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RESEARCH ARTICLE

The Lamina Propria of the Oral Mucosa Harbors a Neural Crest-Like Stem Cell Population Resistant to Hyperglycemia Induced by Diabetes Type II

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ABSTRACT

Oral mucosa stem cells (OMSC) are contained within niches in the lamina propria of the oral mucosa. In vitro, OMSC exhibit a primitive neural crest-stem cell phenotype and differentiate into neural crest-derived cell lineages. Clinical evidence indicate that wound healing of oral mucosa is scarless and negligibly affected by diabetes. Since stem cells play an important role in wound healing we hypothesized that in contrast to adult mesenchymal stem cells, OMSC are negligibly affected by diabetes type II hyperglycemia.

The capacity of murine OMSC (mOMSC) obtained from diabetic db^+/db^+ mice (DmOMSC) to proliferate, express pluripotency-associated markers (PAM) (oct4, sox2, nanog) and neural crest stem cell markers (NCSM) (Klf4, c-Myc, twist, nestin), and differentiate along neural crest cell lineages was compared to that of OMSC derived from background WT C57BL/6 mice (WT-mOMSC) and to those of murine adipose tissue stem cells (mASC) derived from the same animals used for generating mOMSC.

WT-mOMSC capacity to proliferate, express PAM and NCSM and differentiate was higher than that of WT-mASC. Diabetes reduced the proliferation rat of DmOMSC and db^+/db^+ derived mASC (DmASC) by 35% and 42%, respectively. Diabetes did not affect the expression of PAM and NSCSM in DmOMSC and nor their differentiation as compared to WT-mOMSC, but the diabetic state substantially reduced these parameters in DmASC as compared to WT-mASC and DmOMSC. We demonstrated that WT-mOMSC administration enhances wound healing in the db^+/db^+ diabetic wound skin model. To test the effect of diabetes on the therapeutic functionality of DmOMSC, the ability of these cells to enhance diabetic wound healing was assessed in this model. The results indicate that DmOMSC increased the rate of wound healing in db^+/db^+ mice as compared to placebo treated controls. The magnitude of this therapeutic effect is statistically equivalent to that of WT-mOMSC. Collectively, these data demonstrate that: i) diabetes decreased moderately DmOMSC proliferative ability; ii) in contrast to mASC, mOMSC withstand the detrimental effects of diabetes; and iii) the therapeutic properties of DmOMSC are mildly affected by long standing diabetes.

Keywords: Diabetes, Stem cell, Oral mucosa, Hyperglycemia, Wound healing, AGE

Introduction

Diabetes can significantly impact the prevalence and functionality of stem cells in various ways, leading to compromised tissue homeostasis, repair and regeneration. The diabetic microenvironment, characterized by hyperglycemia, oxidative stress, inflammation, and abnormal cytokine levels, can negatively affect both the quantity and functionality of stem cells. Recent studies point to reduced cell proliferation, migration and differentiation and increased apoptosis of bone marrow and adipose tissue-derived mesenchymal stem (stromal) cells in diabetic organisms[1, 2, 3]. Increased levels of advanced glycation end (AGE) products, formed due to long standing hyperglycemia, have been shown to negatively modulate mesenchymal stem cell activity[4], probably by inducing genetic and epigenetic modifications at the chromatin and DNA levels[5, 6]. Diabetes was shown to decrease mesenchymal progenitor subpopulation in the bone marrow[7]. The pool, mobilization, circulation and homing of circulating endothelial cells (cEPC) to wound sites are impaired in diabetes, thereby impeding angiogenesis and neovascularization[8]. Similarly, diabetes impairs the mobilization of hematopoietic cells[3, 9] and of stromal bone marrow stem cells to the tissues and organs in need by down regulating CXCR4, the receptor for CXCL12 and by increasing the amount of adhesion molecules in the stem cell niche[10].

Diabetic foot ulcers (DFUs) are a common but highly morbid complication of long-standing diabetes, carrying high rates of infection and are associated with major amputation and mortality[11]. The pathophysiology at the basis of this complication consists of sensory and motor neuropathy, microvascular disease and minor trauma that lead to wound formation[12]. Peripheral artery disease and the consequent ischemia of the foot is an additional co-etiologic factor for DFU development[13]. However, a less addressed factor is the reduced capacity of the wounded diabetic tissues to undergo repair due to epidermal and dermal resident stem cell dysfunction and the incapacity of bone marrow-derived hematopoietic and mesenchymal stem cells to leave their niche and migrate to wounded tissues[3]. Thus, a logic therapeutic approach would be to deliver ex-vivo expanded mesenchymal stem cells (MSC) at the wound site[14]. Currently, bone marrow and adipose tissue-derived mesenchymal stem cells (BM-MSC and ASC, respectively) or their secretome have been proposed as adequate candidates for the treatment of DFU[15, 16].

We published that human oral mucosa stem cells (hOMSC) are a neural crest-like stem cell population contained within niches in the lamina propria of the adult oral mucosa[17]. hOMSC differentiate in vivo and in vitro into ectodermal, endodermal and mesenchymal derived linages and have been found to have therapeutic capacities in a variety of preclinical models[17, 18, 19, 20, 21, 22]. We have also recently published that allogeneic murine oral mucosa stem cells (mOMSC) enhance skin wound healing in diabetic db^{+}/db^{+}mice[23]. These studies point to the therapeutic potential of autologous oral mucosa stem cells in a variety of chronic diseases as diabetes and neurodegenerative diseases. In the present study we tested whether prolonged exposure of murine oral mucosa stem cells to the diabetic hyperglycemic state in vivo affects their expansion, phenotype and their in vivo therapeutic potential.

Materials and Methods

MATERIALS

The following materials were used for cell culture and expansion, harvesting and freezing:

Fetal Bovine Serum, L-Glutamine Low Glucose DMEM, Low Glucose MEM-Alpha, MEM-Eagle Non-Essential Amino Acids, Ribonucleosides & Deoxyribonucleosides, Pen-Strep-Nystatin, Serum Free Freezing Medium, Sodium Pyruvate, Trypsin-EDTA 0.25%, Phosphate Buffered Saline with Ca,Mg& w/o Ca, Mg were obtained from Sartorius, Beit Haemek, Israel. For FACS, IF, qRT PCR and differentiation experiments: Ascorbic Acid , Bovine Serum Albumin, Dexamethasone, Indomethacin, Isobutylmethylxanthine Isopropanol, Hematoxylin, Oil Red, Triton X-100 were obtaned from Sigma-Aldrich, St. Louis, MO, USA. The following material were obtained from different suppliers: Activin A (PeproTech, Rocky Hill, NJ, USA), DAPI Mounting Medium (Vector Laboratories, CA, USA), KAPA2G Fast HotStart Genotyping PCR Mix (Kapa Biosystems, USA), iScript cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA), SYBR Green (Applied Biosystems, Life Technologies, CA, USA), Tegaderm (3M, USA), TRIzol Reagent (Invitrogen, USA), Hematoxylin (Sigma-Aldrich, St. Louis, MO, USA), KAPA2G Fast HotStart Genotyping PCR Mix (Kapa Biosystems, USA), Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA)

TRIzol Reagent (Invitrogen, USA), Well plates, culture dishes (Nunc, Thermo Fisher Scientific, Denmark)

The following antibodies obtained from R&D Systems, USA were used for flow cytometry and immunohistochemistry: Anti –h/m Oct4 Phycoerythrin Conjugated Rat IgG 2B, Isotype

Control Phycoerythrin Conjugated Rat IgG, Anti – h/m Sox2 Phycoerythrin Conjugated Mouse IgG 2A, Isotype Control Phycoerythrin Conjugated Mouse IgG, Anti-h/m Nanog Phycoerythrin Conjugated Goat IgG, Isotype Control Phycoerythrin Conjugated Goat IgG (R&D Systems, USA).

The following antibodies were obtained from Abcam UK: Anti-h/m Klf4, Anti-h/m C-Myc, Rabbit Polyclonal to Sox17 Antibody, Rabbit Polyclonal to FoxA2 Antibody, Rabbit Polyclonal to Gfap Antibody, Rabbit Polyclonal to S100-β Antibody, Rabbit Polyclonal to Cd31 Antibody, Rabbit Polyclonal to Ki67 Antibody.

Primers for RT PCR (Sigma-Aldrich):
Gapdh forward: 5'-GCACAGTCAAGGCCGAGAAT-3'
Gapdh reverse: 5'-GCCTTCTCCATGGTGGTGAA-3'
Oct4 forward: 5'-GTTGGAGAAGGTGGAACCAA-3'
Oct4 reverse: 5'-CTCCTTCTGCAGGGCTTTC-3'
Sox2 forward: 5'-TCCAAAAACTAATCACAACAATCG-3'
Sox2 reverse: 5'-GAAGTGCAATTGGGATGAAAA-3'
Nanog forward: 5'-GGTTGAAGACTAGCAATGGTCTGA-3'
Nanog reverse: 5'-TGCAATGGATGCTGGGATACTC-3'
Klf4 forward: 5'-TGGGAACTTGACCATGATTG-3'
Klf4 reverse: 5'-GTGCCCCGACTAACCGTTG-3'
C-Myc forward: 5'-TGAGGAGACACCGCCCAC-3'
C-Myc reverse: 5'- CAACATCGATTTCTTCCTCATCT-3'
Snail forward: 5'-TCTGAAGATGCACATCCGAAGCCA-3'
Snail reverse: 5'-AGGAGAATGGCTTCTCACCAGTGT-3'
Nestin forward: 5'-AGGCTGAGAACTCTCGCTTGC-3'
Nestin reverse: 5'-GGTGCTGGTCCTCTGGTATCC-3'
Twist forward: 5'-TCCGCAGTCTTACGAGGAGCT-3'
Twist reverse: 5'-GCTGGACTCCAAGATGGCAAG-3'
Sox17 forward: 5'-CGCTGTAGACCAGACCGCGAC-3'
Sox17 reverse: 5'-TACGGGACTCGCCCTTGGCT-3'
Foxa2 forward: 5'-TGGGAGCGGTGAAGATGGAAGGGCAC-3'
Foxa2 reverse: 5'-TCATGCCAGCGCCCACGTACGACGAC-3'
Gfap forward: 5'-GCCACTGTGAGGCAGAAGCT-3'
Gfap reverse: 5'-TGGCTTCATCTGCTTCCTGTCTA-3'
Bdnf forward: 5'-AGCTCCGGGTTGGTATACTGG-3'
Bdnf reverse: 5'-CCTGGTGGAACTTCTTTGCG-3'
Eaat1 forward: 5'-CGAAGCCATCATGAGACTGGTA-3'
Eaat1 reverse: 5'-TCCCAGCAATCAGGAAGAGAA-3'

Methods

ANIMALS

Two strains of mice were used for the purpose of the below described experiments: C57BL/6 mice were derived from a colony of db^+/db^- mice. The colony was maintained at the SPF facility of Tel Aviv University. The background of the db/db animals is C57BL/6J. Colony maintenance and the experimental protocols described below were approved by the Ethic Committee of Tel Aviv University.

The offsprings of this colony are:

1. 25% homozygous for the Lepr gene - db^+/db^+. These animals manifest morbid obesity, chronic hyperglycemia, pancreatic beta cell atrophy and become hypoinsulinemic. Obesity starts at 3 to 4 weeks of age and at 8 weeks they suffer of overt diabetes. They served as diabetic animals in all the experiments described below. The homozygotic nature of these animals was assessed by PCR.
2. 50% heterozygous animals - db^+/db^-. These animals were used as mating animals for maintaining the colony.
3. 25% of homozygous animals negative for Lepr gene – db^-/db^-. These animals served as the wild type control animals in our experiments. They also served as the source for the generation of mOMSC (murine oral mucosa-derived stem cells).

By using diabetic mice and wild type mice from the same colony with the same background, we actually excluded the possibility of strain interference.

DIAGNOSIS OF DIABETES

The level of glycemia was assessed by obtaining blood from the tail vein of the db^+/db^+ phenotype. A glucometer (Contour, Bayer) served for measuring non-fasting blood glucose levels. An animal with levels >250 mg glucose/dL was considered diabetic. Five to six weeks old animals whom non-fasting glucose levels were between 120- 150 mg glucose/dL were considered prediabetic.

CELL TYPES GENERATION

Wild type, prediabetic and diabetic murine oral mucosa-derived stem cells (WT-mOMSC, Pre-DmOMSC and DmOMSC, respectively) were obtained from the alveolar mucosa and gingival biopsies of the mice. The tissues obtained from oral mucosa biopsies were cut into small fragments that were incubated in Dulbecco`s Modified Eagle`s Low Glucose Medium (DMEM LG) with 10% Fetal Bovine Serum (FBS). Primary cultures obtained in approximately 7-8 days were further expanded in the same medium. mASC were obtained from the adipose tissue of the mice. Adipose tissue was digested in 50 ml of freshly prepared collagenase digestion solution (1 gr collagenase in 50 ml of PBS) at 37°C for 30 minutes. Following incubation, the suspension was centrifuged at 1600 rpm for 5 minutes, the pellet was washed twice with PBS, and seeded in the 25 m^2 flasks. Expansion medium was

added. After one week the cells were harvested, counted and passaged.

Cell cultures were expanded in 75 cm2 flasks in 8 ml DMEM LG, supplemented with 2mM L-Glutamine, 1 mM Sodium Pyruvate solution, 1% Mem-Eagle Non-Essential Amino Acids solution, 1% Pen-Strep-Nystatin solution, 0.2% Mem-Alpha-Ribonucleosides & Deoxyribonucleosides, and 10% Fetal Bovine Serum (FBS). The cells were passaged at approximately 70-80% confluence.

CUMULATIVE POPULATION DOUBLINGS (CPD)
The CPD was determined using the following formula (LOG(10)H-LOG(10)I)/LOG(10)2, where H stands for harvested cell numbers and I stands for plated cell numbers. This experiment was repeated at least 3 times for each mouse phenotype

CLONING EFFICIENCY
Cloning efficiency was determined using the limited dilution method. Cells were plated in 96-well plates in DMEM LG + 10% FBS at a density of 0.33, 1, 2 and 4 cells per well. Fourteen days later the number of empty wells was defined, and their average fraction plotted against the number of cells seeded/wells. Data were analyzed according to the Poisson distribution method. This experiment was repeated three times for 3 healthy donors and three times for 3 diabetic donors.

RT-PCR
Real Time Polymerase Chain Reaction was performed with SYBR Green as the fluorescent reporter to check the presence of genes of interest. First, RNA was isolated from the cells by TRIzol Reagent (Invitrogen, NY, USA) according to the manufacturer`s instructions. RNA concentration was checked using a spectrophotometer for nucleic acid quantitation - NanoDrop. cDNA was prepared using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA) according to the manufacturer`s instructions. The RT-PCR reaction reagents were: SYBR Green (Applied Biosystems, Agentek, Israel) 5 µl, Ultra Pure Water (Bet HaEmek, Israel) 2.4 µl, Primer Forward Sequence (Sigma-Aldrich, Israel) 0.3 µl, Primer Reverse Sequence (Sigma-Aldrich, Israel) 0.3 µl, and cDNA 2 µl which was added to all the wells of the plate except for NTC (No-Template Control) wells. cDNA samples were diluted 10-fold. The real-time thermal cycler was set on 90°C for 20 sec at Stage 1, 95°C for 3 seconds and 60°C for 30 seconds at Stage 2 x40 cycles, 95°C for 15 seconds, 60°C for 1 second, and 95°C for 15 seconds for Melt Curve x1 cycle. Melt curves, amplification plots and standard curves were analyzed with StepOnePlus, RealTime PCR System, (Applied Biosystems, CA, USA) software.

FLOW CYTOMETRY
Flow cytometry was used to analyze the expression of the pluripotential associated stem cell markers and neural crest stem cell markers. Cells were harvested with Trypsin-EDTA, centrifuged, resuspended in blocking buffer consisting of PBS, BSA 0.1%, Sodium Azide 0.01% and then passaged through a 70 µm strainer. Cell suspensions (1 x 10^6 cells/for each marker and each isotype control) were centrifuged, fixed in 100 µl of 1.5% paraformaldehyde in PBS for 10 minutes, washed in 2 ml of blocking buffer, permeabilized in 0.1% Triton X-100 for 10 minutes for intracellular markers, washed again in 2 ml of blocking buffer, resuspended in 100 µl blocking buffer plus PE primary antibodies or PE isotype controls, washed in 2 ml of blocking buffer, and resuspended in 0.5 ml PBS w/o Ca, Mg, and analyzed with a FACScan. The software WinMDI 2.9 and Cyflogic were used to analyze the data. Each experiment was repeated for 3 healthy donors, 3 prediabetic donors and 3 diabetic donors at least 3 times for each donor.

IMMUNOCYTOCHEMISTRY
Immunocytochemistry was used for immunofluorescent identification of intracellular antigens expression. Cells were seeded on cover glasses placed in 24 well plates at a seeding concentration of 2 x 104 / well for overnight. The next day the cells were washed twice in 200 µl of PBS w/o Ca, Mg, fixed in 200 µl of 4% cold paraformaldehyde, washed twice with PBS w/o Ca, Mg, permeabilized in 200 µl of 0.2% Triton X-100, and washed twice in 200 µl of PBS w/o Ca, Mg. Thereafter, cells were incubated with blocking solution consisting of PBS and 1% BSA for 45 minutes, labeled with primary antibodies overnight at 4°C according to the manufacturers instructions for 1 hour at room temperature, washed for 2 minutes 3 times in PBS w/o Ca, Mg and labeled with secondary antibodies for 45 minutes, washed for 2 minutes 3 times in PBS w/o Ca, Mg and mounted with mounting medium containing DAPI. Cultures labeled with only secondary antibodies served as controls. Staining was visualized with a Leica confocal microscope.

In Vitro Differentiation

NEUROECTODERMAL DIFFERENTIATION
mOMSC and mASC were incubated in serum-free medium supplemented with N2, bFGF2, and EGF for 72 hours, and then in serum-free medium supplemented with dibutyryl cyclic AMP, IBMX,

Neuregulin, and PDGF for additional 72 hours. The capacities of mOMSC and mASC to express the glial markers GFAP, BDNF, and EAAT1 at the gene level, and GFAP and s100β at the protein level were assessed by qRT-PCR and immunocytochemistry, respectively. The experiment was repeated at least 3 times each time with cells from a different mouse for each culture phenotype.

ENDODERMAL DIFFERENTIATION

The capacity of mOMSC and mASC to differentiate into definitive endoderm (DE) was assessed by determining the capacity of the cultures to express two characteristic markers of definitive endoderm – Sox17 and Foxa2 after Activin A induction. Differentiation was assessed by determining the change in the expression of these genes compared to baseline, day 0. The experiment was repeated at least 3 times each time with cells from a different mouse for each culture phenotype.

THE DB^+/DB^+ MOUSE WOUND MODEL

The excisional skin wound healing model was used for testing the therapeutic capacity of oral mucosa derived stem cell generated from WT type and diabetic mice[23, 24]. Briefly, following general anesthesia, a biopsy of 6 mm in diameter was made by a punch tool on the dorsal side of the mouse. A silicone ring having a 8 mm internal diameter was sutured to the skin by interrupted 4-0 nylon sutures in order to prevent wound contraction.

The experimental design: A total of 15 db^+/db^- mice suffering of overt diabetes were divided into 3 equal groups: Control group 1 was treated with placebo saline solution; test group 2 was treated with WT-mOMSC; and test group 3 was treated with DmOMSC.

The tested cell types for each group were injected intradermally at the wound periphery at four equally distant sites. The skin was covered by a semi-occlusive dressing Tegaderm. The time to complete wound closure and cell proliferation, neovascularization and new connective tissue formation at wound closure were the endpoints of this experiment. The time of complete wound closures was determined by digitized photography captured every 3-4 days. At wound closure the animals were sacrificed, the skin samples excised peripheral to the silicon ring, fixed and processed for Mallory trichrome staining and immunohistochemistry for Ki67 and CD 31. For this purpose tissue samples were fixed in 10% formalin for 18 hours, washed in PBS thrice for 10 minutes each time, dehydrated in ethanol 100% for 10 minutes, in xylene 100% for 15 minute and embedded in paraffin. The embedded tissues were cut into 5 μm thick sections that were mounted onto microscope slides. The sections were immunostained with antibodies according to the manufacturers` instructions and examined under an Olympus microscope. The number of positive cells/area for Ki67 and CD31 were determined in the center of the healed wound.

STATISTICAL ANALYSIS

Results are expressed as the mean ± standard deviation [SD]. The results were analyzed by One Way ANOVA, Post Hoc Tests, Repeated Measures ANOVA. Values of $p<0.05$ were considered statistically significant.

Results

THE EFFECT OF DIABETES ON mOMSC AND mASC PROLIFERATION

WT-mOMSC and WT-mASC control cultures were generated from the oral mucosa and subcutaneous adipose tissue obtained from the same animal. WT-mOMSC and WT-ASC, Pre-DmOMSC and Pre-mASC and DmOMSC and DmASC test cultures were derived from the respective tissues of the same WT, prediabetic and diabetic animals, respectively. To test the long standing effect of diabetes on the proliferative capacity of each cell type, control and test cultures were grown in low glucose medium. The cumulative population doublings of these cultures, which is summarized in Table 1 indicate that: i) WT-mOMSC exhibit an inherent higher proliferative capacity (2.2 folds) than that of WT-mASC; ii) the proliferative capacity of DmOMSC was by 35% lower than that of WT-mOMSC ($p < 0.05$); iii) the proliferative capacity of DmASC was by 42% lower than that of WT-mASC ($p < 0.05$); iv) the proliferative capacity of DmASC was by was by 61% lower than that of DmOMSC; and v) the proliferative capacity of Pre-DmOMSC and that of Pre-DmASC did not differ from that of their respective WT cognates ($p > 0.05$) and was higher than that of their diabetic cognates ($p < 0.05$).

mOMSC culture	Cumulative population doublings (Mean ± SD)		mASC culture	Cumulative population doublings (Mean ± SD)
WT-mOMSC	23.846 ± 0.863		WT-mASC	10.536 ± 0.274
Pre-DmOMSC	21.595 ± 0.95		Pre-DmASC	9.957 ± 0.27
DmOMSC	15.45 ± 0.96		DmASC	5.995 ± 0.36

Table 1. Cumulative Table population doublings of all the tested cell cultures after 70 days of culture. The experiments were repeated 3 times for each cell population, each time with a new cell batch derived from a new animal.

Since the prevalence of stem cells in culture and their doubling time determine to a large extent the proliferative capacity of the culture, the percentage of clonogenic cells was assessed using the limited dilution technique and the Poisson distribution analysis[15]. The data shown in Table 2 support the results of the cumulative population doublings shown in Table 1, namely the cloning efficiency of WT-mOMSC was by 2.5 folds higher than that of WT-mASC ($p < 0.05$) and that of DmOMSC was by 2.6 fold higher than that of DmASC ($p < 0.05$). The cloning efficiencies of WT-mOMSC and WT-mASC cultures were by 1.19 and 1.25 fold higher than those of DmOMSC and DmASC, respectively ($p < 0.05$ for both comparisons). The prediabetic state did not affect the cloning efficiency of any of the cultures.

mOMSC culture	Clonogenic efficiency (%) (Mean ± SD)		mASC culture	Clonogenic efficiency (%) (Mean ± SD)
WT-mOMSC	62 ± 2.2		WT-mASC	25 ± 1.76
Pre-DmOMSC	58 ± 2.065		Pre-DmASC	25 ± 3.4
DmOMSC	52 ± 2.27		DmASC	20 ± 1.077

Table 2. Cloning efficiency in the 6 types of mOMSC and mASC cultures

THE EFFECT OF DIABETES ON mOMSC AND mASC PHENOTYPE

Human oral mucosa stem cells express pluripotency associated markers such as Oct4, Sox2 and Nanog and neural crest stem cells markers such as Klf4 and c-Myc[17]. In this study the capacity of mOMSC to express similar markers and the long-term effect of the diabetic state on this capacity was assessed at the molecular and protein levels by qRT-PCR and flow cytometry, respectively. The level of expression of the pluripotency associated markers Oct4, Sox2 and Nanog and the neural crest stem cell markers Klf4, c-Myc, snail, twist and nestin was similar for WT-mOMSC and DmOMSC cultures, indicating that the diabetic status did not affect the expression level of these factors (Fig. 1). Opposite results and conclusions were found for WT-mASC and DmASC, namely the expression of pluripotency and neural crest associated markers was by approximately 4 fold lower in the DmASC cultures as compared to those in the WT-ASC cultures ($p < 0.05$ for all comparisons). The expression of the pluripotency associated and neural crest markers was significantly higher, (between 4 to 16 fold; $p < 0.05$ for all comparisons) in mOMSC cultures as compared to mASC cultures (Fig. 1).

Flow cytometry analysis of mOMSC and mASC cultures derived from diabetic and healthy animals supports the findings obtained at the molecular level. The percentage of cells positive for Oct4, Sox2, Nanog, Klf4 and c-MYC was substantially higher in WT-mOMSC and DmOMSC cultures as compared to their counterparts WT-mASC and DmASC (Table 3) ($p < 0.05$ for all comparisons). In contrast to DmASC, in which the percentage of positive cells for the above mentioned markers was statistically significant lower than in WT-mASC, there was no statistical significant difference between the percentage of positive cells for these

markers in DmOMSC as compared to WT-mOMSC ($p > 0.05$ for all comparisons).

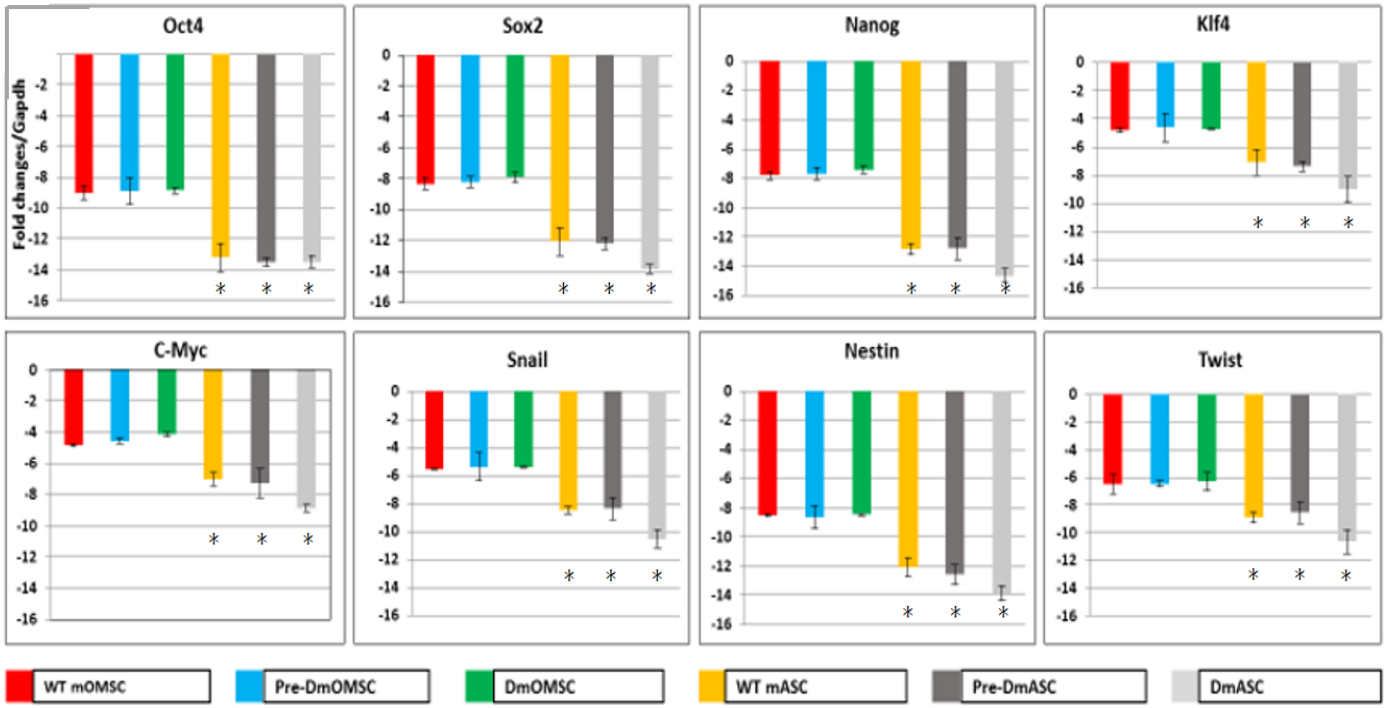

Fig. 1. Expression of pluripotency associated genes Oct4, Sox2 and Nanog and the neural crest stem cells genes Klf4, c-Myc, Snail, Nestin and Twist. The level of expression for each gene is presented as $-\triangle Ct$ vs. the house keeping gene Gapdh. The lower the value of $-\triangle Ct$ the higher the level of expression. The graphs indicate the higher expression of the stem cell genes in mOMSC cultures as compared to mASC cultures and the effect of the diabetic state in vivo on these cells in vitro. Statistical analysis was performed by ANOVA. The asterisk stand for statistical significant differences between the mOMSC and mASC cell types. The bars represent the mean ± SD of the data derived from a sample size of 5 cultures for each cell phenotype, each culture was derived from a different mouse.

	WT mOMSC	Pre-DmOMSC	DmOMSC	WT mASC	Pre-DmASC	DmASC
Oct4	37.83±3.55	36.67±3.51	38.13±1.2	20.83±1.9	20±1.5	13.3±3.5
Sox2	56.6±2.62	55.5±3.28	54±1.73	31±2.65	31.27±2.05	20.4±1.5
Nanog	43.27±1.1	43.17±4.19	46.17±1.04	32±2	32.3±3.04	24.17±2.02
Klf4	86.467±1.7	87±1.32	87±2.65	57.67±2.5	55.67±4.6	37.3±3.5
C-Myc	88.47±1.55	85±2.5	86.67±1.15	57±2	55.3±3.2	32.67±3.51

Table 3. The percentage of positive cells ± SD for the pluripotency and neural crest associated stem cell markers Oct4, Sox2, Nanog, Klf4, and C-Myc as assessed by flow cytometry in the mOMSC and mASC cell lineages is presented. The figures represent the mean ± SD of the data derived from a sample size of 5 mice for each mouse phenotype. The experiments were repeated 3 times for each cell population, each time with a new batch of cells. Statistical analysis was performed by ANOVA.

THE EFFECT OF DIABETES ON mOMSC AND mASC CAPACITY TO DIFFERENTIATE

To test the primitive and neural crest phenotype of mOMSC and the influence of the long-term hyperglycemic state on these mOMSC properties we tested the propensity of healthy, prediabetic and diabetic mOMSC and mASC to differentiate into primitive endoderm and neuroectermal lineages. As previously described for human oral mucosa stem cells,[17] activin A induced the expression of each of the two primitive endoderm transcription factors Sox17 and Foxa2 by approximately 5 folds in WT-mOMSC treated cultures as compared to baseline (Fig. 2A). Immunofluorescent examination revealed nuclear localization of both Sox17 and Foxa2 in these cultures suggesting functionality (Fig. 2B). Activin A also induced the expression of similar levels of Sox17 and Foxa2 with nuclear localization in DmOMSC cultures as in WT-mOMSC ones.

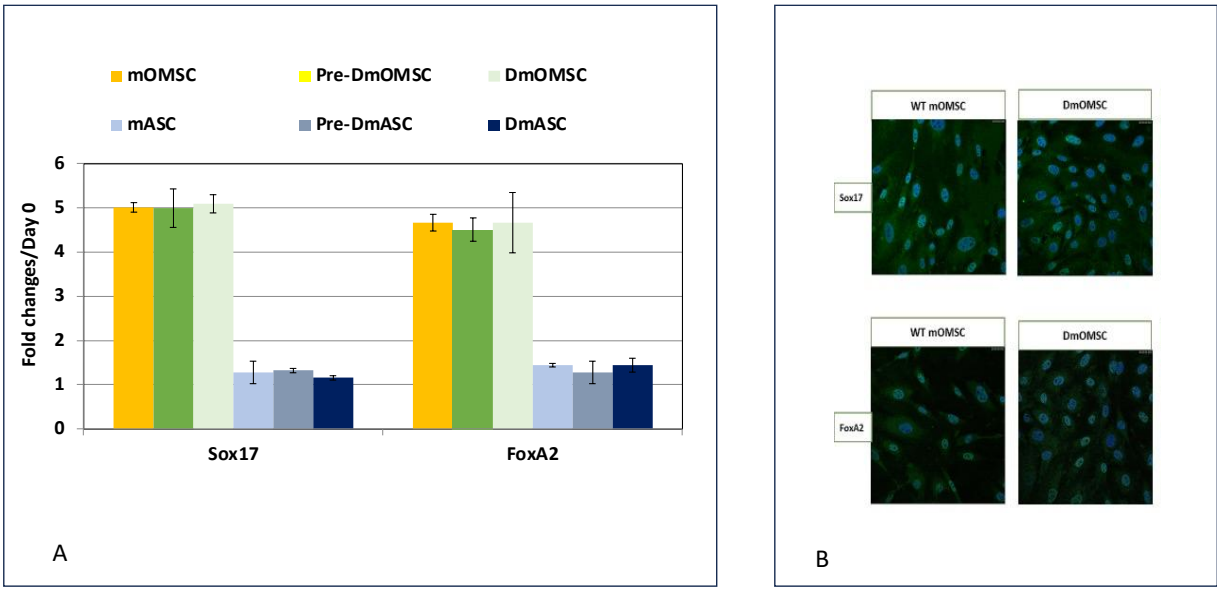

Fig. 2. (A) The effect of the diabetic state on primitive endodermal differentiation of the various mOMSC and mASC cultures as reflected by the expression of the primitive endoderm transcription factors Sox17 and Foxa2. The 3 mOMSC cultures types responded to 5 days activin A induction by similarly increasing the expression of Sox17 and Foxa2 transcription factors. mASC cultures did not respond to activin A induction. The bars represent the mean ± SD of $2^{-\Delta\Delta Ct}$ obtained from a sample size of 5 mice for each culture phenotype. (B) Immunofluorescent nuclear localization of Sox17 and Foxa2 in WT mOMSC and DmOMSC. $2^{-\Delta\Delta CT}$ values lower than 2 were considered not significant.

Neuroectodermal (glial) differentiation of all culture types into glia-like cells was tested by the expression of the glial markers Gfap, Eaat1 and Bdnf at the molecular level and of GFAP and S100β at the protein level (Fig. 3A, 3B). The differentiation regimen increased the expression of Gfap, Eaat1 and Bdnf in WT-mOMSC cultures by 7, 5 and 5.5 folds, respectively and in DmOMSC cultures by 6, 4 and 6 folds, respectively (Fig. 3A). No statistical significant differences were found between the level of expression of these markers in WT-mOMSC cultures as compared to DmOMSC ones indicating that the hyperglycemic state did not affect the neuroectodermal differentiation capacity of DmOMSC. The neuroectodermal differentiation regimen used for mOMSC was unable to induce similar differentiation in WT-mASC cultures and therefore, it was not be possible to assess the effect of diabetes on mASC in this respect. Immunofluorescence examination revealed that GFAP and s100β were localized to the cytoplasm in WT-mOMSC and DmOMSC (Fig. 3B).

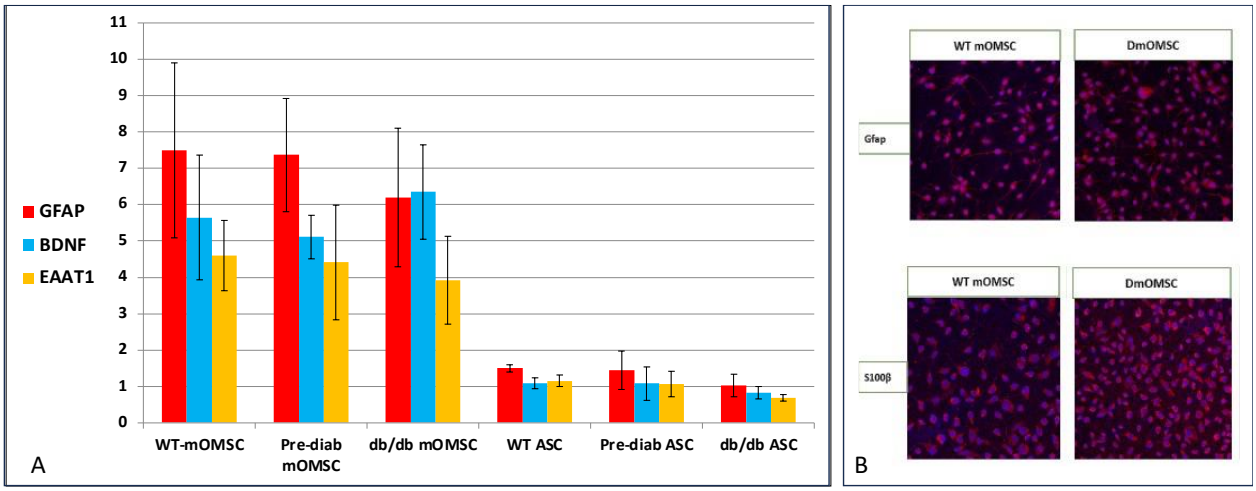

Fig. 3. (A) The effect of the diabetic state on primitive endodermal differentiation of the various mOMSC and mASC cultures as reflected by the expression of the primitive endoderm transcription factors Sox17 and Foxa2. The 3 mOMSC cultures types responded to 5 days activin A induction by similarly increasing the expression of Sox17 and Foxa2 transcription factors. mASC cultures did not respond to activin A induction. The bars represent the mean ± SD of 2^-△△Ct obtained from a sample size of 5 mice for each culture phenotype. (B) Immunofluorescent nuclear localization of Sox17 and Foxa2 in WT mOMSC and DmOMSC. $2^{-\Delta\Delta Ct}$ values lower than 2 were considered not significant.

THE EFFECT OF THE DIABETIC STATE ON THE THERAPEUTIC CAPACITY OF DMOMSC.

We have previously shown that the intradermal implantation of allogeneic mOMSC derived from WT healthy Balb/c mice at the periphery of full thickness dermal wounds in db^+/db^+ mice suffering of overt diabetes enhanced substantially the process of wound healing[23]. To test the effect of prolonged diabetes on mOMSC capacity to accelerate diabetic wound healing, DmOMSC were intradermally implanted at the periphery of similar wounds in db^+/db^+ mice. WT-mOMSC served as controls. Since the db^+/db^+ mice and the WT-mice from which the WT-mOMSC and DmOMSC cultures were generated were derived from the same colony by breeding db^+/db^- males with db^+/db^- females the administration of these cells into db^+/db^+ can be considered an autologous transplantation. The db^+/db^+ mouse was found to exhibit the slowest rate of skin wound healing amongst the various diabetic-mouse models[24]. The natural wound closure time, that is the average time required for a circular wound, 6 mm in diameter, to completely heal in WT healthy mice is 14.85 ± 1.06 days (data not shown). In the placebo treated db^+/db^+ mice with overt diabetes (control group) the wound closure time was 23.75 ± 2.3 days . Implantation of WT-mOMSC at the periphery of similar diabetic wounds in db^+/db^+ mice resulted in a mean wound closure time of 15.14 ± 1.07 days (Fig. 4A), which is by 8 days shorter than the control placebo-treated db^+/db^+ mice ($p < 0.05$) and not statistically different from the natural healing time in WT mice (14.85 ± 1.06). These results indicate that WT-mOMSC have the capacity to overcome the deleterious effect of diabetes on wound healing. The capacity of DmOMSC to bring about to complete wound closure was slightly lower than that of WT-mOMSC, the mean time to closure being 17.4 ± 2.19 days. However, the difference between the time required for DmOMSC and WT-mOMSC to bring about to complete wound healing was not statistically significant ($p > 0.05$). However, the time to complete wound closure in db^+/db^+ mice treated with WT-mOMSC or DmOMSC was significantly shorter (by 8 or 6 days respectively) than that of placebo treated db^+/db^+ mice ($p < 0.05$).

Cell proliferation, angiogenesis and new connective tissue formation play crucial roles in dermal wound healing. We used anti-Ki67 and anti-CD31 antibodies to quantify the number of proliferating cells and the number of new capillaries and small blood vessels in the wound center at wound closure time. The number of Ki67 positive cells in the center of the wounds of placebo-treated db^+/db^+ mice was by 73% and 57% lower than that observed in the center of wounds of animals treated with WT-mOMSC and DmOMSC, respectively ($p < 0.05$). The mean number of positive cells in wounds treated with DmOMSC was lower by 21% than that in wounds treated with WT-mOMSC ($p < 0.05$) (Fig. 4B). The number of CD31 positive vessels in WT-mOMSC and DmOMSC- treated wounds was higher by 60.34% ($p < 0.05$) and 52.27% ($p < 0.05$), respectively than that in placebo-treated diabetic wounds (Fig. 4C). The mean number of CD31

positive blood vessels in wounds treated with DmOMSC was lower by 15% than that in wounds treated with WT-mOMSC ($p < 0.05$). New connective tissue formation was assessed by quantifying the thickness of new connective tissue that stained positively by Masson`s trichrome stain in the center of the closed wound. No differences in the amount of granulation tissue were found between closed wounds of animals treated with either WT-mOMSC or DmOMSC ($p > 0.05$). However, the thickness of the new connective tissue in these groups was by approximately 2 fold higher than that in placebo-treated wounds (Fig. 4D) ($p < 0.05$). Due to the low proliferative potential of DmASC, these cells could not be expanded to the quantities required for their testing in the db+/db+ mouse wound healing mode.

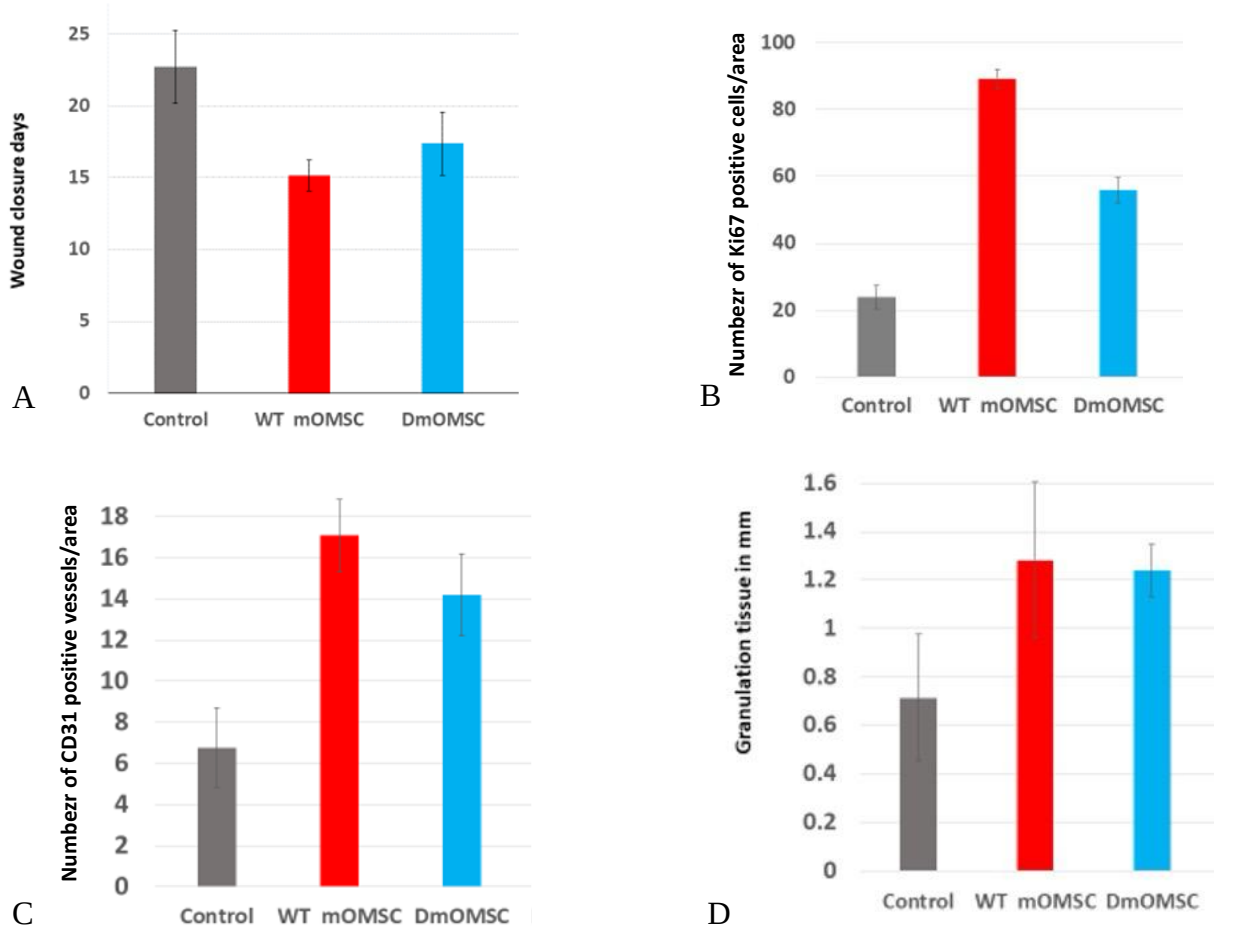

Fig. 4. Diabetes affects negligibly the therepeutic capacity of DmOMSC in the diabetic wound healing model. (A) The graph depicts the number of days untill complete wound closure was achieved in db+/db+ mice treated with either WT mOMSC or DmOMSC or placebo (Control). (B) The diabetic state reduced the capacity of DmOMSC to enhance cell proliferation as compared to WT mOMSC ($p<0.05$) as reflected by the number Ki67 positive cells detected in the center of the wound at closing. (C) DmOMSC exhibit a not significantly ($p>0.05$) lower ability to induce neovascularization in the wound center at wound closure than WT mOMSC as assessed by the number of the CD31 positive blood vessels. (D) The thickness in mm of the new granulation tissue formed under the new epidermis at the closure time was substantially higher in the WT-mOMSC and DmOMSC treated animals as compared to that placebo treated ones ($p < 0.05$). The bars stand for mean ± SD for groups of 5 mice.

Discussion

The incidence and prevalence of diabetes type 2 are continuously increasing in the western countries. This chronic long standing disease is associated with cellular, vascular and neural pathologies that affect the majority of body tissues and organs[25]. Stem cells therapy evolves as a new therapeutic approach for the treatment for diabetic complications. There is accumulating evidence that the long standing hyperglycemic diabetic state impairs the homeostasis of stem cells and their niche and

consequently their capacity to respond to chemotactic cues, migrate, self-renew and differentiate, functions that are crucial for tissue repair and regeneration[26]. Thus, the therapeutic use of autologous stem cells from diabetic patients might be hazarded by the changes in the phenotype and therapeutic abilities of these stem cells. The question remains whether these "diabetic" stem cells might recover during their ex-vivo expansion required for their therapeutic use? Previous reports demonstrate that bone marrow and adipose tissue-derived mesenchymal stem cells derived from diabetic animals exhibit lower abilities to proliferate, secrete trophic factors and differentiate in culture than their cognates derived from healthy animals[27, 28], suggesting that the long standing hyperglycemic state of diabetes imprints a constant signature on the stem cell populations in the diabetic organism. In the present study we tested this hypothesis by expanding the stem cells derived from the oral mucosa and adipose tissue in a hypoglycemic environment for seven consecutive passages. The results obtained for DmASC at the end of the population expansion corroborate with the findings described above, namely DmASC exhibit a lower incidence and proliferative capacity and a lower expression of pluripotency and neural crest associated markers as compared to WT-mASC. Thus, our study further support the notion that long standing hyperglycemia results in permanent genetic and possibly epigenetic changes in the diabetic bone marrow and adipose tissue-derived mesenchymal stem cell populations[29].

Our study demonstrates for the first time that, differently from DmASC, only the incidence and the proliferative capacity of DmOMSC were mildly affected by the diabetic state, whereas the phenotype and the differentiation capacity of DmOMSC were not affected. Furthermore, WT-mOMSC and DmOMSC exhibited an inherent more primitive phenotype than that of WT-mASC and DmASC as reflected by their higher incidence, proliferation rate, pluripotency and neural crest marker expression and their differentiation abilities.

Considering the fact that both types of cells were generated from same animal (WT or db+/db+), these findings suggest that "diabetic" oral mucosa derived stem cells are privileged over other adult "diabetic" stem cell types and consequently their therapeutic effect might be preserved.

To test this assumption we compared the therapeutic potential of DmOMSC to that of WT-mOMSC. The comparison revealed that DmOMSC are slightly (25%) less effective than WT-mOMSC in accelerating complete wound closure in db+/db+ mice and in enhancing cell proliferation and vascularization. These data indicate that the therapeutic capacity of DmOMSC was mainly preserved under the long standing hyperglycemic diabetic state.

The reasons at the basis of the inherent differences between wild type and "diabetic" mOMSC and wild type and "diabetic" mASC is unclear. Possible explanations are i) the different embryonic origin of the cell population in oral mucosa and adipose tissue, namely the neural crest[30] and the somites, respectively; and ii) the fact that in health and disease oral mucosa is continuously exposed to chemical, mechanical and microbial stimuli that exert epigenetic changes in the oral mucosa stem/progenitor cells required to maintain the homeostasis of this highly challenged tissue, which is crucial for the survival and wellbeing of the organism.

Conclusion

Collectively, these data demonstrate that: i) diabetes decreased moderately DmOMSC proliferative ability; ii) in contrast to mASC, mOMSC withstand the majority of the diabetic detrimental effects; and iii) the therapeutic properties of DmOMSC are mildly affected by long standing diabetes. Considering the fact that oral mucosa is a readily accessible source, that a tiny biopsy is sufficient for generating trillions of oral mucosa stem cells[17], which are mainly resistant to the diabetic state it is suggested that oral mucosa stem cells are a favorable therapeutic tool for autologous cell therapy in diabetic patients.

Conflict of interest:

Sandu Pitaru serves as Chief Scientific Officer at Cytora Ltd. and holds equity in the company. Other authors do not declare any conflict of interest.

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