Experimental Cell Research 343 (2016) 168–176

Contents lists available at ScienceDirect

Experimental Cell Research

journal homepage: www.elsevier.com/locate/yexcr

Research Article

In-depth evaluation of commercially available human vascular smooth muscle cells phenotype: Implications for vascular tissue engineering

Sara B.H. Timraz [a], Ilyas A.H. Farhat [b], Ghada Alhussein [a], Nicolas Christoforou [a,c], Jeremy C.M. Teo [a,*]

[a] Department of Biomedical Engineering, Khalifa University, PO Box 127788, Abu Dhabi, United Arab Emirates
[b] Department of Applied Mathematics and Sciences, Khalifa University, PO Box 127788, Abu Dhabi, United Arab Emirates
[c] Department of Biomedical Engineering, Duke University, Durham, NC 27708, USA

ARTICLE INFO

Article history:
Received 28 October 2015
Received in revised form
3 April 2016
Accepted 7 April 2016
Available online 11 April 2016

Keywords:
Smooth muscle cell
Phenotype
Phenotypic modulation
Tissue engineering

ABSTRACT

In vitro research on vascular tissue engineering has extensively used isolated primary human or animal smooth muscle cells (SMC). Research programs that lack such facilities tend towards commercially available primary cells sources. Here, we aim to evaluate the capacity of commercially available human SMC to maintain their contractile phenotype, and determine if dedifferentiation towards the synthetic phenotype occurs in response to conventional cell culture and passaging without any external biochemical or mechanical stimuli. Lower passage SMC adopted a contractile phenotype marked by a relatively slower proliferation rate, higher expression of proteins of the contractile apparatus and smoothelin, elongated morphology, and reduced deposition of collagen types I and III. As the passage number increased, migratory capacity was enhanced, average cell speed, total distance and net distance travelled increased up to passage 8. Through the various assays, corroborative evidence pinpoints SMC at passage 7 as the transition point between the contractile and synthetic phenotypes, while passage 8 distinctly and consistently exhibited characteristics of synthetic phenotype. This knowledge is particularly useful in selecting SMC of appropriate passage number for the target vascular tissue engineering application, for example, a homeostatic vascular graft for blood vessel replacement *versus* recreating atherosclerotic blood vessel model *in vitro*.

1. Introduction

Under physiological conditions, vascular smooth muscle cells (SMC) reside in the tunica (layer) media of the vascular wall and through their contractile apparatus serve as regulators of blood flow and pressure through vasoconstriction and vasodilation [1]. SMC are also pivotal for communicating with adjacent endothelial cells, exchanging information on environmental cues such as blood shear stress, pressure, and components [2,3]. SMC contribution to the initiation and progression of cardiovascular diseases such as atherosclerosis is well-documented [4–6]; when triggered by an atherogenic stimuli like low-density lipoproteins (LDLs), SMC migrate from the tunica media towards the atherosclerotic lesion located at the tunica intima [7,8]. There, the combinatorial upsurge in SMC proliferation rate, and increase in extracellular matrix (ECM) proteins production contribute to the formation of a fibrous capsule around trapped lipids. Progressive enlargement of this occlusive plaque aggravates the disease towards eventual luminal blockage that brings about morbid events [6,9]. Seemingly, SMC residing in the media layer of healthy blood vessels are different from the SMC in the intima of atherosclerotic vessels. The disparity in behavior and function denotes different characteristics between those two populations of SMC.

A primary challenge in the field of vascular tissue engineering is the propensity of SMC to exhibit phenotypic characteristics of the contractile or synthetic phenotype, under different conditions [10,11]. Resident SMC in the tunica media of healthy blood vessels adopt a contractile phenotype, while synthetic SMC are involved in pathogenesis of atherosclerosis and lesion development. The transformation from one phenotype to the other is characterized by an alteration in morphology, contractile apparatus, cellular proliferation rate, capacity of producing ECM proteins and

* Corresponding author. Present address: Khalifa University, F105B, PO Box 127788, Abu Dhabi, United Arab Emirates.
E-mail addresses: sara.timraz@kustar.ac.ae (S.B.H. Timraz), ilyas.farhat@outlook.com (I.A.H. Farhat), ghada.alhussein@kustar.ac.ae (G. Alhussein), nicolas.christoforou@kustar.ac.ae (N. Christoforou), jeremy.teo@kustar.ac.ae (J.C.M. Teo).

http://dx.doi.org/10.1016/j.yexcr.2016.04.004
0014-4827/

migration activity [10,11]. To ensure attaining desired cellular function and response in engineered vascular tissues, control over and maintenance of a contractile SMC phenotype is crucial. Documented *in vitro* research on development of engineered vascular tissue has extensively used isolated primary human or animal SMC [12–14]. Research programs that lack such facilities rely on commercially available primary cells sources. This alternative could potentially benefit the field as a whole, eliminating variability in results through dismissing inconsistent isolation procedures, and simultaneously facilitate comparable results across multiple institutions. Here, we aim to evaluate the capacity of commercially available human primary aortic SMC (ATCC) to maintain their contractile phenotype and determine if spontaneous dedifferentiation towards the synthetic phenotype occurs in response to conventional passaging without any intended exposure to external biochemical or mechanical stimuli. The acquired knowledge from this evaluation is beneficial in optimizing SMC passage number for vascular tissue engineering depending on the target application. It is also of great help in predicting cellular behavior and response to environmental cues such as cytokines and ECM proteins, mechanistically determining factors that maintain, initiate, or reverse phenotypic changes. Our hypothesis is that commercially obtained human SMC are contractile in phenotype and a switch towards the synthetic phenotype occurs with increased passaging; a behavior documented in animal SMC [11,15,16], but not characterized in human-derived SMC, which is our aim here. We assessed phenotypic changes based on proliferation, morphology, expression of the marker contractile proteins, smooth muscle myosin heavy chain (SMMHC) and α-smooth muscle actin (α-SMA), smoothelin, deposition of collagen types I and III and migration activity. This evaluation of commercially available human SMC will add value to the field of vascular tissue engineering, as the results presented here can be harnessed for the design of more robust and tailored engineered blood vessels.

2. Materials and methods

2.1. Cell culture

Primary human aortic smooth muscle cells (ATCC, PCS-100-012) are cryopreserved and provided by the company at passage 2 (as stated by the manufacturer shall we keep this statement?). Cells were maintained at 37 °C and 5% CO_2 in culture media consisting of Dulbecco's Modified Eagle's Medium Nutrient Mixture F-12 HAM (DMEM-F12) supplemented with 50 μg/ml ascorbic acid, 5% fetal bovine serum (FBS), 1% penicillin-streptomycin antibiotic, 10 mM L-glutamine and one vial of endothelial cell growth supplement from bovine neural tissue reconstituted in 1 ml phosphate buffered saline (PBS). Cells were grown until they reached approximately 85% confluence before passaging by enzymatic dissociation using 0.05% trypsin – 0.02% EDTA solution and plating at a density of 2500 cells/cm^2, creating stocks of various passages (minimum 4). The plating density was the minimum recommended and thus will yield the most number of cells before passaging. Passages examined in this study are P5, P7, P8 and P10. All reagents were purchased from Sigma.

2.2. Microscopy

To study the effect of passaging on the proliferation rate of human SMC, number of cells at each passage was quantified, through manual counting, over days from bright field microscopic images obtained at random regions. Furthermore, population doubling rate (PDT) was calculated using $t_2-t_1/(3.32*(\log(n_2)-\log(n_1)))$, where n_1 is initial cell count at time t_1 and n_2 is final cell count at time t_2. To assess cell morphology, bright field images at random regions were acquired. Area, major axis, minor axis and perimeter of individual cells were quantified using image processing software FIJI [17]. Aspect ratio, defined by the ratio of the major axis to the minor axis of a bounding ellipse, was quantified for each cell. Aspect ratio is an indicator of cell elongation; the higher its value the more elongated a cell is. Cell shape index (CSI), calculated as $(4\pi*\text{area})/\text{perimeter}^2$, was measured to quantify elongation. A value approaching 0 indicates a more linear and elongated morphology, while a value approaching 1 means the cell spreads out and adopts a less elongated shape [18].

On day 5, cell cultures were fixed using 2% paraformaldehyde (PFA) at room temperature (RT) for 30 minutes. Permeabilization of cells and blocking of non-specific binding sites were achieved by incubating cells with 0.2% triton X-100 (Sigma) in PBS plus 10% FBS for 30 min. Subsequently, cells were incubated with anti-α-SMA primary antibody (Abcam, ab7817), anti-smoothelin antibody (Abcam, ab8969), anti-smooth muscle myosin heavy chain 11 antibody (SMMHC) (Abcam, ab125884), anti-collagen I or anti-collagen III antibodies (Abcam, ab34710, ab7778) or with anti-Ki67 antibody (Abcam, ab16667). Afterwards, cells were incubated with Alexa Fluor 568 donkey anti-rabbit IgG H+L (Life Technologies, A10042) or Alexa Fluor 568 goat anti-mouse IgG2a (Life Technologies; A21134). Nuclei were counterstained with DAPI. Images were acquired using an inverted fluorescent microscope (Zeiss Axiovert Z10, Carl Zeiss Microscopy GmbH, Germany) at a fixed exposure time.

2.3. Gene expression analysis

Primers were designed using NCBI primer-BLAST. In order to avoid polymerization of non-specific DNA amplimers, when applicable primers were required to span an exon-exon junction and the primer pair was required to be separated by at least one intron on the corresponding genomic DNA. Total RNA was collected using the RNeasy Mini Kit (Qiagen, 74104). Quantitative RT.PCR analysis was performed on an ViiA™ real time PCR System (Applied Biosystems) using the GOTAQ® one-step RT-qPCR kit (Biorad, A6023). We used the provided software to analyze the raw data and then additional analysis was performed on Microsoft Excel. The primers used for this experiment are the following: ACTN2_Fw: GACTCTGTGCCCTCATCCAC, ACTN2_Rv: AGGGGTGTTCACGATG TCTTC, MYH11_Fw: AGTATCACGGGAGAGCTGGA, MYH11_Rv: TTGGCTCCCACGATGTAACC, SMO_Fw: ATCGTGGGAGGCTACTTCCT, SMO_Rv: GGAAGCCAAAAATGCCCAGG, ACTB_Fw: ACAGAGCCTC GCCTTTGCCGAT, ACTB_Rv: CATGCCCACCATCACGCCCTG.

2.4. Beta galactosidase assay

Activity of β-galactosidase enzyme is linked to senescence of mammalian cells [19,20]. In order to analyze senescence in SMC at different passage numbers, β-galactosidase was detected through the application of the fluorogenic substrate 3-carboxyumbelliferyl β-D-galactopyranoside, using a commercially available kit (Abcam, ab189815). Samples containing cell suspension were pipetted into a 96-well plate ($n=4$). Blank wells with only reaction buffer served as negative control. Fluorescence intensities were measured using a microplate reader (Tecan Group Ltd, Infinite 200 Pro).

2.5. Deposited collagen I quantification

For each passage of interest, cells were grown to 85% confluency and after enzymatic dissociation and 3 washes of 1X PBS, a fixed volume of PBS was used to collect cell deposited ECM from the cell culture flasks with the aid of a cell scraper. A commercial ELISA kit (R&D Systems, DY6220) was then used to quantify the