UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ODONTOLOGIA DE BAURU

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Relation between the function and expression of VEGFR1 and the vasculogenic differentiation of dental pulp stem cells

Relação entre a função e expressão do VEGFR1 e a diferenciação vasculogênica de células-tronco da polpa dentária

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Tese apresentada a Faculdade de Odontologia de Bauru da Universidade de São Paulo para obtenção do título de Doutor em Ciências no Programa de Ciências Odontológicas Aplicadas, na área de concentração Odontopediatria.

Orientadores: Profa. Dra. Thais Marchini de Oliveira e Prof. Dr. Jacques Eduardo Nor

BAURU
2019

Bergamo, Mariel Tavares de Oliveira Prado
Relation between the function and expression of VEGFR1 and the vasculogenic differentiation of dental pulp stem cells / Mariel Tavares de Oliveira Prado Bergamo– Bauru, 2019.
63 p.

Tese (Doutorado) – Faculdade de Odontologia de Bauru. Universidade de São Paulo

Orientador: Prof. Dr. Thais Marchini de Oliveira

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ERRATA

DEDICATÓRIA

Dedico esse trabalho a duas pessoas extraordinárias: minha mãe e meu marido, que são exemplos de fé, perseverança, profissionalismo e determinação. Obrigada por todo apoio, carinho, paciência e orações. Amo vocês

AGRADECIMENTOS ESPECIAIS

Agradeço a minha orientadora **Profa. Dra. Thais Marchini de Oliveira,** por sempre acreditar em mim. Você é para mim, não só exemplo de pesquisadora, dentista, professora, esposa, mas agora também de mãe. Seu mais novo posto de: mãe da Manu nos mostrou novamente sua garra, perseverança e amor, que agora será dividido entre os filhos científicos e a nossa amada e sonhada Manu. Obrigada por ser incrível e não medir esforços para ajudar, orientar, ensinar e acolher seus alunos. Obrigada por sonhar junto comigo o Doutorado Sanduíche e me ajudar na busca e concretização desse projeto. Você marcou minha vida profissional e pessoal. Levarei para sempre sua amizade, sua perseverança e seus ensinamentos. Te admiro muito! Obrigada por tudo!!

Agradeço ao meu orientador **Prof. Dr. Jacques Eduardo Nor**, pelos ensinamentos e pela oportunidade dada a mim de trabalhar na melhor universidade do mundo, em um laboratório altamente equipado e com pessoas incríveis. Quando via seu nome nos artigos, sabia que se referia a um pesquisador brilhante, mas não tinha ideia do ser humano humilde, professor amigo, pai orgulhoso do Lucas e querido que havia por trás. Obrigada por tudo, professor. Serei eternamente grata.

AGRADECIMENTOS

A **Deus,** por sempre cuidar de mim e desenhar os caminhos mais lindos para mim. Por sua proteção, amor e cuidado todos os dias de minha vida

Ao **meu marido Yuri**, por ser esse companheiro fiel, por suas orações, cuidado e amor em todos os momentos. Essa conquista é nossa!

Aos **meus pais, irmãs e sobrinha**, pelo apoio, carinho, amor, orações e torcida.

Aos **meus sogros e cunhada**, pelo incentivo, carinho e orações.

A **minha família, tias, tios, primos e primas**, em especial aos meus tios Souza e Célia, por todas as orações e carinho comigo desde de minha infância até agora.

À minha querida e inspiradora orientadora, **Profa. Dra. Thais Marchini de Oliveira Valarelli**, por todos os ensinamentos profissionais e pessoais. Por ser essa pessoa incrível e acreditar em mim quando nem mesmo eu acreditei. Você é um exemplo de determinação, perseverança e amor. Te admiro muito.

Ao meu querido supervisor no exterior, **Prof. Dr. Jacques Eduardo Nor**, pela oportunidade, confiança e por me permitir vivenciar a melhor experiência em minha vida até agora. O senhor é uma pessoa extraordinária e sua humildade e inteligência inspiram a todo momento.

A profa. **Dra. Cidinha**, por todos os ensinamentos compartilhados, pelo entusiasmo característico e o exemplo de fé inabalável. Deus abençoe a senhora e que sua energia e profissionalismo sempre contagiem os seus alunos como me contagiou.

Ao **Prof. Dr. Carlos Ferreira dos Santos,** diretor da Faculdade de Odontologia de Bauru, pelo incentivo e pelos ensinamentos dispensados a mim desde a época da minha graduação até agora. Te admiro Professor.

Aos **pacientes**, que contribuíram para o desenvolvimento da ciência e aos pais que nos permitiram cuidar com amor e respeito do bem mais precioso que eles têm: seus filhos.

Aos queridos**docentes do Departamento de Odontopediatria,** tanto pelos ensinamentos teóricos, práticos e científicos como pelo convívio diário.

Aos **funcionários do Departamento de Odontopediatria**, pelo convívio diário e cuidado com os alunos e pacientes da clínica de odontopediatria.

Aos **meus amigos de pós-graduação** do Departamento de Odontopediatria, por todos os bons momentos e risadas compartilhadas. Desejo um futuro brilhante a vocês.

Ao meu grupo de pesquisa da FOB, amigos do CIP e técnicos do laboratório, principalmente **Luciana, Natalino, Nathália, Nádia, Cíntia,** pelo carinho, amizade e ensinamentos compartilhados durante todos esses anos. Aos queridos técnicos **Thiago e Evandro Dionísio**, por toda ajuda com os ensaios e pela amizade.

A minha amiga **Andrea Mantesso**, por me acolher e me ensinar, por ser minha professora particular e amiga a todo tempo. Tenho muito respeito e admiração por você. Obrigada por todo carinho seu, do Jake e da Olivia.

A minha família mineira **Larrisa e Ernane** e a Minha família Baiana **Isabella e Cassiano**, não existirão palavras para agradecer todo o cuidado e amor de vocês conosco durante esse uma no em Michigan. Amamos vocês.

Aos meus amigos de Michigan **Christie, Karol, Takafumi e família, Jussara, Zhang, Kristy, Alexandra, Marilia e Marcos, Camila, Aron, Fabiana e Duda, Mariana,** pelas conversas, ajuda e todo carinho que tiveram comigo. Christie, Takafumi e Jussara vocês são incríveis e agradeço a Deus por ter me dado suas amizades.

À **Fundação de Amparo a Pesquisa do Estado de São Paulo** pelo auxílio financeiro (Processo #2017/11396-3 e #2018/13675-0).

A vida é uma corrida que não se corre sozinho. E vencer não é chegar, é aproveitar o caminho sentindo o cheiro das flores e aprendendo com as dores causadas por cada espinho".

Bráulio Bessa

RESUMO

O objetivo desse estudo foi avaliar o papel da expressão do Receptor 1 do Fator de Crescimento Endotelial Vascular (do inglês Vascular Endothelial Growth Factor 1 – VEGFR1) na diferenciação das células-tronco da polpa dentária (do inglês Dental Pulp Stem Cell – DPSC) e Células-tronco de dentes decíduos humanos esfoliados (do inglês Stem cell from Human Exfoliated Deciduous tooth – SHED) em células endoteliais. Células foram separadas em células com alta (positivo/VEGFR1^{HIGH}) e baixa (negativo/VEGFR1^{LOW}) expressão do VEGFR-1 através de Citometria de Fluxo e cultivadas em alphaMEM suplementado com 20% de Soro Fetal Bovino (SFB) ou Meio de Crescimento Endotelial (EGM2-MV) suplementado com 50ng/mL de rhVEGF (controle ou meio de diferenciação) e 0 ou 25µg/mL de Bevacizumab (Avastin® ou Bevacizumab). Os outros testes foram feitos in vitro para avaliar a proliferação celular e a diferenciação endotelial das células-tronco: SRB, ensaio de formação tubular, RT-PCR, Western Blot e Imunofluorescência. Para avaliar esse processo in vivo, matrizes receberam SHED com altos e baixos níveis de expressão de VEGFR1 e foram transplantadas para a região subcutânea do dorso de ratos imunocomprometidos e depois de 28 dias as amostras foram retiradas e ensaios de HE, imunohistoquímica e imunofluorescência foram realizados. A contagem de novos vasos sanguíneos foi feita no software ImageJ e as análises estatísticas foram feitas usando Teste T não pareado ou ANOVA a um critério seguido pelo Teste de Tukey e a significância estatística foi considerada $p<0,05$. Os resultados mostraram que SHED VEGFR1^{LOW} teve maior taxa de proliferação no período de 72h independente do meio de cultura. A diferenciação de SHED/DPSC em células endoteliais in vitro foi confirmada através da expressão de marcadores de células endoteliais e formação de brotamentos por essas mesmas células. SHED VEGFR1^{HIGH} apresentou maior formação de brotamentos in vitro e maior quantidade de microvasos sanguíneos in vivo, mostrando o importante papel deste receptor na diferenciação vasculogênica da SHED e DPSC.

Palavras-chave: Células-Tronco. Engenharia Tecidual. Angiogênese. Fator de Crescimento Endotelial Vascular. Polpa Dentária.

ABSTRACT

Relation between the function and expression of VEGFR1 and the vasculogenic differentiation of dental pulp stem cells

This study aimed to evaluate the role of the Vascular Endothelial Growth Factor Receptor 1 (VEGFR1) expression at the endothelial differentiation of Dental Pulp Stem Cell (DPSC) and Stem Cell from human deciduous tooth (SHED). Cells were sorted by High (positive/ VEGFR1^{HIGH}) and Low (negative/VEGFR1^{LOW}) levels of VEGFR1 expression through Flow Cytometry and cultured in alpha-MEM supplemented with 20% Fetal Bovine Serum (FBS) or Endothelial Growth Medium (EGM2-MV) with 50ng/mL rhVEGF (control or differentiation medium) and 0 or 25µg/mL of Bevacizumab (Avastin or Bevacizumab). The following tests were performed in vitro to evaluate cell proliferation and endothelial differentiation of stem cells: SRB, Sprouting Assay, RT-PCR, Western Blot, and Immunofluorescence. To evaluate this process in vivo, scaffolds seeded with SHED expressing high and low levels of VEGFR1 were transplanted into the subcutaneous dorsum of immunodeficient mice and after 28 days the samples were retrieved for, HE staining, immunohistochemistry, and immunofluorescence. The new blood microvessels formation were counted through ImageJ software, the statistical analyses were performed using unpaired T Test or One-Way ANOVA followed by Tukey Test, and the threshold of statistical significance was set at $p < 0.05$. The results showed that the SHED VEGFR1^{LOW} has more proliferation rate in 72h regardless of culture media. The differentiation of SHED/DPSC in endothelial-like cells in vitro was confirmed through the expression of endothelial cells markers and sprouting formation by those cells. SHED VEGFR1^{HIGH} generated more quantity of sprouts in vitro and more quantity of microvessel in vivo, showing the important role of this receptor in vasculogenic differentiation of SHED and DPSC.

Key words: Stem Cells. Tissue Engineering. Angiogenesis. Vascular Endothelial Growth Factor

LIST OF ILUSTRATION

LIST OF ABBREVIATIONS

% = Percentage

°C = Degree Celsius

µg = Microgram

µm = Micrometre

CD31/PECAM-1 = Platelet endothelial cell adhesion molecule

cDNA = Complementary DNA

CO_2 = Carbon dioxide

DAB = 3,3'-Diaminobenzidine

DNA = Deoxyribonucleic acid

DPSC = Dental Pulp Stem Cell

DMEM = Dulbeccos Modified Eagle`s Medium

DTT = Dithiothreitoll

EGM2-MV = Microvascular Endothelial Cell Growth Medium-2

et al = and others

FBS = Fetal Bovine Serum

Flk-1/VEGFR-2 = Vascular endothelial growth factor receptor 2

Flt-1/VEGFR-1 = Vascular endothelial growth factor receptor 1

GAPDH = Glyceraldehyde-3-phosphate dehydrogenase

HDMEC = Human Dermal Microvascular Endothelial Cells

HE = Hematoxilin-Eosin

IgG = Immunoglobulin G

MEMα = Minimum Essential Medium Eagle - Alpha Modification

mL = Millilitre

ng = Nanogram

NP 40 = nonyl phenoxypolyethoxylethanol

PBS = Phosphate Buffered Saline

pH = Potential of Hydrogen

PlGF = Placenta growth factor

PLLA = Poly-L-lactic acid

p-STAT3 = phospho-STAT3

RNA = Ribonucleic acid

RNAm = Messenger RNA

rpm = rotations per minute

RT-PCR = Reverse transcription polymerase chain reaction

SCID = Severe combined immunodeficient mice

SDS = Sodium dodecyl sulfate

SRB = Sulforhodamine B

SHED = Stem cells from human exfoliated deciduous teeth.

TBST = Tris Buffered Saline with Tween 20

Tie-2= Tyrosine-protein kinase receptor TIE-2

VEGF = Vascular endothelial growth factor

Ve-cadherin= Vascular endothelial cadherin

SUMARY

1 Introduction

1 **INTRODUCTION**

Tooth decay is a common disease in children (1). The carie disease in younger patients (less than 6 years old) is known as Early Childhood Caries (ECC), but there are some specific considerations before checking this classification that becomes hard to establish the prevalence of it in the world (2). However, according to data collected in three periods (1988 to 1994,1999 to 2004, and 2011 to 2012) in National Health and Nutrition Examination Survey (NHANES), the tooth decay and carie experience in preschool students decreased in this last evaluation, but it still is a concern and the prevalence of this disease can be greater because there are a misunderstand about the terminology and diagnosis criteria of the ECC (2).

Dental caries in younger patients is a problem that should be solved because it can advance, and a superficial decay can become a deep decay and some methods should be made to control its progress. Although, some restorative and reparative materials have been studied to heal the root after pulpotomy and have been obtained a high level of the clinic and radiographic success, in the pulpectomy treatment there are no final conclusions about the best material to replace the pulp inside the root (1). It becomes important looking for other therapies and ways to replace or to give conditions to the pulp tissue healing itself. One important and rising method that has been studied a lot is the Tissue Engineering and Regenerative Endodontics and promising results in pulp and dentin regeneration have been obtained (3–9).

The engineering tissue involves three important points: scaffold, stem cells and growth factors and leads the stem cells to differentiate into a pulp-like tissue able to replace the former pulp tissue which suffered some injuries through carie disease or trauma. In respect of the stem cells from pulp tissue, it is known that they are a heterogeneous population of cells with a multipotent ability (10). That cells can differentiate in adipocytes, neural cells, chondrocytes, odontoblast, and endothelial cells (3,5,6,8,11,12).

Stem cells from Human Deciduous Teeth (SHED) and Dental Pulp Stem Cell (DPSC) are two kinds of postnatal stem cells from the dental pulp of deciduous and permanent teeth, respectively (13,14). They have the self-renew, plasticity, and differentiation ability and besides that, its obtaining is easier compare to the other sources, which makes them interesting in tissue engineering (4,13).

Angiogenesis has an important role in the tissue engineering, promoting the oxygen and nutrients supply for tissue. Vascular Endothelial Growth Factor (VEGF) and its family is involved in angiogenesis, cell proliferation and differentiation of stem cells in endothelial-like cells (5,12,15,16). The ability of SHED and DPSC to differentiate in endothelial cells is related to the presence of a Vascular Endothelial Growth Factor Receptor 1 (Flt-1/VEGFR-1), where the VEGF binds and activates MEK1/ERK signaling (5,12), so the STAT 3, stemness signaling, is inhibited and the stem cells should differentiate in endothelial cells.

It becomes important for the achievement of tissue engineering to have a vascular network capable to maintain the oxygen and blood supplies to the tissue, so it is fundamental to understand the vasculogenic process in that case. We hypothesized that not all stem cells as SHED and DPSC can differentiate in endothelial cells (Figure 1), so have some specific subpopulation of the stem cells that can do it. The other hypothesis is the dental pulp stem cells with high expression of VEGFR1 are prone to differentiate into endothelial cells.

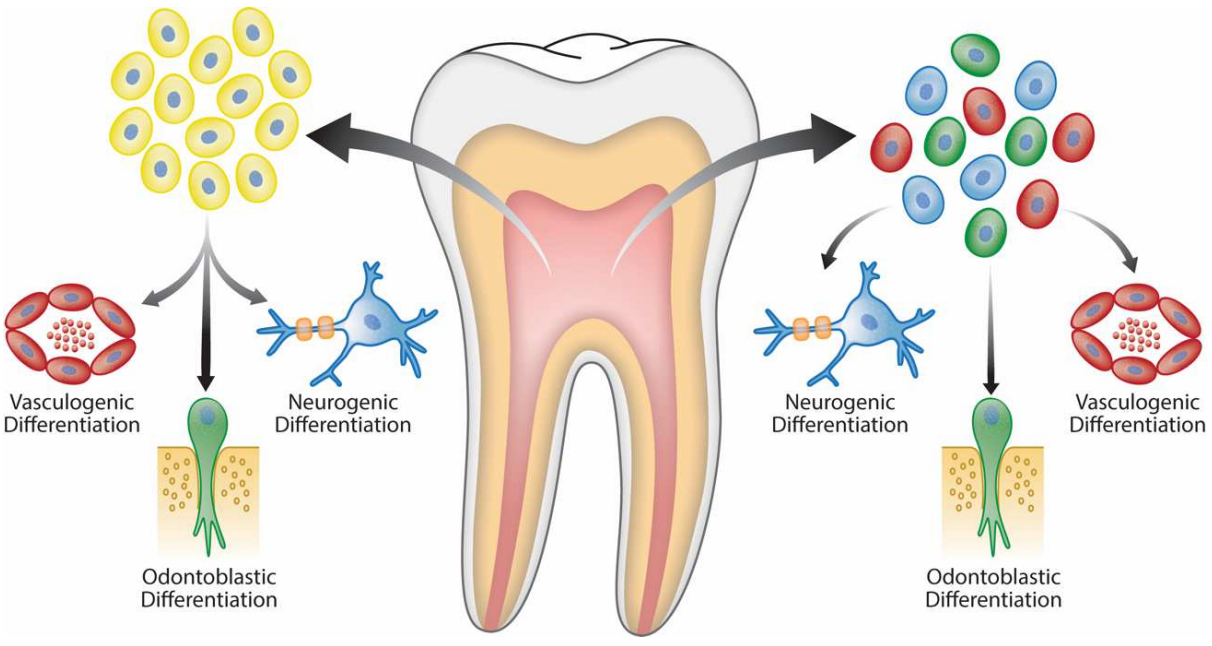

Figure 1 – Scheme showing two hypotheses: the first one is all stem cell from dental pulp is able to differentiate into vascular, odontoblast, and neuronal cells (right); and the second is there are some pulp stem cells with specific features which become them able to differentiate themselves into a certain kind of cell (left).

2 Paper

2 **PAPER**

Relation between the function and expression of VEGFR1 and the vasculogenic differentiation of dental pulp stem cells

Introduction

Dental caries in younger patients is a problem that should be solved because it can advance, and a superficial decay can become a deep decay and some methods should be made to control its progress. Although, some restorative and reparative materials have been studied to heal the root after pulpotomy and have been obtained a high level of the clinic and radiographic success, in the pulpectomy treatment there are no final conclusions about the best material to replace the pulp inside the root (1). It becomes important looking for other therapies and ways to replace or to give conditions to the pulp tissue healing itself. One important and rising method that has been studied a lot is the Tissue Engineering and Regenerative Endodontics and promising results in pulp and dentin regeneration have been obtained (2–9).

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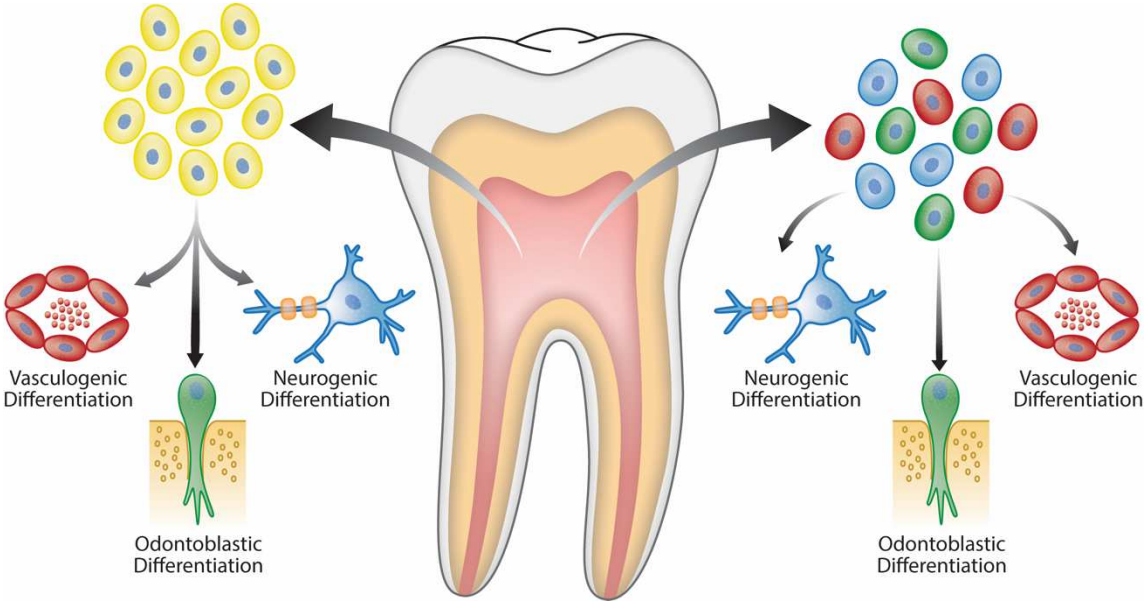

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Material and Methods

Cell Culture

SHED and DPSC were kindly provided by Songtao Shi and were cultured in Minimal Essential Media (MEMα - Invitrogen, Carlsbad, California) 20% Fetal Bovine Serum (FBS - Thermo Fisher Scientific, USA), 1% Antimycotic and Antibiotic Solution (Anti-Anti - Gibco, Grand Island, NY, USA) at 37°C, 5% CO_2 until those get 90% of confluence in the flask. Human Dermal Microvascular Endothelial Cells (HDMEC,

Lonza, Walkersville, MD, USA) were cultured in Endothelial Cell Medium (ECM - ScienCell, Carlsbad, CA, USA) supplemented with 5 % FBS and were used as positive control for endothelial cell markers. SHED and DPSC were treated with Endothelial Growth Medium (EGM2-MV) supplemented with 50ng/mL of rhVEGF (R&D Systems, Minneapolis, MN, USA) (it was referred here as Control Group and this medium was named differentiation medium) and with 0 or 25 ug/mL Bevacizumab (referred here as Bevacizumab Group).

Semi-quantitative RT- PCR

The total RNA from SHED/DPSC was isolated by Trizol (Invitrogen) and the Total RNA concentration was measured by NanoDrop(Thermo Scientific, USA). The assay was performed using Superscript II Reverse Transcriptase (RT - Invitrogen) according to the manufacturer's instructions. The primers used for this experiment were: VEGFR-1(sense 5'-actcccttgaacacgagagttc-3', antisense 5'-gatttctcagtcgcaggtaacc-3'), VEGFR-2 (sense 5'-gctgtctcagtgacaaacccat-3', antisense 5'-ctcccacatggattggcagagg-3'), Tie-2 (sense 5'tacacctgcctcatgctcag-3', antisense 5'-gcagagacatccttggaagc-3'), CD31 (sense 5'-tactcagtcatggccatggt-3', antisense 5'-ttggccttggctttcctcag-3'), VE-cadherin (sense 5'-cctggtataacctgactgtg-3', antisense 5'-tgtgatggtgaggatgcaga-3'), and GAPDH (sense 5'-gacccctcattgacctcaact-3', antisense 5'-caccaccttcttgatgtcatc-3').

Cell sorting by flow cytometry

The cells were harvested into a FACS tubes (Corning, USA) at the density 10^6 cells/tube, washed with PBS 1X and incubated with Human VEGF R1/Flt-1 PE-conjugated (RD Systems, Minnesota, EUA) at dark and room temperature for 35 minutes, using the concentration 14µL/10^6 cells. After this time, the cells were washed and resuspended in Stain Buffer (BD Bioscience, San Jose, CA, EUA). The cells were sorted by high and low-expression of VEGFR-1 ($VEGFR1^{HIGH}$/ $VEGFR1^{LOW}$). As negative control was used cell without staining and cell stained with Monoclonal Mouse IgG (RD Systems, Minnesota, USA). The analyses were performed in FlowJo Software.

Sulforhodamine B (SRB) Assay

SHED and DPSC were seeded at the density 2.5x10^{3}cell/well in a 96-well plate. After the period of adhesion, they were fixed with 10% trichloroacetic acid and were incubated at 4ºC for 1 hour. After, the cells were washed, dried, stained with 0.4%SRB solution, and incubated at room temperature for 30 minutes. In order to remove the unbound excess dye, the cells were washed with 1% acetic acid and after to dry the dye was solubilized with trizma-base. The plates were read on the microplate reader at 565nm. Data were obtained from 8 wells per condition.

Western Blot

The protein from DPSC/SHED was collected, lysed it in NP-40, and its concentration was quantified according to absorbance emitted in a spectrophotometer at 595 nm of wavelength (Genios, Techan). The protein lysate was loaded in a 08% SDS Gel and after the gel was transferred to a cellulose membrane. The membrane was blocked in 5% milk for 30 minutes, incubated with a primary antibody and kept overnight in 4° C. Primary antibodies were the following: VEGFR-1/Flt-1, VEGFR-2/FLK-1, TIE-2, CD31, VE-cadherin, and GAPDH. The next day, the membrane was washed 2 times with TBST, incubated with a secondary antibody for 2 hours, washed again for twice, and followed by the use of SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, Rockford, IL, USA).

Differentiation Assay in Vitro

Immediately after the sorting of SHED in VEGFR1^{HIGH}/ VEGFR1^{LOW}, 2x10^5 cells were seeded in Petri's Dish with normal medium alpha-MEM supplemented with 20%FBS and lead to adhere overnight. At next day the medium was changed for Endothelial Growth Medium (EGM2-MV) supplemented with 50ng/mL of rhVEGF (RD Systems, Minneapolis, MN, USA) (differentiation medium) and the medium was replaced each 2 days. To confirm the endothelial differentiation of SHED after 3, 5 and 9 days in differentiation medium, western blot was performed to check the presence of endothelial markers as VEGFR-2, CD-31, Ve-cadherin, and Tie-2 on SHED.

In vitro Sprouting Assay

SHED and DPSC at a cell density of 1.0 x10^4 cell/well were seeded in a 12-well plate pre-coated with Growth Factor Reduced Matrigel (BD Bioscience, Bedford,

MA, USA). The cells were cultured for 10 days in an Endothelial Growth Medium (EGM2-MV) supplemented with 50ng/mL of rhVEGF (RD Systems, Minneapolis, MN, USA) with the presence or not of 25μg/mL of Bevacizumab (Avastin® - Genentech, CA, USA). The medium was replaced each 2 days. The groups were divided into VEGFR1^{HIGH} Control, VEGFR1^{HIGH} Bevacizumab, VEGFR1^{LOW} Control, and VEGFR1^{LOW} Bevacizumab. The sprouts were counted under a light microscope with high magnification (100X), considering 12 fields per well in 3 wells per condition. The experiment was performed three times in three independent weeks.

Immunofluorescence

After the sorting of cells in DPSC/SHED VEGFR1^{HIGH} and VEGFR1^{LOW}, 5x10^4 cells were seeded in chamber slides and were fixed 24h after. The monolayer of cells was washed and incubated overnight with primary antibody Flt-1 (C-17) (rabbit polyclonal IgG; # SC-316; Santa Cruz Biotechnology, Santa Cruz, CA, USA). At the second day the samples were washed and received the secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (H+L) (# A11034; Life Technologies Corporation, Eugene, Oregon, USA) to visualize the VEGFR-1 and the nuclei were stained with Vectashield Mounting Medium for fluorescence with DAPI (#H-1200; Vector Laboratories, Burlingame, CA, USA).

Scaffold Assay

Scaffolds of PLLA (Poly-L-lactic/chloroform) were prepared and were cut in 6mmX6mm. Cells were sorted in SHED VEGFR1^{HIGH} and VEGFR1^{LOW} through Flow Cytometry (PE fluorescence) and the quantity of 7.7 x 10^6 cells mixed with Matrigel (1:1) were seeded per scaffold. A total of 6 scaffold per condition were transplanted bilaterally (Right = VEGFR1^{HIGH} and Left = VEGFR1^{LOW}) in a subcutaneous into the dorsum of severe combined immunodeficient mice (SCID) immediately after the plating of the cells. The scaffolds were retrieved after 28 days and were fixed in 10% formaldehyde for 24h at 4ºC and prepared for histology. Histologic sections (5-µm-thick) were stained with hematoxylin-eosin or kept unstained for immunohistochemistry assay.

Immunohistochemistry

The slices pass through a process of deparaffinization with a series of bath of Xylene, Ethanol 100%, Ethanol 95%, Ethanol 75%, and water(DDW). After adding

Trypsin (Merck, Germany) at concentration 1mg/1ml for 1 hour at 37º C. The first day was used the solutions 1xPBS-0,1%Triton-X-100 and 3%H_2O_2, and background Sniper (Biocare Medical, USA) for 20 minutes each at room temperature, followed by the addition of primary antibody. The antibodies used were CD-31(Rabbit anti-CD31 IHC Antibody, Bethyl Laboratories, Montgomerry, TX) and Factor VIII related antigen/Von Willebrand factor Ab-1 (Thermo Scientific, Waltham, MA USA). At the second day was performed the washing with Wash Buffer (Dako North America, Carpinteria, CA, USA) and the solutions MACH 3 Rabbit/Mouse Probe (Biocare Medical, USA), MACH 3 Rabbit/Mouse HRP-Polymer (Biocare Medical, USA), Betazoid DABI Chromogen Kit (Biocare Medical, USA) were added to the slices for 20 min each, except the DABI that just kept contact with the slice for a few minutes. At the final wash, Vecta Mount (Vector Laboratories, CA) was added. A total of 8 pictures (200x) were taken randomly into a scaffold area and the new microvessels were counted by a calibrated researcher using the Image J software.

Statistical analysis

All statistical analyses were executed by GraphPad Prism software (GraphPad, San Diego, CA). The Shapiro-Wilk normality test was applied in the quantitative measurements. Data were analyzed by Test T unpaired or one-way ANOVA followed by Tukey test. The level of significance was set at 5%.

Results

Expression of Endothelial Cell Markers by DPSC, SHED and HDMEC cultured in normal medium and DPSC and SHED morphology after cultured in differentiation medium.

SHED and DPSC cultured in alpha-MEM supplemented with 20% FBS express Tie-2 and VEGFR-1 mRNA and at protein level they express only VEGFR-1. Tie-2 protein was overexposed when the western blot was performed, and it showed none signals of the expression of this protein on studied cells. In respect of the endothelial cell markers, those cells don't show VEGFR-2, Tie-2, CD-31, and Ve-cadherin expression when cultured into normal medium (Figure 2). When that cells were seeded in 3D Matrigel and cultured with a medium of differentiation (EGM2-MV supplemented with 50 ng/ml of VEGF) their cell morphology changed and the formation of new sprouts increased (Figure 2) in comparison with the other cells

which received the differentiation medium supplemented with Bevacizumab (Avastin), a potent inhibitor of vasculogenesis through its binding to VEGF, blocking the possible VEGF-VEGFR1 binding (Figure 2). The HDMEC cells present high levels of important receptors as VEGFR1 and VEGFR-2, whereas DPSC and SHED present lower levels of VEGFR-2. SHED showed twice as much VEGFR1 as DPSC (Figure 3).

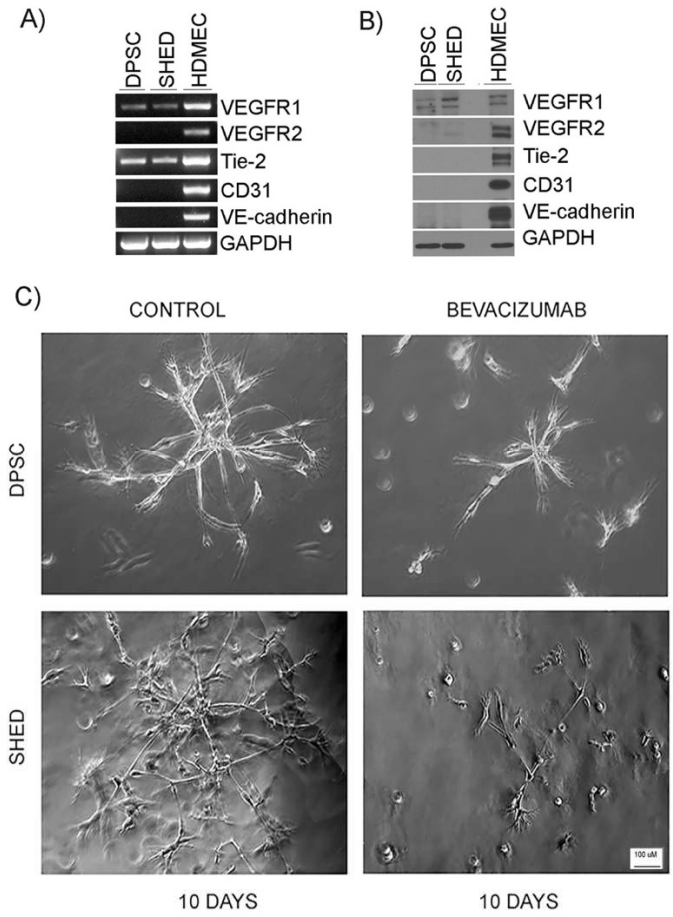

Figure 2 – RT-PCR and Western Blot: Analyses of VEGFR1, VEGFR2, Tie2, CD-31, VE-cadherin, and GAPDH expression in DPSC and SHED cultured in medium alpha MEM 20 % FBS (A,B). HDMEC was used with positive control. SHED and DPSC were seeded into a pre-coated well with 3-D Matrigel and cultured with EGM2-MV supplemented with 50 ng/mL rhVEGF (Control) and 0 or 25μg/ml of Bevacizumab (Avastin®) for 10 days.

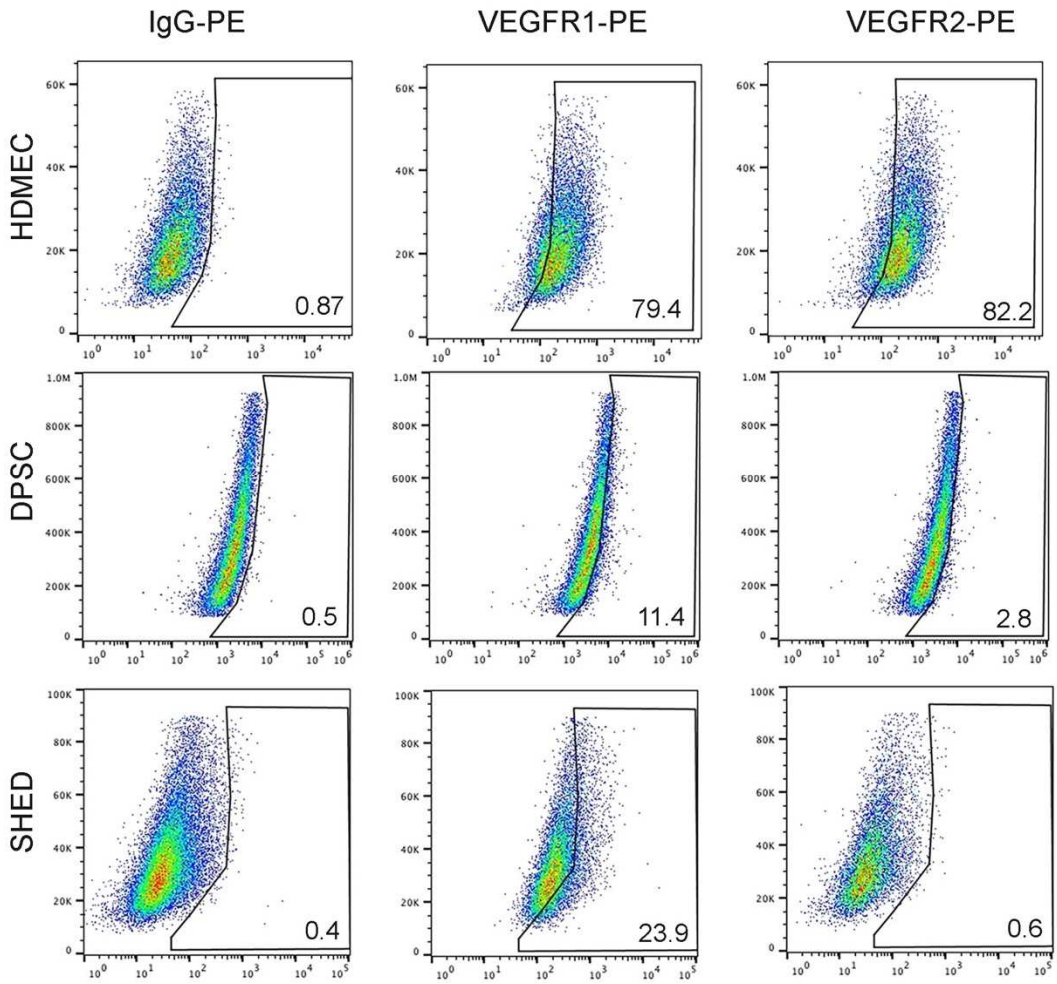

Figure 3 – Flow cytometry analysis of VEGFR1 and VEGFR2 expression on SHED, DPSC, and HDMEC. Cells were analyzed using anti-VEGFR1 and anti-VEGFR2 conjugated PE antibody and IGG-PE monoclonal antibody was used as a control. The data were analyzed at FlowJo Software.

Sorting of the SHED/DPSC according to VEGFR-1 expression

In order to evaluate if the levels of VEGFR1 expression in SHED and DPSC increase the vasculogenic differentiation of DPSC and SHED, the sorter was performed, and the same percentage of positive and negative cells was selected (Figure 4). To set up the machine and decrease the background, the population was selected according to Figure 5. The proliferation of $VEGFR1^{LOW}$ presents no difference with the proliferation rate of $VEGFR1^{HIGH}$ at 24h and 48h when cultured in alpha-MEM or EGM2-MV, but at 72h the $VEGFR1^{LOW}$ has more cell than $VEGFR1^{HIGH}$ for the two kinds of medium.

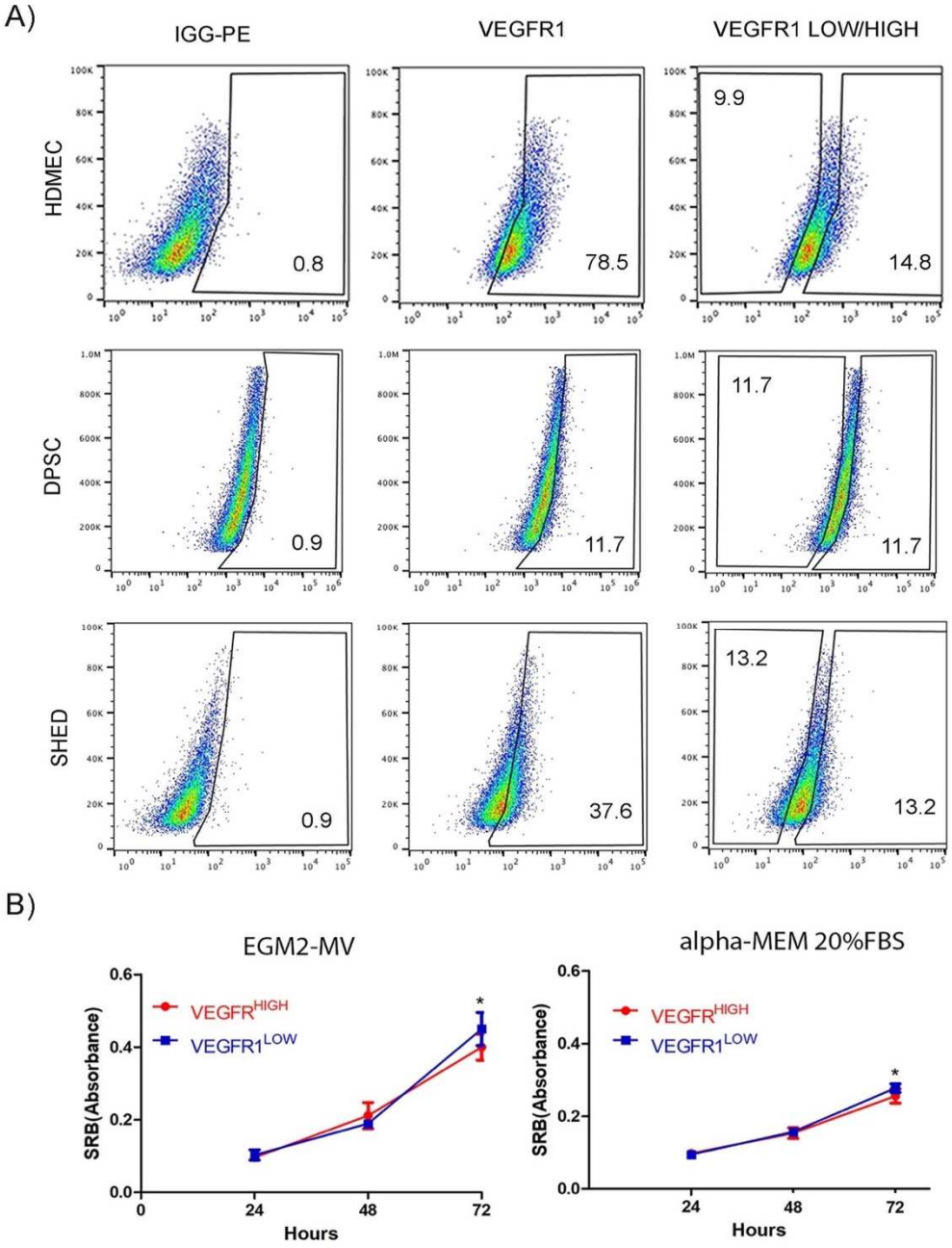

Figure 4 –HDMEC, SHED, and DPSC were sorted in VEGFR1^{High} and VEGFR1^{Low}, using Flt-1/VEGFR1 PE-conjugated antibody (R & D Systems) in a Synergy Sorter Machine (Sony Biotechnology), IgG-PE and unstained cells were used as the negative control (A). Evaluation of cell proliferation rate between SHED VEGFR1^{HIGH} and VEGFR1^{LOW} cultured into Endothelial Growth Medium (EGM2-MV) supplemented with 50ng/ml of rhVEGF (B) and alpha-MEM supplemented with 20%FBS(B) at 24h, 48h, and 72h. The proliferation assay is based on data from 8 well per condition in 2 independent experiments.

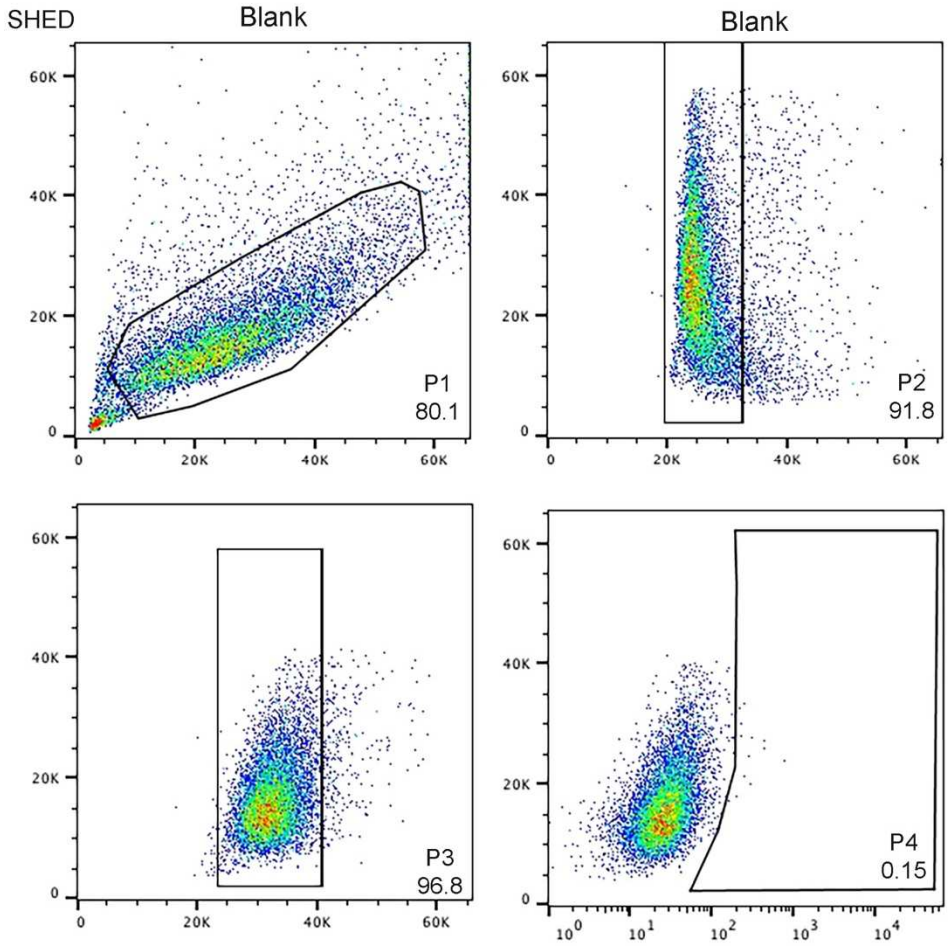

Figure 5 – Selection of the population from P1 to P4 with the aim to remove debris (P1) and double cells (P2, P3), purify the population and decrease the background at the final population (P4). This figure is about SHED unstained (Blank) which works as a negative control and shows the machine the normal cell's fluorescence.

Endothelial Differentiation in vitro checked by endothelial markers expression and cell morphology by SHED/DPSC

After 5 days cultured in differentiation medium, SHED starts to express the endothelial markers VEGFR-2, Ve-cadherin, and Tie2, but the CD-31 expression is only seen at 9 days after. There is greater quantity of endothelial markers in cells with high levels of VEGFR1 than the cells with low levels of this same receptor (Figure 6-A). Tie-2 expression is weak for $VEGFR1^{HIGH}$ and absent at $VEGFR1^{LOW}$ (Figure 6-A). When sorted SHED and DPSC were seeded in matrigel and cultured with differentiation Medium and medium supplemented with 25μg/ml of Bevacizumab, it is posible to see the morphology of those cells and the difference in the quantity of sprouts formation in vitro between cells. The VEGFR1 positive cell

and cultured only with differentiation medium seem produce more quantity of sprouts than VEGFR1 negative cells and those that received treatment with Bevacizumab (Figure 6 – B).

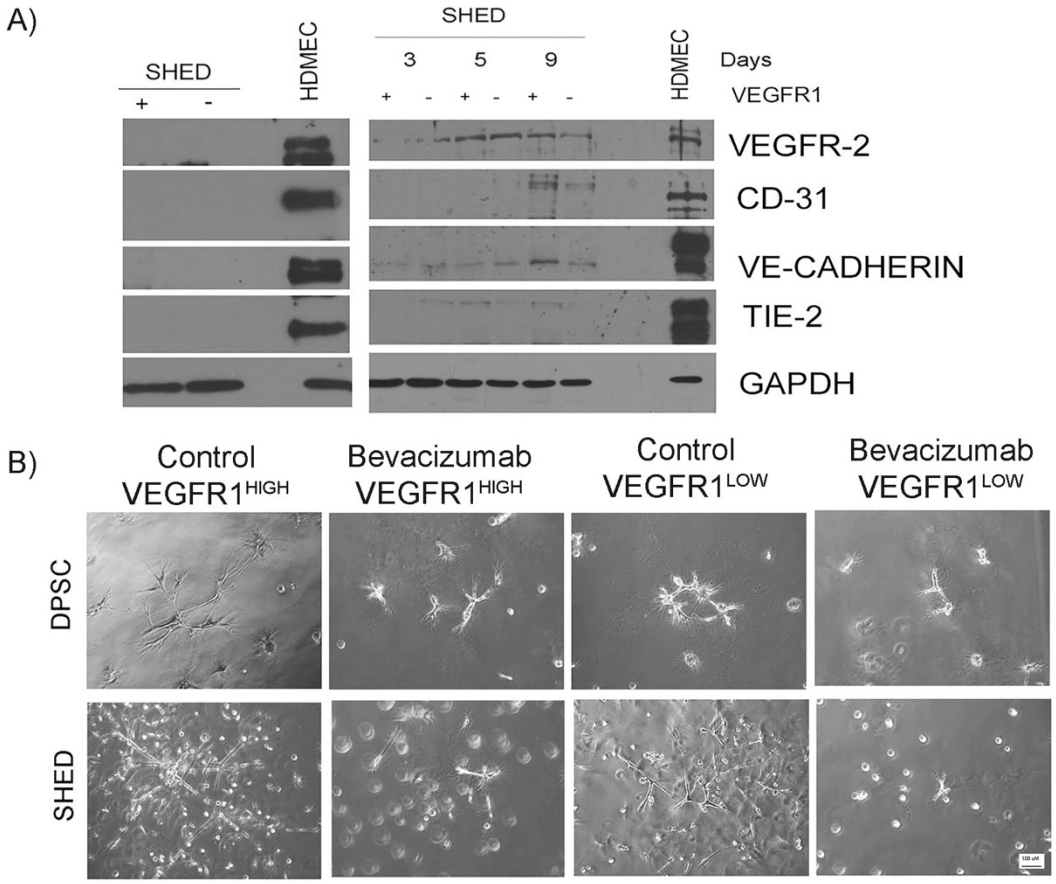

Figure 6 - In vitro endothelial differentiation of SHED. SHED doesn't express endothelial markers (A – right), but when cultured for 9 days in EGM2-MV supplemented with 50ng/ml of VEGF, those cells present VEGFR-2, CD-31, and Ve-cadherin expression (A- left). SHED VEGFR1^{HIGH} (VEGFR1 positive) exhibited a greater quantity of those proteins than VEGFR1^{LOW} (VEGFR1 negative). The quantity of sprouts production is higher in positive cells than negative cells for VEGFR1 (B) for both SHED and DPSC.

Time Course of sprouting formation by SHED and DPSC with High and Low Levels of VEGFR-1

Overtime the quantity of sprouts formation was greater for VEGFR1^{HIGH} SHED/DPSC than VEGFR1^{LOW} SHED /DPSC, and the medium which received

Bevacizumab (Avastin) had an important role in the decrease of the sprout formation (Figure 7, 8 and 9).

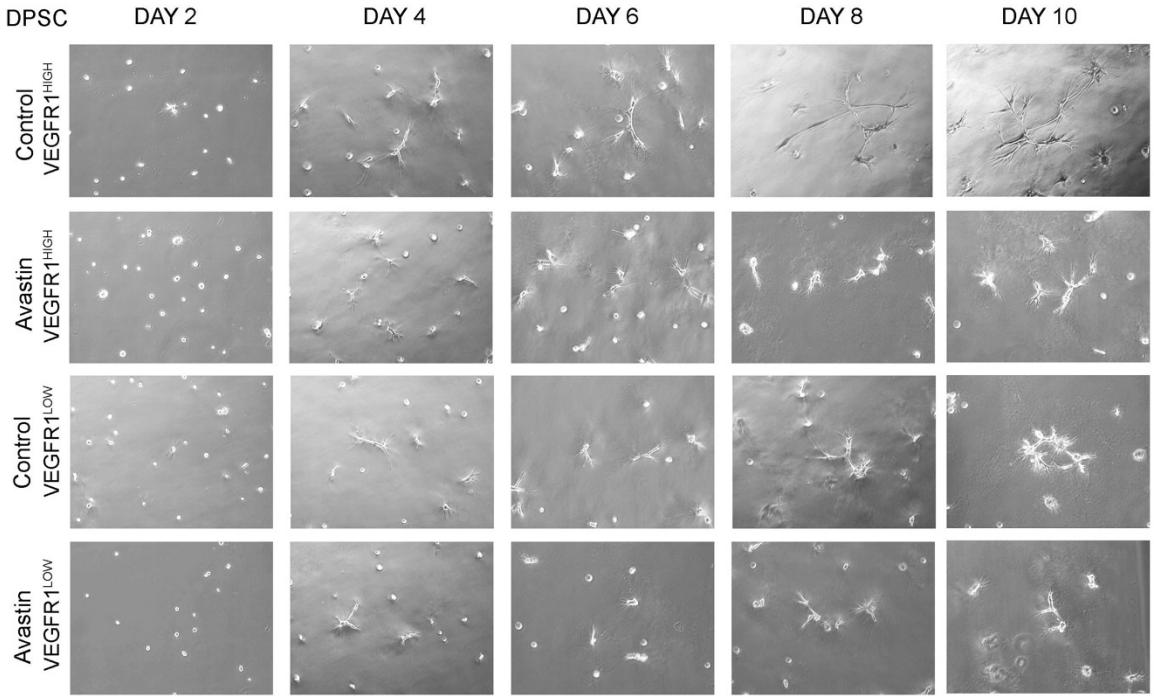

Figure 7- Cell morphology over time of VEGFR1^{High}/VEGFR1^{Low} DPSC, seeded into a pre-coated well with Matrigel and treated with EGM2-MV with 50ng/mL rhVEGF (Control) and 0 or 25µg/mL of Bevacizumab (Avastin®) for 10 days (bar= 100x).

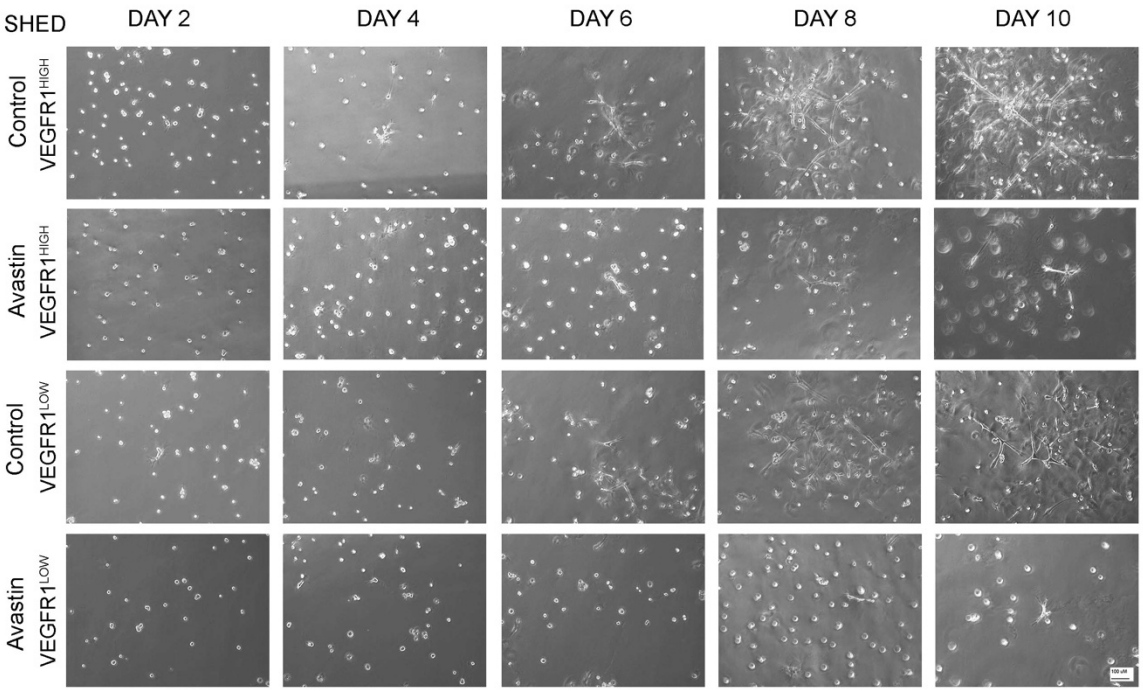

Figure 8- Cell morphology over time of VEGFR1^{High}/VEGFR1^{Low} SHED, seeded into a pre-coated well with Matrigel and treated with EGM2-MV with 50ng/mL rhVEGF (Control) and 0 or 25µg/mL of Bevacizumab (Avastin®) for 10 days (bar= 100x).

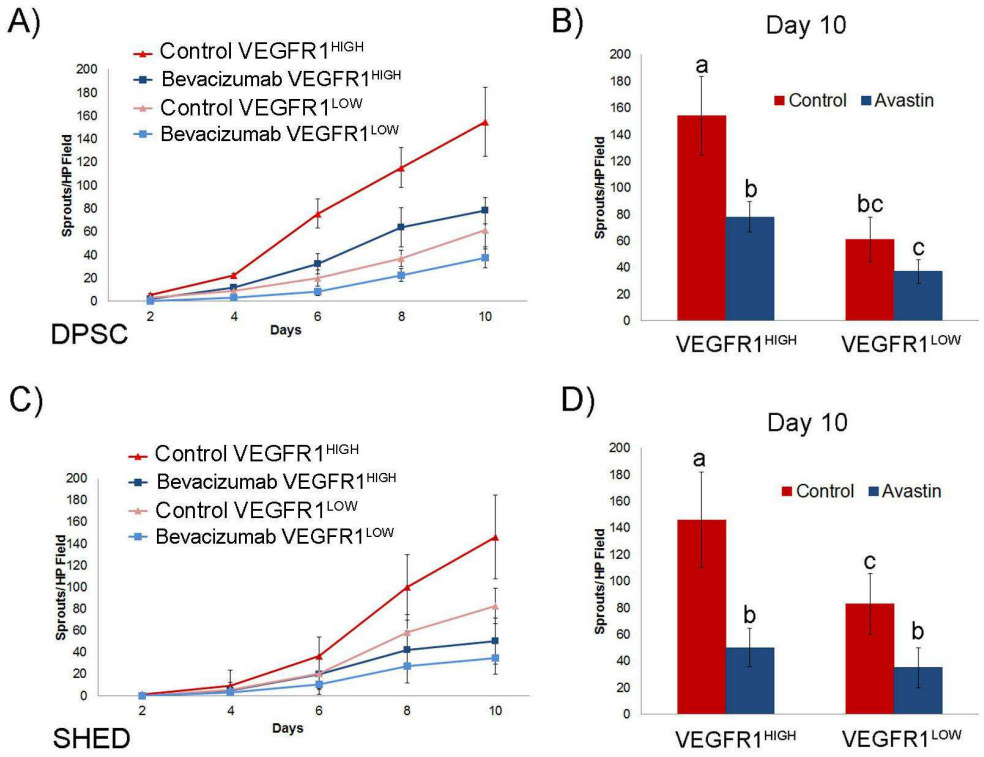

Figure 9 –High levels of VEGFR1 expression in cells in vitro is related to increase VEGF-induced capillary sprouting of SHED and DPSC. Evaluation over time of DPSC (A, B) and SHED (C, D) seeded in Matrigel and cultured in EGM2-MV with 50ng/mL rhVEGF (Control) and 0 or 25µg/mL of Bevacizumab (Avastin®) for 10 days. The groups were divided according to the High (VEGFR1^{HIGH}) and Low (VEGFR1^{LOW}) expression of that receptor. Data were analyzed in 12 microscopic fields previously selected from triplicate wells per condition, and data of the quantity of sprouts are shown as the average ± standard deviation.

Time course of VEGFR1 expression on SHED and DPSC after sorting

The sorting maintains its features of the VEGFR1^{HIGH} SHED/DPSC to have more expression of that receptor than VEGFR1^{LOW} SHED/DPSC for a period of time. To evaluate this cell ability to keep the VEGFR1 expression different between the positive and negative group, immunofluorescence was performed after one day of sorting and flow cytometry after 4 and 10 days of sorting. The immunofluorescence assay revealed a weak difference between the groups (Figure 10-A). The flow cytometry assay exhibited an increase of the percentage of the population of cells VEGFR-1 positive overtime for both groups (VEGFR1^{HIGH} and VEGFR1^{LOW}), but the group VEGFR1^{LOW} kept its lower percentage of VEGFR-1 expression (Figure 10-B).

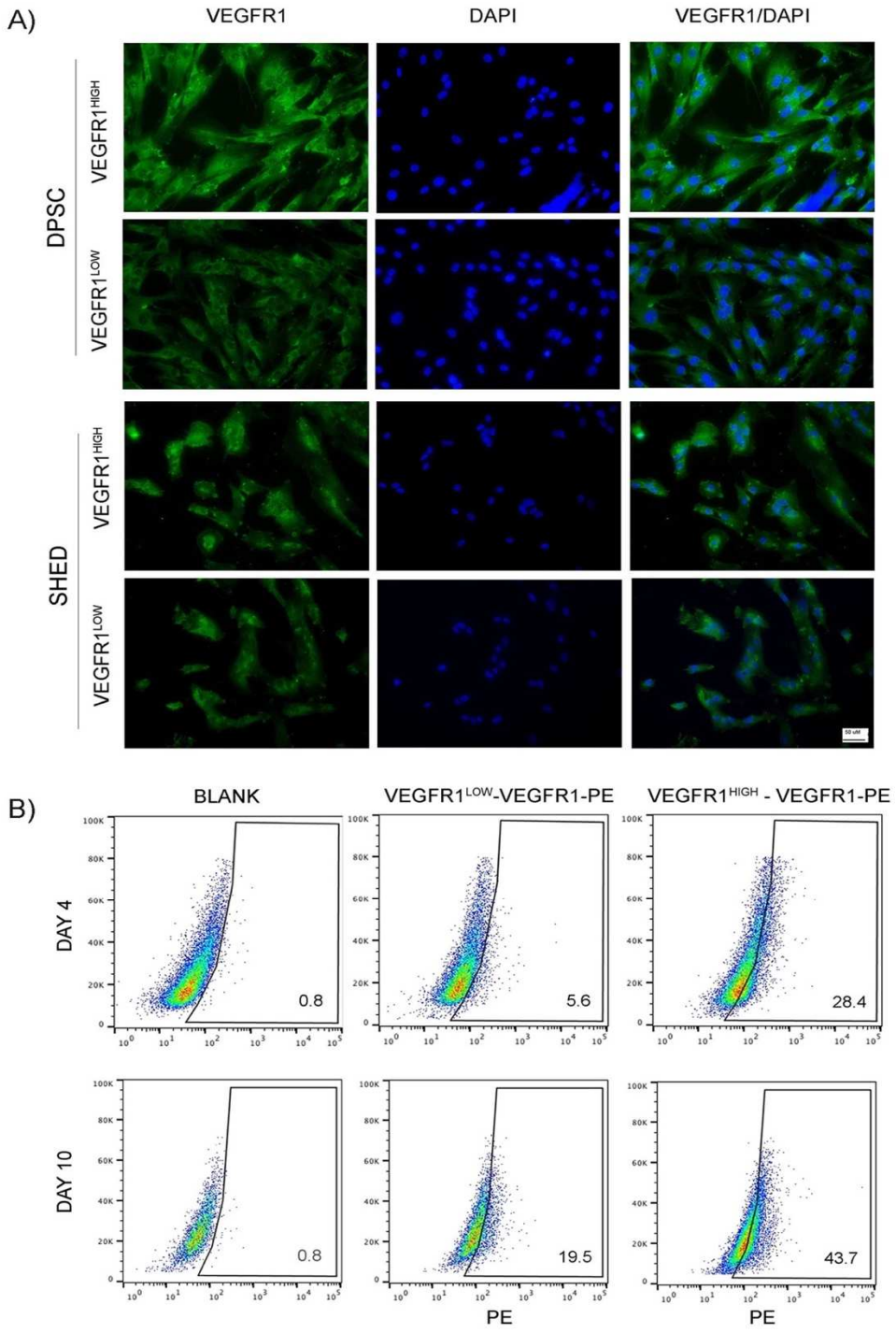

Figure 10 – DPSC and SHED were sorted in VEGFR1^{High}/VEGFR1^{Low} through Flow Cytometry. The cells were kept into the culture for one day and after they were fixed and the Immunofluorescence Assay was performed for VEGFR1 and DAPI (bar=20μm) (A). The same cells that were sorted, were kept in culture with alpha MEM supplemented with 20%FBS and they were analyzed through Flow Cytometry after 4 and 10 days. HDMEC was used as a positive control (B).

Analysis of the morphology between cells unsorted, cells with VEGFR1^{HIGH} and VEGFR1^{LOW} for SHED and DPSC

SHED and DPSC unsorted and sorted according to the levels of VEGFR1 expression were cultured in differentiation medium for 11 days. Data from 3 independent experiments. If we evaluating the pictures at the same magnification, SHED seems to lead more time to differentiate than DPSC, because DPSC exhibits sprouts at day 5, while SHED just starts to exhibit bigger sprouts at day 7. It is important emphasize that SHED is smaller than DPSC and it is possible to see at the microscope good quantity of smaller sprouts on every area of the well (Figure 11 and 12).

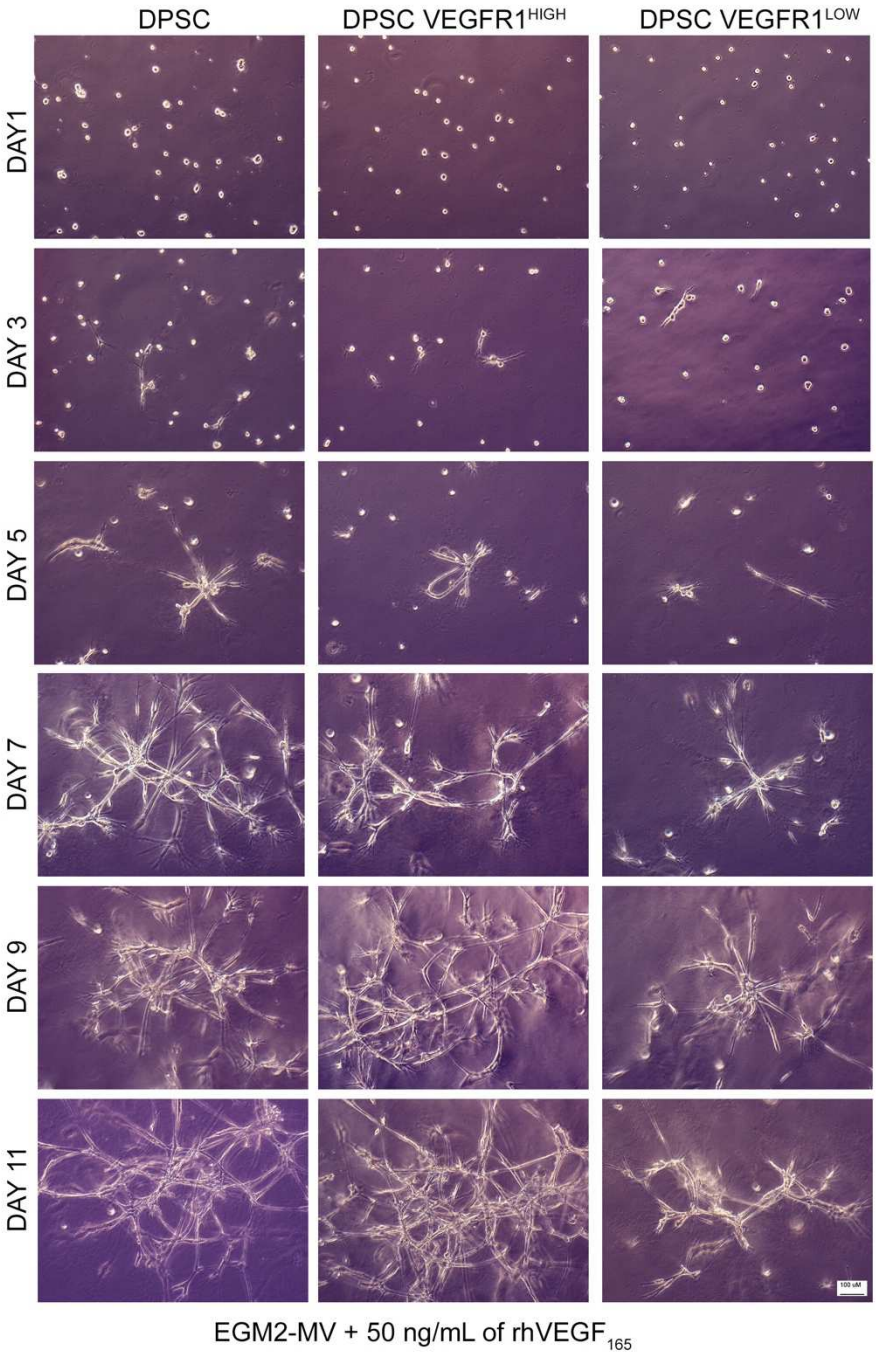

Figure 11 – Evaluation over time of DPSC unsorted and sorted in VEGFR1^{High}/VEGFR1^{Low} through Flow Cytometry. The cells were seeded in a pre-coated well with Matrigel and cultured in EGM2-MV with 50ng/mL rhVEGF for 11 days(bar=100x).

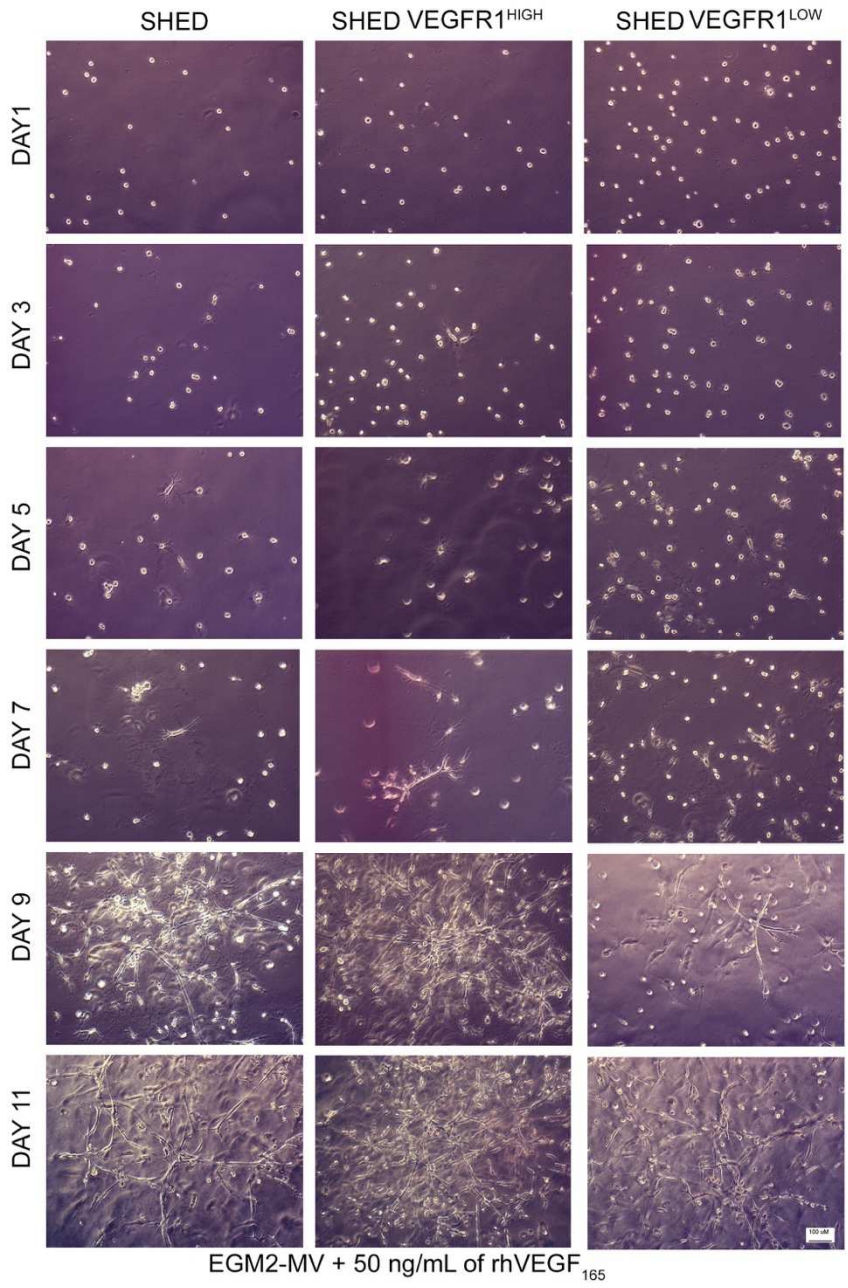

Figure 12 – Evaluation over time of SHED unsorted and sorted in VEGFR1^{High}/VEGFR1^{Low} through Flow Cytometry. The cells were seeded in a pre-coated well with Matrigel and cultured in EGM2-MV with 50ng/mL rhVEGF for 11 days(bar=100x).

In vivo Assay

The in vivo assay revealed more quantity of blood microvessel formation for scaffolds which received cells VEGFR1^{HIGH} than which one that received VEGFR1^{LOW} suggesting that the higher levels of this receptor is involved with the differentiation of a greater quantity of cells and formation of new blood vessels in

vivo. The immunohistochemistry assay was run and microvessel positive for factor VIII and anti-human CD31 were counted using the ImageJ Software, 8 randomly area per scaffold (n=6) were selected resulting in the total of 48 areas per group. The cells with high levels of VEGFR1 showed greater number of blood vessel formation than one with low levels of the same receptor (Figure 13). The immunofluorescence assay for the same slices, and with the same purpose: to show blood vessels markers in the different groups, didn't exhibit big difference between the two groups in the confocal microscope (Figure 14). It is possible to see some green fluorescence around the blood vessel for both groups, but the signal is weak (Figure 14).

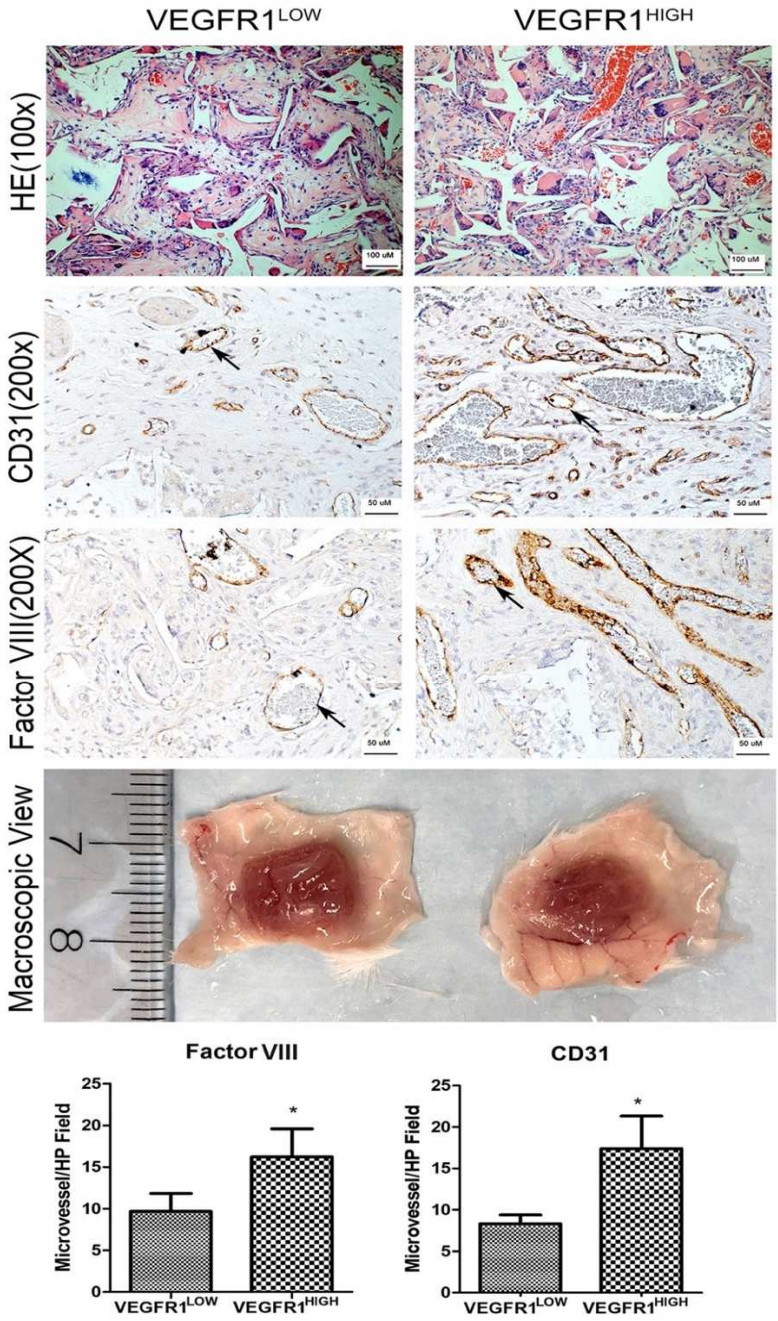

Figure 13 – SHED with high levels of VEGFR-1 is more prone to produce greater quantity of blood vessels in vivo. Hematoxylin and eosin staining after 28 days of scaffold implantation (bar:200x). Immunohistochemistry for anti-human CD-31 and Factor VIII performed at slices from scaffolds with $VEGFR1^{High}/VEGFR1^{Low}$ (bar: 200x). Macroscopic View of the scaffold retrieved from immunodeficient mice after 28 days of implantation. The graphs are in respect of the quantity of microvessel after the in vivo implant into immunodeficient mice. The data were collected in 8 randomly different areas from the slices (n=6), in a total of 48 pictures per condition (200x). Black arrows show the CD31-positive and Factor VIII- positive blood microvessels.

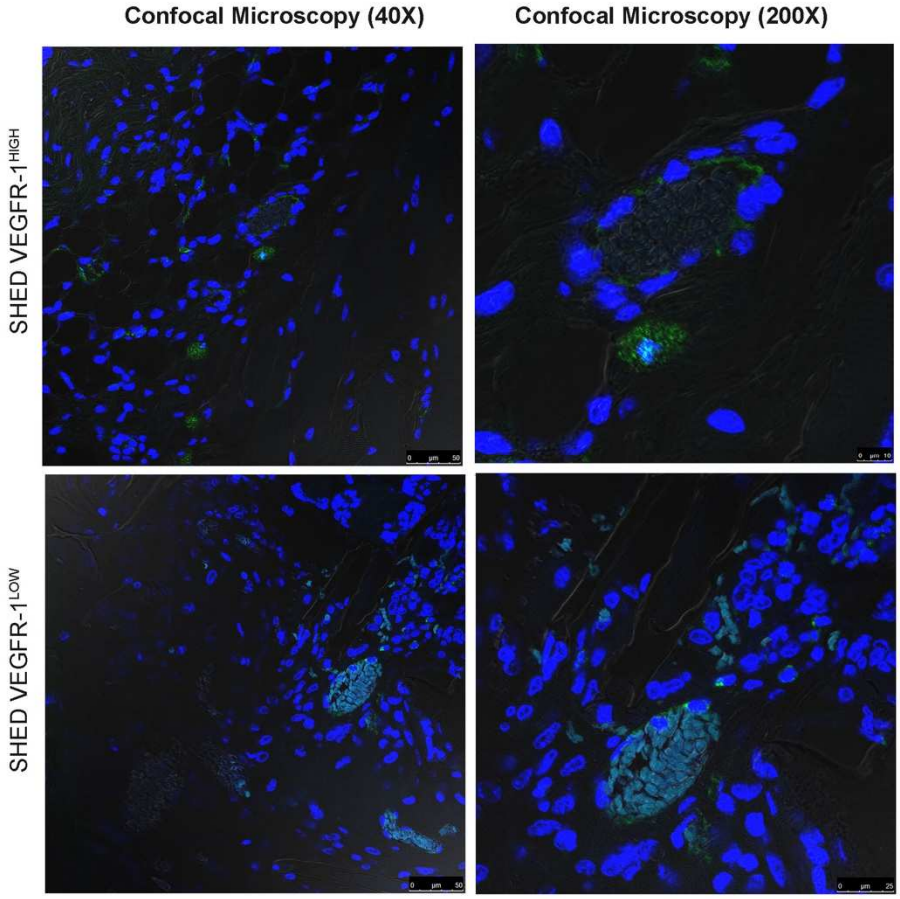

Figure 14 – Scaffolds seeded with SHED VEGFR1^{High}/VEGFR1^{Low} and transplanted into the subcutaneous of immunodeficient mice. The samples were retrieved after 28 days, fixed, and analyzed by Immunofluorescence Assay for anti-human CD-31(green) and DAPI (blue) (bar: 40x and 200x).

Discussion

This work shows the importance of the presence of VEGFR1 in the differentiation process of Stem Cells from dental pulp in endothelial-like cells and its relationship with angiogenesis. Stem cells with higher function and expression of VEGFR1, when cultured in medium supplemented with VEGF in vitro, showed more quantity of sprouting formation and more quantity of microvessel development in vivo.

The presence of VEGFR-1 on undifferentiated SHED/DPSC and after the differentiation leads us to think that this receptor keep relation with a possible role in endothelial differentiation from SHED and DPSC. SHED and DPSC when cultured in

normal medium for stem cells (alpha MEM) showed VEGFR-1 and Tie-2 expression in RNAm level, but at the protein level just exhibited the VEGFR1 expression, suggesting a possible pos-transcriptional regulation of the Tie-2 in the DPSC and SHED what results in no expression of this protein. On the other hand, when those cells were cultured in differentiation medium (EGM2-MV supplemented with 50ng/mL of VEGF) for 5 days or more, they exhibited a different morphology with the sprout formation and started the expression of endothelial cells markers as VEGFR-2, Tie-2, CD-31 and Ve-cadherin confirming the differentiation of this cells in vitro. When cells are characterized as mesenchymal stem cells (MSC) they should present positive stem mesenchymal markers and negative for endothelial markers as CD-31 and VEGFR-2 , however the presence of these markers confirms the endothelial differentiation of stem cells (5,12,15,17,18).

The formation of new vessels from the current ones is known as angiogenesis and it allows the correct supply of oxygen and nutrients arrives into the tissue.The angiogenesis is an important step for the regenerative process mainly in pulp repair, but this process is also involved in tumor development, inflammatory and vascular disease. There are some methods to control the formation of new vessels by blocking one of the main pro-angiogenic growth factors: VEGF. This growth factor can be regulated naturally with the presence of sVEGFR1 or with anti-angiogenic drugs as Bevacizumab (Avastin), in both cases these molecules bind to VEGF and decrease the quantity available of this growth factors hence decrease the VEGF/VEGFR1 interaction and blocking the next step which would result in angiogenesis (19–21). In our study we choose the bevacizumab and the results are solid because its use decreases the sprout development by SHED and DPSC even in the presence of differentiation medium.

During the process of pulp regeneration, the cells from dental pulp pass through some cellular events that is followed by angiogenic and neurogenic outcomes (8). It is important to understand the pathways involved in the angiogenesis process, and it is known the role of VEGFA and its main receptors as VEGFR-1 and VEGFR-2 in this process in endothelial cells (21). The main receptor involved in angiogenesis in this cells is the VEGFR2, but VEGFA binds to VEGFR-1 (soluble or not) with more affinity than VEGFR-2, so in this way, the quantity of VEGFA whose binds to the VEGFR-2 and the proliferation of these cells are decreased and the angiogenesis process is regulated (21,22). Our paper shows that

on undifferentiated SHED and DPSC the VEGFR2 receptor is not expressed, but this receptor is expressed when cells are differentiated. One the other hand the VEGFR1 is expressed in those undifferentiated and differentiated cells. This suggest in the first moment the vasculogenic differentiation by dental pulp stem cells is started through VEGF/VEGFR-1 binding and at second moment the differentiated cells express VEGFR2 and it added to the vasculogenic process.

Some previous papers showed the importance and the role of VEGFR-1 in the process of endothelial differentiation of dental pulp stem cells through gene silencing of VEGFR-1 and its isoforms which resulted in a inhibition of the expression of endothelial cells markers and of the formation of blood vessels in vivo(5,12). This study didn't use the technique of silencing gene because the aim was evaluating the lower expression/function of this receptor instead of the complete removal of it. In order to sort the DPSC and SHED in cells with higher/positive and lower/negative expression of VEGFR-1, the flow cytometry technique was used.

One limitation of this study was the most important step: the sorting of cells through flow cytometry. So, some parameters had to be considered as the confluence of the flask (80-90%), the enzymatic solution used to detach cells from cell culture flask, the number of cells cultured and seeded inside each flow tube, temperature and time of incubation, and concentration of antibody. After, the other issues appeared as how much time leads the $VEGFR1^{LOW}$ cells to get back the VEGFR1 expression? Is the proliferation rate of $VEGFR1^{LOW}$ and $VEGFR1^{HIGH}$ cells the same? The flow cytometry time course and SRB assays answered those questions. The flow cytometry showed that until day 10 after sorting the cells $VEGFR1^{LOW}$ exhibited lower expression of VEGFR1 than $VEGFR1^{HIGH}$, but the expression of this receptor increased overtime. The SRB assay showed that the $VEGFR1^{LOW}$ and $VEGFR1^{HIGH}$ presented the same rate of proliferation at 24h and 48h, but at 72h the proliferation rate of $VEGFR1^{LOW}$ was higher, but it was not enough to be a bias in this study because even the negative population showing higher proliferation rate, they not presented greater quantity of sprouting formation at in vitro assays.

Our study showed that SHED exhibited higher levels of VEGFR-1 expression than DPSC and the photos of time course of SHED and DPSC showed greater quantities of sprout formation at day 10 for SHED. That way, it is expected a lower production of sprouts for DPSC than SHED, in other words SHED should present

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SHED when stimulated has the capacity to differentiate in endothelial cells and to yield new blood vessels in vivo (5,12,17).The cells with a higher function of VEGFR1 showed better performance in vitro and in vivo. In vitro, the morphology of cells showed bigger sprouts and better distributed spatially in the well than cells with lower function of VEGFR1. In our in vivo assay was used the CD-31 and Factor-8 to show the new vessel formation that was clearly identified with immunohistochemistry. This study highlights the importance of this receptor and his function in the endothelial differentiation process of stem cells from dental pulp.

Conclusion

Not all stem cells from dental pulp can differentiate in endothelial cells. Our study showed that one subpopulation of the stem cells which has high levels of VEGFR1 expression and function seems to be related to events of vasculogenic differentiation. This result was checked by a greater number of tubular formations by differentiated stem cells in vitro and more quantity of new blood vessel formation in vivo. These results suggest an important role of this receptor in pulp stem cell differentiation in endothelial cells and open new opportunities to study deeper the pulp tissue regeneration at the molecular level.

Acknowledgments

The Department of Cariology, Restorative Sciences, and Endodontics at the University of Michigan School of Dentistry (Nor Lab) and FAPESP (#2017/11396-3; #2018/13675-0) supported this work. This paper is a doctoral thesis developed in at the University of São Paulo and at the University of Michigan.

REFERENCES

*Same of the chapter REFERENCES.

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