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Phenotypic changes in cultured smooth muscle cells: limitation or opportunity to tissue engineering of hollow organs?

Alexander Huber, PhD and Stephen F. Badylak, DVM, PhD, MD

McGowan Institute for Regenerative Medicine, University of Pittsburgh, 450 Technology Drive, Suite 300, Pittsburgh, PA 15219, Phone (412) 624-5253, Fax (412) 624-5256

Alexander Huber: hubera@upmc.edu; Stephen F. Badylak: badylaks@upmc.edu

Abstract

Smooth muscle cells (SMCs) are typically used as a cell source for the reconstruction of hollow organs by conventional tissue engineering techniques. However, the necessity and advantage for the use of tissue specific SMCs is unknown. The present study investigated the phenotypic changes that occur following isolation and *in vitro* expansion of rat SMC populations isolated from three different tissues: the aorta, esophagus, and urinary bladder. rSMCs were isolated by enzymatic dispersion and expanded by conventional cell culture techniques yielding microscopically homogeneous populations. SMC phenotypes were monitored according to their expression of marker proteins during the first two passages. Two of the three SMC populations (rSMC-a and rSMC-e) showed a marked change in their marker protein profiles during the first two passages which resulted in a homogenous phenotype that was neither fully contractile nor fully synthetic. SMCs from the urinary bladder did not show such a shift. Differences between the three rSMC populations were observed with regard to proliferative activity and gene expression patterns suggesting the retention of some tissue-specific cell characteristics. In summary, phenotypic changes in SMCs occur as a result of conventional cell isolation and expansion techniques, thus questioning the necessity of a tissue specific cell source for regenerative medicine applications.

1. Introduction

The therapeutic replacement or regeneration of functional tissue following loss due to trauma, neoplasia, or congenital defects is challenging at best. The field of tissue engineering/regenerative medicine has emerged as a potential strategy for the replacement of various tissues including osteochondral (Frohlich *et al.*, 2008, Kandel *et al.*, 2008, Soucacos *et al.*, 2008), urogenital (Atala, 2009), cardiovascular (Habib *et al.*, 2008, Yuasa and Fukuda, 2008), respiratory (Nichols and Cortiella, 2008), hepatic (Fiegel *et al.*, 2009), and lymphatic (Hitchcock and Niklason, 2008) tissue among others. Conventional tissue engineering approaches include the use of structurally and functionally defined scaffold materials of natural or synthetic origin in combination with allogeneic or autogeneic cells of a single or a small number of different cell types. As such, the construction of even small-scale tissue engineered devices for clinical application requires the isolation and expansion of large numbers of cells while maintaining cellular phenotype and physiology without inducing events of cellular senescence (Hayflick and Moorhead, 1961) and dedifferentiation (Shi *et al.*, 2008, Yu *et al.*, 2007).

Correspondence to: Stephen F. Badylak, badylaks@upmc.edu.

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Smooth muscle cells (SMCs) play an important role in the functionality of numerous tissues and organs, including the vasculature, gastrointestinal tract, urinary bladder, respiratory tract, and the reproductive tract. Several cellular tissue engineering approaches have investigated the use of SMCs in the reconstruction of hollow organs including the esophagus (Nakase *et al.*, 2008, Saxena *et al.*, 2009), intestine (Nakase *et al.*, 2006), internal anal sphincter (Somara *et al.*, 2009), and blood vessels (Zhang *et al.*, 2009). There is surprising heterogeneity with regard to the developmental origin and phenotypic characteristics of SMCs (Owens *et al.*, 2004, Rensen *et al.*, 2007). SMCs have been characterized as showing either a proliferative “synthetic” phenotype or a biomechanically active, but quiescent, “contractile” phenotype. Phenotypic plasticity (“phenotypic switching”) can occur reversibly and transiently between these two forms *in vitro* and *in vivo* (Aikawa *et al.*, 1997, Thyberg *et al.*, 1995, Thyberg *et al.*, 1997) and has been observed in response to biochemical and mechanical cues (Beamish *et al.*, Shen *et al.*, 2006).

The use of SMCs, or for that matter any cell type, as part of a tissue engineering strategy for functional tissue replacement requires the reliable expansion of cells *in vitro* while minimizing the processes of dedifferentiation and/or controlling the processes of cellular redifferentiation towards the desired cellular phenotype. The events associated with SMCs' plasticity have mostly been studied in vascular SMCs. However, the actual process of cellular dedifferentiation, phenotypic changes, and senescence associated with *in vitro* cell expansion have been largely unexplored. Similarly, the methods to either prevent or proactively induce such changes by biochemical or biomechanical means are unknown.

The present study investigated the phenotypic changes that occur in rat smooth muscle cells harvested from three different tissues (aorta, esophagus, and urinary bladder), during the processes of initial cell isolation and early stages of cell maintenance and expansion using conventional cell culture techniques.

2. Material and Methods

2.1 rSMC Isolation and *in vitro* Cell Culture

Rat Smooth Muscle Cells (rSMCs) were isolated under sterile conditions from three different tissues; aorta, esophagus, and urinary bladder by enzymatic dispersion technique (Sreejayan and Yang, 2007). Briefly, following tissue isolation, all connective, adipose tissue and the adventitia were removed. The tissues were opened longitudinally (esophagus: along the greater curvature side) and the luminal surface layers (aorta: endothelium; esophagus/urinary bladder: epithelium, mucosa, and submucosa) were removed mechanically by scraping with a scalpel blade. The tissues were washed three times in Krebs Ringer solution (Sigma, St. Louise, MT, USA containing 0.015 mM NaHCO₃, 3.0 mM CaCl₂, 100 U/ml Pen-Strep). The tissues were minced and digested 30 min at 37°C under agitation in Krebs Ringer solution containing 15 U/ml elastase (Calbiochem), 20 U/ml papain (Sigma, St. Louise, MT, USA), 200 U/ml collagenase type II (Gibco), 0.4 mg/ml trypsin inhibitor (Soybean) (Sigma, St. Louise, MT, USA), and 1.7 mg/ml of bovine serum albumin (Sigma, St. Louise, MT, USA). Single cell suspensions were prepared by trituration through a glass pipette. Undigested tissue fragments were removed by centrifugation. Cells were plated at 2.5×10^4 cells/cm² in complete culture medium (RPMI 1640 + 10% fetal bovine serum + ab/am) in a T25 tissue culture-treated cell culture flask in a humidified incubator, 37°C, 5% CO₂ in air. Culture media was replaced after 24 hrs to remove dead cells and grown until 90% confluent changing the media every 2-3 days.

At near confluency, cells were collected by trypsinization and expanded further in a T75 tissue culture flask. Again at near confluency, cells were collected by trypsinization and expanded further in a T175 tissue culture flask.

Cells were cultured for two passages following isolation. At each subculturing step, a subset of cells (5.0×10^4 cells) was subcultured on collagen type I-coated glass coverslips (diameter: 2.0 cm) for immunocytochemical evaluation.

2.2 Immunohistochemical Staining

The expression of marker proteins for contractile and synthetic phenotypes of SMC was evaluated by conventional immunocytochemical techniques for cells grown on collagen type I-coated glass coverslips. All samples were processed in parallel to enable comparable processing and staining times between samples. Cells were fixed and permeabilized in ice-cold methanol for 5 min and rehydrated in phosphate buffer solution. Samples were blocked with solution consisting of PBS and 10% of the appropriate serum at room temperature for 30 min. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide solution. Slides were then incubated sequentially with primary antibody (Ab) at 4°C overnight. Primary Abs against smooth muscle actin (1:200; Dako Cytomation, Carpinteria CA, USA), Smoothelin (1:100; Santa Cruz Biotech Inc., Santa Cruz, CA, USA), and cellular retinol binding protein 1 (1:50; Santa Cruz Biotech Inc., Santa Cruz, CA, USA) were applied and followed by biotinylated anti-goat, anti-mouse, or anti-rabbit Ab, respectively. After washing, slides were incubated with avidin-biotin conjugate of horseradish peroxidase (ABC; Vector Labs, Burlingame, CA, USA) in PBS for 30 min at room temperature. To identify the resulting peroxidase activity, the slides were treated with 3,3'-diaminobenzidine (DAB) solution (Vector Labs, Burlingame, CA, USA). The slides were counterstained with hematoxylin and mounted on glass slides with mounting medium (Vector). The cells were visualized and photographed by conventional light microscopy (Nikon E600, Nikon Instr. Inc., Melville, NY, USA).

2.3 Cell Proliferation Assay

The rate of rSMC proliferation was determined as described previously (Rago *et al.*, 1990). Briefly, following trypsinization, cells were suspended in complete growth media. Cell standards were prepared by serial dilutions ($1-125.0 \times 10^4$ cells in 100 μ l) on a 96 well cell culture plate (Corning, Inc., Corning, NY, USA). Cells were left to adhere for 8-10 hrs in the incubator (37°C, 5% CO₂ in air) before further processing. Experimental plates were prepared by culturing 5.0×10^4 cells in 100 μ l complete culture medium in a 96 well plate. Cells were incubated for 1-3 days. At each endpoint, the culture medium was removed and the cells were frozen at -80 °C.

On the day of the assay, 100 μ l dH₂O was added to each well and incubated at room temperature for 60 min. The plates were frozen at -80 °C and thawed to reach room temperature before adding 100 μ l of 20 μ g/ml Hoechst 33258 in TNE buffer (10.0 mM Tris, 1.0 mM EDTA, 2.0 M NaCl, pH 7.4). Sample emissions (460nm) were measured on a microplate reader (SpectraMax M2, Molecular Devices, Inc., Sunnyvale, CA, USA) at an excitation of 350 nm. Readings were normalized to medium alone.

2.4 Gene Expression Analysis by RealTime-PCR

The gene expression activity of marker proteins for contractile and synthetic phenotypes of SMC was evaluated by qPCR in a two-step protocol. Total RNA was isolated from the explanted cells using RT TRI-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) according to the manufacturer's instructions. The isolated total RNA was purified using an RNAeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA was synthesized from 1.0 μ g of total RNA in a volume of 20 μ l using a Superscript RT III kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

Quantitative real-time PCR was performed using primers specific for genes of contractile or synthetic marker proteins (Huber Table1). Gene primer sequences were custom designed using Beacon Designer 7.2 primer design software (PREMIER Biosoft International, Palo Alto, CA) or as described elsewhere. Primers for the housekeeping gene Glyceraldehyde 3-phosphate dehydrogenase (gapdh) were purchased as part of a housekeeping gene primer kit (Rat Housekeeping Gene Primer Set, Real Time Primers, Elkins Park, PA, USA), and gapdh expression was used to normalize reactions. 1.0 µg of cDNA was mixed with the appropriate primers and 2× SYBR Green Master Mix (BioRad, Hercules, CA, USA) in a total volume of 25.0 µl. All reactions were performed in triplicate and were monitored using an iQ5 Real-Time Detection System (BioRad, Hercules, CA, USA). Absolute gene expression levels normalized to the housekeeping gene were expressed in their respective threshold cycles for each gene of interest.

3. Results and Discussion

Cell based tissue engineering approaches require the reliable isolation and expansion of cells *in vitro*, and the maintenance of the cells' tissue-specific phenotype while preventing undesirable events such as cellular senescence and de- and transdifferentiation. A variety of cell types have been used for the reconstruction of complex tissues and organs as part of a conventional cellular tissue engineering approach. For instance, SMCs have been suggested as a useful cell type for the reconstruction of hollow organs including the esophagus (Nakase *et al.*, 2008, Saxena *et al.*, 2009), intestine (Nakase *et al.*, 2006), internal anal sphincter (Somara *et al.*, 2009), and blood vessels (Zhang *et al.*, 2009). However, previous research has demonstrated the phenotypic plasticity of SMCs to transiently switch between a contractile (functional) and synthetic (proliferative) phenotype as indicated through the gradual expression of several marker proteins (Beamish *et al.*, Rensen *et al.*, 2007).

The present study evaluated the effects of conventional cell isolation and expansion techniques on the phenotype of rat SMCs isolated from three different tissues (aorta, esophagus, and urinary bladder) during early stages of cell expansion. Conventional cell isolation and pre-plating techniques resulted in the successful establishment of *in vitro* cell cultures of SMCs from rat aorta (rSMC-a), esophagus (rSMC-e), and urinary bladder (rSMC-ub). The isolated cell cultures were homogeneous in their microscopic appearance (Huber Figure1).

These cells dedifferentiated within two passages on tissue culture-treated culture flasks towards phenotypically homogeneous cell populations showing a distinct alteration in the expression of SMC marker proteins as determined by immunocytochemical staining (Huber Figure2). Cells from all three tissues stained positive for the presence of alpha smooth muscle actin at the early and later stage of cell expansion. No SMC population stained positive for the presence of the CRBP-1, a synthetic marker, nor for the expression of smoothelin, a late contractile marker protein. While urinary bladder SMCs showed a low level of calponin expression, esophageal SMCs and aortic SMCs showed increasing levels of calponin between passages (rSMC-a: none → moderate; rSMC-e: weak → moderate). While a clear distinction between the synthetic and contractile phenotype is difficult due to the absence of a set of clear-cut markers and the nature of transient marker gene expression, the synthetic marker cellular retinol binding protein-1 (CRBP-1), and contractile markers such as alpha-smooth muscle actin (Aikawa *et al.*, 1997), calponin, and smoothelin (Christen *et al.*, 1999) have been described as a good indicator for the phenotypic state of SMCs. As a result, the expanded rSMC populations isolated as part of this study were determined to be of neither a fully synthetic, nor fully contractile phenotype, as demonstrated by lack of CRBP-1 and smoothelin, respectively.

In spite of showing a remarkable level of similarity between the three rSMC populations with respect to their microscopic appearance and the presence of marker proteins, tissue-specific differences between the three rSMC populations were observed with regard to the cells' proliferative activity and gene expression patterns. rSMC-a showed an almost fourfold greater rate of cell proliferation, duplicating every 33.1 hours, in comparison with both rSMC-e and rSMC-ub cell populations that duplicated every 128.2 and 123.7 hours, respectively (Huber Figure3).

In addition, gene expression of structural proteins as well as marker proteins associated with phenotypic differentiation of SMCs, including collagens type I and III, elastin, smooth muscle actin, calponin, smooth muscle myosin heavy chain, and smoothelin, as determined by quantitative RT-PCR showed distinct tissue-specific differences between the three SMC populations (Huber Figure4). rSMC-ub showed the highest level of gene expression for alpha actin (*acta2*), while rSMC-a had the lowest levels, with rSMC-e having an intermediate level of alpha actin gene expression. Similar expression levels were observed for collagen type I (*col1a1*, *col1a2*), collagen type III (*col3a2*), retinol binding protein 1 (*rbp-1*), smoothelin (*smtn*), calponin (*cnn1*), and smooth muscle myosin heavy chain (*myh11*). The expression of elastin was the highest in rSMC-a cultures, the lowest in rSMC-ub and intermediate in rSMC-e cultures. Stated differently, each type of SMC showed a distinct gene expression profile.

This study focused only on a small subset of SMC-specific marker genes and proteins for the evaluation of a phenotypic shift during SMC expansion *in vitro*. Although these have previously been described as reliable markers for the evaluation of SMC phenotypic populations, a more detailed understanding in the molecular changes affecting these during SMC dedifferentiation in culture is desirable to evaluate SMC cell states. Such a study would also allow for a more detailed analysis of the effects of the environmental cues (i.e. the microenvironmental niche) upon cell phenotype outlined above.

The phenotypic switch observed in SMC populations may be used to control the tissue-specific phenotype of cells as part of a cellular tissue engineering approach by controlling environmental conditions during the cell expansion, tissue assembly, and neotissue maturation. Methods to maintain cell phenotype may include soluble factors (e.g. growth factors), cell culture environments (e.g. synthetic and natural scaffold materials), and biomechanical stimulation (e.g. flow or mechanical stretch). A more detailed understanding of the effects of these and other environmental cues on SMC phenotype is needed to allow for optimization of tissue engineering/regenerative medicine strategies for hollow organ reconstruction. Future experiments will use various environmental conditions to control and regulate tissue-specific SMC phenotypes.

Previous experiments in the tissue engineering of hollow organs including blood vessels (Furukawa *et al.*, 2002, Nelson *et al.*, 2008, Yang *et al.*, 2005, Zhang *et al.*, 2009), esophagus (Marzaro *et al.*, 2006, Nakase *et al.*, 2008, Saxena *et al.*, 2009, Zhu *et al.*, 2005, Zhu *et al.*, 2006) and urinary bladder (Azzarello *et al.*, 2009, Baumert *et al.*, 2007, Gabouev *et al.*, 2003, McManus *et al.*, 2007, Nakanishi *et al.*, 2003, Pariente *et al.*, 2002, Pattison *et al.*, 2005, Zhang *et al.*, 2004, Zhang *et al.*, 2000) following the conventional cellular tissue engineering paradigm have employed the use of SMCs from their respective tissue-specific cell source. However, the controlled regulation of SMC phenotype may allow the use of SMCs from one tissue, e.g. bladder SMCs, for the *in vitro* reconstruction of another tissue, e.g. esophagus. Therefore, the source of SMCs for tissue engineering applications may be irrelevant if a cell expansion phase is included as part of the strategy for creating a replacement organ. Experimental findings in the use of intestinal and urinary bladder SMCs in urinary bladder wall replacements in the rabbit seem to support this view (Lai *et al.*,

2006). Consequentially, the phenotypic plasticity of certain cells, in particular the plasticity of SMCs, may be exploited as part of a tissue engineering approach by using cells from an easily obtainable tissue location, e.g. via a skin biopsy, without the need to isolate cells from the functionally compromised tissue that is to be reconstructed.

The extent of phenotypic changes within a particular cell type are of great importance with respect to the paradigm of cellular tissue engineering. While a certain, yet reversible, degree of dedifferentiation may be tolerable, it is fundamental that tissue engineered constructs are structurally and functionally complementary to the target host tissue. Such tissue specificity will require the strict control of cellular phenotype of each cell type used in a tissue engineering approach throughout the tissue engineering process, from initial cell isolation, expansion, and seeding, to neotissue assembly, maturation, and transplantation. The careful design of the appropriate, tissue-specific cell culture conditions including the application of biochemical stimuli (i.e. growth factors and cell culture supplements) and biomechanical stimuli will need to be taken into consideration for any tissue engineering approach. This must not be seen as an insurmountable obstacle but as an essential control mechanism ensuring the quality of engineered tissues and organs.

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Figure 1. Microscopic Appearance of Rat Smooth Muscle Cells grown in cell cultures *in vitro* as isolated by enzymatic dispersion technique from aorta (rSMC-a, A), esophagus (rSMC-e, B), and urinary bladder (rSMC-ub, C); scale bar = 150 μ m.

Figure 2.

Immunocytochemical Staining for the Presence of Marker Proteins. Cultures of rSMCs grown on collagen-coated glass coverslips were stained immunocytochemically for the presence of alpha-smooth muscle actin (α -SM actin), calponin, cellular retinol binding protein-1 (CRBP-1), and smoothelin in cell cultures following the first and second passage after cell isolation. positive controls (pos. ctrl.) from rat aorta (a), esophagus (b), and urinary bladder (c). Scale bars = 150 μ m.

	fold change per 24 hrs	duration per duplication [hrs]
rSMC-a	0.73*	33.1*
rSMC-e	0.19	128.2
rSMC-ub	0.19	123.7

* p<0.05

Figure 3.

Rates of Proliferation: 5.0×10^4 rSMCs were grown on tissue culture-treated multi-well plates. The level of cell proliferation was assessed over a period of three days by Hoechst 33258 staining of DNA and expressed in relative change to day 1 (n = 2).

Figure 4.

Expressional Profiling of rSMCs grown in vitro. Expanded rSMCs were evaluated for their expression of structural and functional gene associated with SMC phenotype including collagen I (col1a1 and col1a2), collagen II (col3a2), elastin (eln), alpha-smooth muscle actin (acta2), calponin (cnn1), and smooth muscle myosin heavy chain (myh11), retinol binding protein-1 (rbp-1), and smoothelin (smtn). Results presented as absolute threshold levels (Ct), normalised to gapdh, n = 3.

Table 1

Primer Sequences for RT-PCR.

gene	GenID	forward primer (5'-3')	reverse primer (5'-3')	reference
acta2	81633	AGCCAGTCGCCATCAGGAAC	TCATCACCCAGCAAAGCCCG	Bouchez <i>et al.</i> , 2008
coll1a1	29393	ATCAGCCCAAAACCCAAAGGAGA	CGCAGGAAGGTCAGCTGGATAG	Heinemeier <i>et al.</i> , 2007
coll1a2	84352	TTCCCGGTGAATTCGGTCTC	ACCTCGGATTCCAATAGGACCAG	Nozaki <i>et al.</i> , 2005
coll3a1	84032	TGGGATCCAATGAGGGAGAA	CTCATGGCCTTGGCGTGTITG	Petrick <i>et al.</i> , 2009
eln	25043	CCTGTCCCTGACTCCCAATTA	CAGTGTGAGAAAGTCGTCGGA	Gray <i>et al.</i> , 2008
cnn1	65204	GCCAGGTGTATGATCCCAAGT	ATTGTGGGTGGGCTCG	Hendriks-Balk <i>et al.</i> , 2008
myh11	24582	GAA GCAGCCAGCATCAAG	CAGCACAGAAAGCCTCATTTG	Beacon
rbp-1	25056	AGGCATAGATGACCGCAAGT	TCATCACCCCTCAATCCACTG	Mezaki <i>et al.</i> , 2007
smtn	289734	CCAGAGGCTCCTCTAACACTAAGAG	TTGGCTCTTGATTTTGGGTGGCTG	Ross <i>et al.</i> , 2006