's medium (KO-DMEM) supplemented with 10% FBS was used as basal medium, demonstrating that SHEDs can be expanded with the highest efficiency in this medium⁴⁷.

However, these culture media, supplemented with animal products, might contain toxic proteins or immunogens, capable of triggering an immune response, and animal pathogens, that increase the risk of contamination in humans⁴. This problem induced some Authors^{4,27} to test viability, proliferation, and immunocytochemical characteristics of the cells cultured in serum-free media, and those cultured in conventional serum-containing medium. These studies reported different results. Hirata et al⁴ using D-MEM with antibiotic and antimycotic additives supplemented by several combinations of grow factors, particularly 1% insulin-transferrin-selenium-X (ITS-X), noted a good survival rate, a better proliferation rate and the strongest expression of all the stem cell markers. Otherwise Tarle et al²⁷ found that the proliferation of cells in four different serum-free media (K-M) was significantly less than the proliferation of cells in standard serum-containing media (FBS-M). However when the pre-coated fibronectina (FM) tissue culture plates were used to provide growth and attachment support for cells grown in the serum-free medium, the SHEDs were able to proliferate at higher rates than cells cultured in serum-containing medium. Nevertheless the osteogenic potential and the multipotency were similar between SHEDs cultured in K-M versus those cultured in FBS-M²⁷.

The supplementation of basic fibroblast growth factor (bFGF) in culture medium seem to play a significant role in self-renewal ability and in stemness maintenance of SHEDs. In an *in vitro* study³⁶ colony forming unit efficiency and stem cell marker mRNA expression were increased by the short-term (48h) bFGF treatment and by the later passage of the long-term bFGF supplementation, though in this latter case the effect was not robust.

Characterization of SHEDs

All the studies selected confirmed that SHEDs had a morphology indistinguishable from human

embryonic stem cells in cultures (adherent cells with spindle-shaped, fibroblast-like appearance), and had a high proliferation ability.

The presence of stem cells population in SHEDs, larger than that present in DPSCs, was demonstrated by the expression of ABCG2 multidrug-transporter (the marker of side population) that was higher in SHEDs than in DPSCs teeth²⁵.

The proliferation rate of SHEDs was significantly higher than that of dental pulp stem cells (DPSCs) and bone marrow-derived mesenchymal stem cells (BMMSCs), suggesting that they represent a more immature population of multipotent stem cells^{2,5,10,13,17,25,29,36,45}.

In contrast with these data, in a study²⁰ in which stem cells isolated in the pulp of human third molar and in the deciduous incisors were compared, DPSCs tended to exhibit more proliferation capacity, significantly higher expansion rate, higher colonogenic activity and better growth curve than SHEDs.

In addition, SHEDs presented mesenchymal markers, such as CD105 (endoglin), CD90, CD146, and CD44 (cell adhesion receptor), expressed several growth factors (FGF, TGF-β, CTGF, NGF, BMP), which were involved in the cell proliferation and in the abundant extracellular matrix formation, and did not express hematopoietic markers, lymphocyte or leucocyte antigens, which excluded the contamination with other cells^{5,8,13,17,22,25,26,45,48-52}.

The stem cell markers of SHEDs seemed to be influenced by mechanical stress. After exposing SHEDs to a continuous pressure ranging from 0 to 2.5 g cm² for 2 h, Rex-1, Sox-2, Oct-4 and Nanog mRNA levels were increased and the increase in Rex-1 mRNA expression was in a force-dependent manner⁴⁶. In addition the mechanical stress dramatically induced the release of inflammatory cytokines IL-6 and IL-8 mRNA expression but not IL-β and TFN-α, suggesting the role of mechanical stress in the regulation of stemness via IL-6-Rex-1 interaction⁴⁶.

The proteins highly expressed in cytoplasmic and nuclear fractions of SHEDs, identified by proteomic analysis, revealed a proteomic profile very similar to that of MSC-like cells derived from other tissues⁵¹.

Furthermore, SHEDs seemed to have immune properties. Indeed in a *vitro* study⁵³ SHEDs were able to suppress the activation of human T lymphocytes, even if, comparing them to BMMSC, the suppression was alleviated and the mechanisms of immunosuppression were different.

Differentiation *in vitro*

The selected studies showed that SHEDs present a similar multilineage differentiation capacity to other MSCs, with the ability to differentiate *in vitro* and *in vivo* in osteoblasts, hepatocytes, adipocytes, neural cells, chondrocytes, myocytes, skin cells and odontoblasts.

Osteoblasts

All the analyzed texts reported that SHEDs can differentiate in osteoblasts.

The protocol was described for the first time by Miura⁷ as previously reported. After 7 days, the culture medium was switched to α -MEM supplemented with 10% FCS, 2 mmol/L L-glutamine, 10^{-7} mol/L dexamethasone sodium phosphate, 100 μ mol L-ascorbate-2-phosphate (ASC-2P), 1.8 mmol/L monopotassium phosphate (KH_2P_0_4) to give a final phosphate concentration of 2.9 mmol/L^{7,49,59}. After two weeks recombinant human BMP-4 was used to induce osteogenic differentiation. Western blot analysis showed an up-regulated expression of the osteogenic and angiogenic markers (CBFA1, ALP, MEPE), the bone sialoprotein (BSP) and the dentin sialophosphoprotein (DSPP), after induction⁷.

A similar culture protocol was used for the induction of odontogenic/osteogenic differentiation in SHEDs, isolated by two different methods (enzymatic dissociation vs direct outgrowth), in order to evaluate *in vitro* mineralized tissue formation and expression of differentiation markers³. SHEDs isolated using either method, when exposed to dexamethasone sodium phosphate, monopotassium phosphate, and β -glycerophosphate, gave rise to 3D mineralized structures, although SHEDs isolated by enzymatic dissociation expressed the mineralization rate and the amount of mineralized matrix higher compared to SHEDs isolated by direct outgrowth³.

To obtain a differentiation into osteoblasts Laino et al³³ sorted and replaced in the culture medium with a 20% FBS the selected SHEDs population, showing a significant positivity for c-kit STRO-1 and CD34. At day 30 of culture, sorted cells started to form aggregated centers, and at day 36 induced the formation of hemispheric ossification centers, that at day 40 grew up to build rounded 3D structures. The 3D woven bone samples, thus obtained, showed a strong positivity to alkaline phosphatase (ALP), alizarin red, calcein, other than to specific antibodies, and were remodeled in a lamellar bone containing osteocytes after *in vivo* transplantation into immunosuppressed rats.

The influence of the extracellular matrix stiffness on osteogenic differentiation of SHEDs was investigated using three different grades of stiffness (hard, medium, soft) of an elastic polydimethylsiloxane (PDMS) substrate, with a range from bone to connective tissue⁴². On a cell culture matrix with a bone-like stiffness, the proliferation of SHEDs increased, whereas it significantly decreased on a connective tissue-like surface. In addition a rigid surface in combination of dexamethasone increased the expression of osteogenic markers in SHEDs.

As a SHED-culture model, the three-dimensional (3D) dense collagen scaffold, made by plastic compression of pre-formed, highly hydrated collagen gels, was also used in a *in vitro* study⁵⁴. The increase in calcium and phosphate concentration, under osteogenic conditions, within the cell-seeded scaffolds compared with acellular scaffolds demonstrated that 3D scaffolds promote osteogenic/odontogenic cell differentiation and mineralization⁵⁴.

The osteogenic differentiation of SHEDs was inhibited by high-dose basic fibroblastic growth factor (bFGF) treatment through activation of the extracellular signal-regulated kinase (ERK) 1/2 pathway, resulting in a reduced mineralized nodule formation⁵⁵. The bFGF inhibited ALP mRNA expression, ALP enzymatic activity and mineral deposition via the FGFR and MEK signaling pathway, and could attenuate the Notch-signaling-induced mineralization⁵⁶.

The comparison between osteo-differentiation of SHEDs and dental pulp stem cells (DPSCs), periodontal ligaments stem cells (PDLSCs), and bone marrow mesenchymal stem cells (BMMSCs) was analyzed by some studies.

The osteogenic differentiation was stimulated in SHEDs and in DPSCs by using a media supplemented with 10% FBS, 10^{-6} mol/L dexamethasone, 10 mmol/L β -glycerol phosphate, and 100 mmol/L of L-ascorbic acid-2 phosphates, and both types of cells were able to differentiate into osteoblasts but SHEDs exhibited better differentiation capability^{5,17,34}.

In order to evaluate the ability of SHEDs to differentiate into osteoblasts, the expression status of cadherins (adhesive molecules in fibroblast-like cells derived from dental pulp of deciduous teeth) was evaluated in dental pulp cells derived both from permanent and deciduous teeth²¹. After 4 weeks in osteogenic differentiation medium, the level of R-cadherin was strongly expressed in DPSCs but not in SHEDs, while the

status of N-cadherin was comparable in both types of cells. Because the expression of R-cadherin was related to with the status of osteogenic differentiation, and the cells expressing R-cadherin did not express osteoblast-specific marker (osterix and osteocalcin), the Authors concluded that the R-cadherin expression restricts the multipotency of dental pulp cells and that the SHEDs were more able than DPSCs to differentiate into osteoblasts.

The influence of retinoic acid (RA) and dexamethasone (Dex) on the proliferation and osteogenic differentiation and on the osteogenic potential of SHEDs and PDLSCs were investigated and compared by Chadipiralla et al⁵⁷. The study has demonstrated that the treatment of RA significantly up-regulates the osteogenic expression and ALP activity in SHEDs after only 7 days, whereas the matrix mineralization was seen after 21 days, and that the supplementation with insulin enhanced RA-induced osteogenic differentiation of SHEDs. In PDLSCs the treatment of RA up-regulates only the Alp gene expression, though the significantly higher cell proliferation resulted in greater calcium deposition after 3 weeks of culture. The influence of RA on the osteogenic differentiation of SHEDs and PDLSCs has been proved to be stronger than those of Dex⁵⁷.

The comparison among SHEDs and BMMSCs culturing in medium for osteogenic differentiation showed a similar osteogenic potential and no apparent difference was detected between the two cell culture⁵⁰.

In one study focused on the characterization and mechanism of differentiation into mineralized tissue-forming cells of SHEDs as compared with BMMSCs⁴⁴, the gene expression profile revealed that SHEDs were involved in the bone morphogenetic proteins (BMP) signaling pathway. Moreover after BMP-2 stimulation, the expression levels of osteogenic related genes increased in both types of cells and the BMP-4 protein was expressed strongly in SHEDs but only mildly in BMMSCs, as confirmed by immunofluorescence.

The transcription factors TP53 (gene that induces cell cycle arrest, apoptosis, senescence, DNA repair, or changes in metabolism) and SP1 (a human transcription factor involved in gene expression in the early development of an organism) influenced the processes of cell proliferation and osteogenic differentiation in SHEDs and in dental follicle cells (DFC)⁵⁸. The transfection of

SP1 had no significant influence on the ALP activity of DFCs and SHEDs after 7 days of differentiation, while the overexpression of TP53 did not affect the proliferation but increased the ALP activity of DFCs and SHEDs significantly. The microarray data suggested that the influence of TP53 overexpression was more pronounced on DFCs, whereas the overexpression of SP1 had a stronger impact on SHEDs.

Adipocytes

The capacity of SHEDs to achieve an adipocytic differentiation was described in most of the studies.

Adipogenesis was obtained in the confluent cultures of SHEDs using inductive mixtures with differing components different in components (isobutyl-methylxanthine, indomethacin, hydrocortisone, dexamethasone insulin) and dosages^{5,7,8,17,20,21,33-35,45,49,59}.

Adipogenic differentiation was detected by the accumulation of lipid droplets, that were visualized by staining with oil red O reagent, and by the expression of adipogenic markers including peroxisome proliferators activated-receptor-alpha (PPAR-alpha), peroxisome proliferators activated-receptor-gamma (PPAR-gamma), and Lipoprotein lipase (LPL)^{20,35}.

The comparison among SHEDs and DPSCs confirmed a higher adipogenic differentiation capacity of SHEDs than DPSCs^{5,17,21}.

In contrast with the above results, Koyama et al reported that the expression of the adipogenic markers were observed in SHEDs, in DPSCs, and in BMMSCs, but that the development of lipid-laden adipocytes failed in SHEDs and in DPSCs whereas BMMSCs developed extensive sheets of abundant lipid-laden adipocytic clusters³⁵.

Neural Cells

The neural-differentiation potential of SHEDS was documented in many studies because these cells under non-neuronal inductive conditions expressed the neural markers, probably due to the neural crest cell origin of dental pulp^{7,49,59,60}.

Interestingly, neural differentiation of SHEDs took place about 4 times slower than that observed for neural differentiation of IDPSCs, both spontaneous or chemically induced with retinoic acid and dimethylsulfoxide⁹.

After 4 weeks of culture in the presence of B27 supplement, basic fibroblast growth factor and epidermal growth factor, SHEDs lost their fibroblastic appearance and gained neural-like

morphology, developing multiccytoplasmic processes, and showed the increased expression level of β III tubulin, GAD, and NeuN while expression levels of glial cell markers (nestin, GFAP, NFM, CNPase) remained unchanged^{7,49}.

To obtain mixed neuronal/glial cell culture a new neuronal differentiation protocol was proposed⁶¹. After standard cultivation medium, the cells were cultivated in SFM medium for 5-7 days and in the neurogenic medium for 7-10 days, then they were exposed for 14-21 days to 1.5 mM dibutyryl cAMP, 10 ng/ml nerve growth factor (NGF), 10 ng/ml glial cell line-derived neurotrophic factor (GDNF), and 10 ng/ml brain-derived neurotrophic factor (BDNF).

Furthermore, it has demonstrated that SHED-derived sphere-like (formed in neurogenic culture medium), after incubation with a cocktail of cytokines including sonic hedgehog (SHH), fibroblast growth factor 8 (FGF8), glial cell line-derived neurotrophic factor (GDNF), and forskolin, were able to generate a cell population contained specific dopaminergic neurons^{49,62}.

To evaluate the propensity toward neuronal lineage, SHEDs were compared with DPSCs and dental follicle cells (DFCs).

The comparison among SHEDs and DPSCs showed that the neurospheres formation and the expression of neuroectodermal markers, nestin, and neuronal marker were higher in the differentiate DPSCs into neuron-like cells, as compared with SHEDs, probably for the great expression of nestin present in DPSCs, that seems to be essential for induction of neurospheres⁵.

The comparison among SHEDs and DFCs showed that both these types of cells in standard culture medium had similar morphologies and similar gene expression patterns for stem cell markers, but only SHEDs expressed a marker of retinal stem cells (Pax6), that could indicate a good neural cell differentiation potential⁶³. After culture in different serum-replacement media (SRM), differentiated DFCs and SHEDs presented different neural cell marker expression patterns. In SRM supplemented with B27 culture, gene expression of the late neural cell marker microtubule-associated protein 2 (MAP2) was up-regulated in DFCs and down-regulated in SHEDs. In the presence of B27 supplement, basic fibroblast growth factor and epidermal growth factor, SHEDs formed neurosphere-like cell clusters and they expressed the glial fibrillary acidic protein (GFAP), that were weakly or not expressed in DFCs⁶³.

Chondrocytes

Chondroblast differentiation of SHEDs was obtained using inductive mixtures with differing components (insulin transferin selenium+, L-ascorbic acid-2 phosphate, sodium pyruvate, L-proline, transforming growth factor- β , dexamethasone, HEPES, BMP6, FBS, bovine serum albumin, penicillin, streptomycin) and dosages^{5,20,34,35,45,59}.

Kerkis et al⁹ obtained a chondrogenic differentiation also from IDPSCs replaced in a culture medium supplemented with retinoic acid and dimethylsulfoxide. The first signs of differentiation were detected after 1 week of culture.

The chondrogenic differentiation of SHEDs and IDPSCs was demonstrated through the presence of anti-chondroitin sulfate proteoglycan aggregation (ACSP-AG), anti-fetal cartilage proteoglycan antibodies and toluidine blue staining^{9,20,37}.

Both in the micromass cultures of SHEDs and DPSCs treated with BMP₂ the chondrocyte nodules appeared, but the levels of chondrogenic markers (type II collagen, type X collagen, and Sox 9) were significantly lower in DPSCs than in SHEDs³⁵.

Myocytes

The myogenic differentiation was induced supplementing control medium with 5% horse serum, 50 M hydrocortisone, 0.1 M dexamethasone and 2% FBS, and was characterized at 7 days by the formation of multinucleated myotubes, stained with cristal violet^{37,44,45}.

In IDPSC, spontaneous differentiation was also observed through manipulation of the seeding cell density and the time of the transfer into chemically defined medium (D-MEM supplemented with 20% Knockout Serum Replacement)⁹. SHEDs plated at low density (10^3) after 6 days were capable to differentiate into skeletal muscle, forming a large number of fully developed myofibrils with cross-striated morphology, as the immunostaining analysis with antibody against titin and α -actinin (sarcomeric) confirmed⁹. Instead SHEDs seeding at high density (10^6) were able to form multicellular smooth muscle nodules and spheroids, that expressed actin (differentiation into smooth muscle)⁹.

Endothelial Cell

SHEDs exposed to endothelial cell growth medium (EGM-2MV) containing rhEGF, hydrocortisone, 5% FBS, rhbFGF, R3-IGF-1, ascorbic acid, and 2 ng/mL rhVEGF, supplemented with

50 ng/mL rhVEGF 165 differentiated into VEGFR2-positive and CD31-positive endothelial cells *in vitro*⁴¹. In the regulation of the endothelia differentiation of SHEDs the key role was done by the VEGF/MEK1/ERK signaling pattern⁴¹.

Odontoblasts

To obtain odontoblast differentiation, SHED cultures were provided with D-MEM supplemented with 0.5 M vitamin D3, 50 mg/ml ascorbic 2-phosphate, 10 nM dexamethasone, and 10 mM β -glycerol phosphate²⁴. After three weeks of culture RT-PCR analysis showed the expression of odontoblast-specific genes, such as Dentin matrix protein (DMP1) and dentin sialophosphoprotein (DSPP) whereas the area of mineralized matrix was stained by alizarin red staining²⁰.

As previously cited concerning osteoblasts, SHEDs, isolated by two different methods (enzymatic dissociation vs direct outgrowth) showed different characteristics after odontogenic differentiation. SHEDs isolated by enzymatic dissociation produced quickly mineralized tissue but retained their spindle-shaped morphology, whereas SHEDs isolated by direct outgrowth, despite the lower mineralization rate, acquired the phenotypic characteristics of functional odontoblasts, with a typical cell elongation and polarization of the cell bodies vertical to the dentin-like matrix produced³.

SHEDs have a strong tendency to induce into odontoblasts as demonstrated after BMP-2 stimulation, by the high expression of dentin sialophosphoprotein (DSPP), and the high level of genes associated with odontogenesis and tooth morphogenesis^{48,64}.

Furthermore it was investigated the role in odontogenesis of mammalian target of rapamycin (mTor) kinase, that is the catalytic subunit of two distinct signaling complexes: target of rapamycin complex 1 and 2 (TORC 1 and 2)³⁸. The inhibition of TORC1 (regulator of protein synthesis, director of protein translation, necessary for cell growth, cell proliferation, and the cell cycle), seriously restricted the synthesis of dentin sialophosphoprotein (DSPP) and inhibited deposition of a mineralized matrix, while the inhibition of Torc 2 (involved in cytoskeleton reorganization and cell survival) enhanced mineralization³⁸.

To explore the possibility of endodontic regeneration, the adherence of SHEDs within dental pulp constructs was examined in the *in vitro* study. The dental pulp constructs, in which

SHEDs were seeded in two types of 3-D scaffolds (open-cell polylactic acid and bovine collagen) were inserted using various treatments into cleaned and shaped root canals of the extracted human teeth. The SEM micrographs showed the adherence of the dental pulp constructs to root canals was similar in all of the experimental groups, also if the adhesion to cleaned and shaped root dentin of SHEDs without any scaffold suggested that pulp stem cells did not need scaffolds for transplantation into teeth⁶⁵.

Hepatocytes

The differentiation of SHEDs in hepatocytes was treated for the first time by Ishkitiev et al²⁸ that compared SHEDs and DPSCs cultured for 3-4 weeks at 37°C in 5% CO₂ in a D-MEM supplemented with 10% fetus calf serum, 100 U/mL penicillin, and 100 mg/mL kanamycin. To obtain hepatic differentiation, when the cells reached 70% confluence, the culture medium was supplemented with 20 ng/mL recombinant human hepatocyte growth factor and 2% fetal calf serum for 5 days, and with a mixture of 10 ng/mL Onco-stat M, 10 nmol/L dexamethasone, and 1% Insulin-Transferrin-Selenium-X for another 15 days.

After exposition to the differentiation medium for 22-28 day, both SHEDs and DPSCs changed morphology from spindle shaped, fibroblast like cells, to polygonal and parenchymal-like cells. After differentiation, both cultures showed cells positive for specific hepatic markers (alpha-fetoprotein, albumin, and hepatic nuclear factor 4 alpha) and for insulin-like growth factor 1, besides urea production and the storage of glycogen indicated that these cells were functionally close to hepatic cells. No significant differences between SHEDs and DPSCs were observed, but an high contaminations of non-differentiated cells were detected²⁸.

For this reason, two years later the same Authors¹⁸ developed a protocol that use CD117 (membrane receptor for the stem cell factor related to mesenchymal, endothelial, and endodermal lineage cells) antibody and magnetic separation techniques for separating stem cell from human dental pulp and serum-free medium for their differentiation. After differentiation immunofluorescent analyses showed that both cell cultures were positive for all tested hepatic markers and, both cell types differentiated into a high-purity hepatic lineage. In the same year the Authors¹⁹ tested also the effect on hepatogenic differentiation of hy-

drogen sulfide (H₂S), that is a gas physiologically produced in many tissues including the liver, and discovered that hepatic markers, and urea and glycogen production were expressed more in the test groups exposed to H₂S at physiological concentrations than in the control group H₂S free control group.

Pancreatic Cells

Govindasamy et al³⁴ tested the potential of SHEDs to differentiate into pancreatic cell lineage resembling islet-like cell aggregates (ICAs). Differentiation of SHEDs in ICAs was carried out in 3 stages: undifferentiated SHEDs were re-suspended in Serum-free medium-A (containing Dulbecco's modified Eagle's medium Knock Out, 1% BSA Cohn fraction V, fatty acid free, 1x-insulin-transferrin-selenium, 4 nM activin A, 1mM sodium butyrate, and 50 µM 2-mercaptoethanol), and plated in a Petri dish; on the third day the medium was changed to SFM-B (containing DMEM-KO, 1% BSA, ITS, and 0,3 mM taurine); on the fifth day the medium was shifted to SFM-C (containing DMEM-KO, 1,5% BSA, ITS, 3 mM taurine, 100 nM glucagon-like peptide-1, 1 nM nicotinamide, and 1 x non-essential amino acids). The differentiation of SHEDs in ICAs was confirmed by the dithizone-positive staining (10 mg/mL dimethyl sulfoxide concentration) and by the expression of C-peptide, Pdx-1, Pax4, Pax6, Ngn3, and Isl-1.

Moreover, day 10 ICAs exhibited *in vitro* functionality releasing insulin and C-peptide in a glucose-dependent manner.

When the yield of ICAs generated with the same three-step protocol from SHEDs and DPSCs was compared, SHEDs produced more ICAs compared with DPSCs, with better acceptable size, although size differences were not significant between two groups⁶⁶.

The capacity of CD117+ SHEDs and CD117+DPSCs to differentiate into pancreatic cells was investigated *in vitro* by Ishkitiev et al⁶⁷ applying the same protocol used in hepatic differentiation. The cells were characterized with real-time reverse-transcription PCR for a panel of embryonal lineage markers. The number of expressed genes in the mesodermal lineage markers, endodermal lineage markers and axis/symmetry/segmentation markers was higher in SHEDs compared with DPSCs. On the other hand a higher expression of the ectodermal lineage markers, stem cell/embryonic developmental markers and other pluripotency markers

groups was found in DPSCs⁶⁷. After pancreatic differentiation *in vitro*, expression of pancreatic-specific endocrine markers glucagon, somatostatin and pancreatic polypeptide, and esocrine marker amylase-2a was lower in DPSCs compared with SHEDs while the expression of insulin remained weak in both cell cultures⁶⁷. To monitor pancreatic differentiation the expression of transcription factors related to embryonic development of pancreas was investigated. SHEDs after differentiation expressed PDX1 (a key factor for starting the development of all cell types in the pancreas), HHEX (factor required for proper embryonic developmental of entire pancreas), MNX1 (endodermal transcription factor in developing β-cells), NEUROG3 (a key factor for the developmental of endocrine pancreas), PAX4 (required for the normal development of β- and δ-cells), PAX6 (required for the development of the pancreatic endocrine cell types except α-cells), and NKK6-1 (a β-cell differentiation factor)⁶⁷.

Use in Animal Model

Transplantation

All *in vivo* studies we have analyzed, with the exception of those on Systemic disease and one about the bone tissue recovery following transplantation of IDPSC in sheep affected by induced osteonecrosis of femoral head, proposed to transplant SHEDs into immunosuppressed mice or rats, because these animals are deficient in thymus-derived lymphocytes, have a decreased cellular-mediated immune response, and accept tissue grafts from other species without the usual subsequent rejection of the foreign tissue⁶⁸. The ability of nude mice to support the growth of transplanted cells make these animals a good resource for the evaluation of the new developed tissues.

For *in vivo* transplantation SHEDs were combined with different scaffolds: hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder^{7,32,59} polylactoglycolide scaffold¹⁵, Peptide Amphiphile nanofibers⁴⁰, collagen membrane³⁵, ceramic bovine bone¹⁷, tooth slice/scaffold, combined with collagen membrane⁶ or without any scaffold³³.

Osteoblasts

To investigate *in vivo* the potential to induce bone formation, SHEDs mixed with 40 mg of hydroxyapatite/tricalcium phosphate (HA/TCP) ce-

ramic powder were transplanted into immunocompromised mice⁷. SHEDs proved to be unable to differentiate directly into osteoblasts but, forming an osteoconductive template, they were capable of inducing recipient murine cells to differentiate into osteocytes and osteoblasts⁷. In addition all transplanted single-colony-derived SHED lines were able to induce bone formation on the surface of HA/TCP, although in varying quantities (approximately 40% of the clonal cell strains induced a significant amount of new bone, whilst the 60% induced a very limited amount of bone)⁷.

In contrast with these results, SHEDs mixed with 40 mg of HA/TCP ceramic powder as a carrier were able to repair with substantial bone formation critical-size calvarial defects in immunocompromised mice and, after 6 months post-transplantation, SHEDs maintained bone continuity and complete calvarial repair³². Furthermore SHEDs actively contributed to bone formation and not only induced recipient murine cells to differentiate into osteogenic, as confirmed by human *alu* in situ hybridization³².

When SHEDs, treated with high dose of b-FGF in vitro and mixed with 40 mg of HA/TCP ceramic powder, were subcutaneously transplanted into immunocompromised mice showed a reduced expression level of MSC markers STRO-1, CD146, CD90, and CD73 and a lower osteogenic differentiation, suggesting that b-FGF treatment partially attenuate SHED differentiation⁵⁵.

The osteogenesis capacity of SHEDs was demonstrated implanting into subcutaneous of immunocompromised mice aggregates of SHEDs and DPSCs with ceramic bovine bone (CBB)¹⁷. SHEDs transplants showed the formation of bone-like structures on the surface of the CBB, that were located away from the murine host osteogenic cells, with no haematopoietic marrow elements¹⁷. On the contrary DPSCs transplants mainly formed connective tissue with small amounts of mineralized tissue, implying a lower capacity for osteogenic differentiation than SHEDs¹⁷.

The transplantation of IDPSC in sheep affected by induced osteonecrosis of femoral head was also investigated¹¹. Feitosa and coll. demonstrated that IDPSC were able to proliferate and to give a better bone regeneration than that observed in the control group.

Neural Cells

Neuronal developmental potential was studied *in vivo* by injecting SHEDs, cultured in the neur-

al-differentiation medium, into the dentate gyrus of the hippocampus of immunocompromised mice⁷. Histological examination demonstrated that SHEDs survived inside the mouse brain for more than 10 days and that they continued to express neural markers⁷.

The capacity of SHEDs and IDPSCs to promote locomotor functional recovery in spinal cord injury (SCI) was evaluated in a rat model^{60,69}.

In the study of Taghipour et al⁶⁰ SHEDs and IDPSC transplantation in acute contused injured spinal cord (SCI) resulted in significant locomotor improvement (higher in IDPSCs than in SHEDs), that could be attributed to their production of trophic factors and the differentiation potential to neural cells. However, when compared with the SHED group, the IDPSC group showed more myelin basic protein (MBP) and chondroitin sulfate proteoglycan NG2 expression – oligodendrocyte markers – and less glial fibrillary acidic protein (GFAP) – astrocyte marker – in comparison with the SHED group⁶⁰.

Three major neuroregenerative activities of SHEDs were identified by Sakai et al⁶⁹, by transplanting them into a completely transected rat SCI model: inhibition of the apoptosis of neurons, astrocytes, and oligodendrocytes, which improved the preservation of neuronal filaments and myelin sheaths; regeneration of the transected axons by directly inhibiting multiple axon growth inhibitors, including chondroitin sulfate proteoglycan and myelin-associated glycoprotein; replacement of lost or damaged cells by differentiating into mature oligodendrocytes. Remarkably, the animals that received transplantation of BMSCs or skin-derived fibroblasts exhibited substantially less recovery of locomotor function⁶⁹.

The effects of SHEDs compared with those of BMSCs on ischemic tissue injury in the rat brain after permanent middle cerebral artery occlusion (pMCAO) was also investigated¹³. SHEDs, administered intranasally in rats subjected to pMCAO, promoted the migration and differentiation of endogenous neuronal progenitor cells in the subventricular zone, promoted the angiogenesis and neurogenesis, and ameliorated ischemic brain injury¹⁴. It was interesting that progressive improvement in motor disability and decrease in infarct volume became significant in the SHED group with respect to the BMSC group¹⁴.

Odontoblasts

The ability of SHEDs to differentiate into odontoblasts *in vivo* was tested transplanting the mixtures of SHEDs and hydroxyapatite/tricalcium phosphate (HA/TCP) ceramic powder into immunocompromised mice⁷. The human-specific-alu-positive odontoblasts, yielded by SHED transplants, were directly associated with a dentin-like structure formation on the surface of HA/TCP carrier, but they were unable to regenerate a complete dentin-pulp-like complex as do DPSCs *in vivo*⁷. However when the single-colony-derived SHED strains were transplanted, only one fourth of the clones demonstrated to be able to generate ectopic dentin equivalent to that generated by multicolony-derived SHEDs⁷.

To demonstrate the capacity to differentiate into odontoblast-like cells, SHEDs seeded in the tooth slice/scaffold were transplanted into the subcutaneous tissue of the dorsum of immunodeficient mice. The porous biodegradable scaffold (poly-L-lactic acid) filled the space of the pulp chamber, in close contact with predentin layer, in the tooth slice retrieved from extracted third molars. In this tissue construct, after a period of 14–28 days, the cells adjacent to the predentin showed findings of active dentin-secreting odontoblasts, including expression of DSP, eccentric polarized nuclear position at the basal part of the cell body, the presence and position of cell-cell gap junctions, a well-developed rough endoplasmic reticulum, a well-developed Golgi's complex and numerous vesicles^{32,39}. SHEDs were also able to differentiate into endothelial-like cells, as demonstrated by β -galactosidase staining of cells lining the walls of blood-containing vessels in tissues with LacZ tagged SHEDs, and the blood vessels were capable of anastomosing with the host vasculature^{32,39,41}.

On the contrary, SHEDs cultured in scaffold without dentin did not express markers of odontoblastic differentiation, such as dentin matrix protein (DMP-1), dentin sialophosphoprotein (DSPP), and matrix extracellular phosphoglycoprotein (MEPE)⁶⁴.

Skin Cells

To examine the effect on wound healing of SHEDs, the 8-mm full-thickness skin defects created on the dorsal surface of nude mice were treated respectively with phosphate-buffered saline (control group), basic fibroblast growth factor (b-FGF), SHEDs and SHEDs with b-FGF^{23,70}.

In SHED group the wound healing was accelerated, via increasing collagen production, compared with the control group, but there were no statistically significant differences between the SHED group and b-FGF group, whereas in the presence of b-FGF, SHEDs enhanced more efficient wound healing^{23,70}.

The role of SHEDs in the treatment of photoaging and wound-healing was also investigated inducing wrinkles in hairless mice after an 8-weeks regimen of UV-B irradiation². The subcutaneous injection of SHEDs and the intradermal injection of SHED-derived conditioned medium (SH-CM) in the restrict area were able to improve the induced fine wrinkles and to increase dermal thickness, also if SHEDs were more effective than SH-CM².

No items found for hepatocytes, adipocytes, chondrocytes and myocytes.

Treatment of Systemic Diseases

SHEDs and IDPSCs were utilized for treatment of systemic diseases in animal models, such as neurodegenerative diseases (Alzheimer, Parkinson and Amyotrophic Lateral Sclerosis), Perinatal Hypoxic-Ischemic Brain Injury (HI), Type 1 Diabetes, Lupus Erythematosus (SLE)-associated disorders, Dermatoheliosis, Muscular Dystrophy, osteoporotic disorder and osteonecrosis of the femoral head, and a significant improvement of all these disorders was found.

The therapeutic effect of SHEDs in *neurodegenerative diseases* was investigated, transplanting into the striatum of parkinsonian rats basic medium (negative control), intact SHEDs and SHED-derived spheres, induced into dopaminergic neurons after incubation with a cocktail of cytokine (SHH, FGF8, GDNF, and forskolin)⁶². Partial improvement of apomorphine-evoked rotation, starting 2 weeks after transplantation and sustained for up to 6 weeks, was observed in the group transplanted with SHED-derived spheres, and was similar to the intact SHED group for 2–4 weeks after transplantation, but significantly different after 4 weeks⁶². This behavioral recovery was not observed in the control group, that received basic medium without cytokine, and there were no significant differences in the 3 groups after 8 weeks of transplantation⁶².

The intracerebral administration of SHEDs into the injured brain of the day 5 mice significantly reduced the HI-induced brain-tissue loss and improved neurological function and the survival

rate. The engrafted SHEDs rarely differentiated into neural lineage, but their transplantation inhibited the expression of pro-inflammatory cytokines, increased the expression of anti-inflammatory ones, and reduced apoptosis of neurons⁷¹.

The management of type 1 diabetes was studied by transplanting subcutaneously ICAs derived from SHEDs, packed in immune-isolatory biocompatible macro-capsules, into streptozotocin (STZ)-induced diabetic mice⁶⁶. 90% of transplanted mice survived and were restored to normoglycemia within 2 weeks after operation and maintained normoglycemia for 2 months, while their body weight and glucose level in urine reverted to normal levels⁶⁶.

Furthermore, the removal of macro-capsule-containing ICAs did not cause hyperglycemia, indicating endogenous pancreatic regeneration, confirmed by histopathological investigations⁶⁶.

Systemic SHED infusion in SLE murine model was utilized to study the immunomodulatory functions and to evaluate the therapeutic efficacy to treat *Systemic Lupus Erythematosus (SLE)-like autoimmune disorders*^{59,72}. SHEDs transplantation was able to reduce levels of autoantibodies to Ana and anti-dsDNA IgG, decrease the peripheral immunoglobulins, such as IgG1, IgG2a, IgG2b, IgM, improve renal function, and prolong the lifespan^{59,72}.

In comparison to BMMSCs, SHEDs provided more significant immunomodulatory effects in up-regulating level of the ratio between regulatory T cells (Tregs) and Th17 cells, and in reducing Th17 cells levels in the peripheral blood. In addition, as seen in BMMSCs, SHEDs were capable of recovering trabecular bone and inhibiting osteoclast activity^{59,72}.

Ueda et al (2010)² investigated, for the first time, the interaction between SHEDs and Human Dermal Fibroblast (HDF) for the treatment of Photoaging (*Dermatoheliosis*) in nude mice after an 8-week regimen of UV-B irradiation. Cells cultured in D-MEM containing 20% mesenchymal cell growth supplement and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin, and 0.25 mg/mL amphotericin B), possessed high proliferation ability, were enriched with extracellular matrix, and expressed several growth factors such as fibroblast growth factor, transforming growth factor, connective-tissue growth factor, nerve growth factor, and bone morphogenetic protein. The Authors concluded that SHEDs should be constitutionally suited for photoaging treatment.

To analyze the IDPSCs ability for migration, engraftment, myogenic potential, and expression of *human dystrophin in affected muscles*, IDPSCs were transplanted in four golden retriever dogs with muscular dystrophy (GRDM) by local (muscular) or systemic (arterial) injection without any immunosuppression⁴⁴. IDPSCs were capable of migrating and engrafting into GRDM dog and they were able to form chimeric canine-human muscle, although the expression of human dystrophin was modest and limited to several muscle fibers. Therefore the clinical benefit observed in treated animals would seem to be due to the immunomodulatory effect of IDPSCs and not due to dystrophin expression in the host muscles. Moreover, no signs of immune rejection were observed, and systemic multiple deliveries (monthly arterial injections) seemed more effective than local injections⁴⁴.

SHEDs were also able to improve *osteoporotic disorder* in nude mice's long bones. Seven days after the infusion into bones an increment of BMD and new trabecular bone were observed. Flow cytometry revealed that CD4⁺, IL-17⁺, IFN γ , Th17 cells were significantly reduced in bone marrow cells compared to the control and osteogenic analysis showed that Alizarin Red-positive area was shared larger in SHED than Control. These data indicated the immunomodulatory functions of SHED to improve osteoporotic disorder⁵⁹.

IDPSCs were used in the cellular therapy in (sheep) ovine with *osteonecrosis of the femoral head* induced by intra-bone injections of ethanol. The histological data suggested that the trabecular bone was better organized and the bone regeneration was faster in the sheep that received inoculation of IDPSCs than in the animals submitted to central decompression alone¹¹.

Stem Cell Banking

Tooth banking creates a unique opportunity to store SHEDs with a view of treating diseases or injuries that could occur during one's life. Furthermore, SHEDs are complementary to stem cells from cord blood, but the cost of storage is much lower.

The teeth (only those with red pulp, with not apical abscesses, tumors or cysts) are transferred into a container with hypotonic phosphate buffered saline solution to prevent the drying. This container is placed inside another metal container and it had to arrive in the tooth bank 40 within hours. When the tooth arrived

in the bank the stem cells are isolated and cultured in accord with the previous described protocol. Then for the cryopreservation, the cell culture maintained semiconfluent are resuspended in a medium containing 20% FBS, 70% DMEM, and 10% dimethylsulfoxide (DMSO) at 5×10^5 cells/ml, and the temperature was slowly and gradually decreased by 1°C per minute until -70°C ⁸. Then, the cells were transferred to liquid nitrogen vapor at less than -150 - 196°C or less. The cells can remain cryopreserved for a long time and, when needed, they can be thawed into a 37°C water bath for 2 minutes, washed twice with 20% FBS and 70% DMEM, and placed into culture⁹.

Also the dental pulp separated from exfoliated deciduous teeth can be cryopreserved. The pulp removed en bloc mechanically is mixed with a cryopreserved medium (containing 10% DMSO and 90% FBS) at 4°C , kept overnight at -80°C and then transferred into liquid nitrogen and store for 25-30 months⁵⁹.

To simplify the initial cryopreservation protocol, delaying complex processing procedures at the moment of actual use, a new method to cryopreserve SHEDs inside the whole teeth, without the tooth mechanical fracture and in vitro expansion, was proposed⁷³. All the whole teeth were pierced on the crown-root connection with an Nd/YAG laser beam, for making micro-channels to allow the cryopreservation solution to reach the dental pulp, and cryopreserved at -80°C with DMSO⁷². *In vitro* analysis showed that morphology, immunophenotype, viability and proliferation rate of SHEDs extracted from teeth cryopreserved with laser piercing were similar to those of cells isolated from fresh teeth. Conversely, SHEDs isolated from teeth cryopreserved without laser piercing showed a significant loss of cell viability and a very low proliferation rate⁷³.

Conclusions

Stem Cells from Human Exfoliated Deciduous Teeth, described for the first time more than 10 years ago, display multifactorial potential such as strong and high proliferative capacity, easy accessibility, high viability and multilineage differentiation capacity. Their retrieval is relatively simple and non-invasive, no risks for developing immune reactions or rejection following transplantation exist and no immunosuppressive therapy is needed.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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