Differentiation of smooth muscle progenitor cells in peripheral blood and its application in tissue engineered blood vessels*

Shang-zhe XIE¹, Ning-tao FANG¹, Shui LIU¹, Ping ZHOU², Yi ZHANG²,
 Song-mei WANG¹, Hong-yang GAO¹, Luan-feng PAN^{†‡1}

(¹Laboratory of Molecular Biology, Shanghai Medical College, Fudan University, Shanghai 200032, China)

(²Key Laboratory of Molecular Engineering of Polymers, Ministry of Education, Department of Macromolecular Science, Fudan University, Shanghai 200433, China)

[†]E-mail: lfpan@shmu.edu.cn

Received Aug. 22, 2008; revision accepted Nov. 11, 2008; CrossCheck deposited Nov. 7, 2008

Abstract: Background: A major shortcoming in tissue engineered blood vessels (TEBVs) is the lack of healthy and easily attainable smooth muscle cells (SMCs). Smooth muscle progenitor cells (SPCs), especially from peripheral blood, may offer an alternative cell source for tissue engineering involving a less invasive harvesting technique. Methods: SPCs were isolated from 5-ml fresh rat peripheral blood by density-gradient centrifugation and cultured for 3 weeks in endothelial growth medium-2-MV (EGM-2-MV) medium containing platelet-derived growth factor-BB (PDGF BB). Before seeded on the synthesized scaffold, SPC-derived smooth muscle outgrowth cell (SOC) phenotypes were assessed by immuno-fluorescent staining, Western blot analysis, and reverse transcription polymerase chain reaction (RT-PCR). The cells were seeded onto the silk fibroin-modified poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (SF-PHBHH_x) scaffolds by 6×10^4 cells/cm² and cultured under the static condition for 3 weeks. The growth and proliferation of the seeded cells on the scaffold were analyzed by 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide (MTT) assay, scanning electron microscope (SEM), and 4,6-diamidino-2-phenylindole (DAPI) staining. Results: SOC displayed specific “hill and valley” morphology, expressed the specific markers of the SMC lineage: smooth muscle (SM) α -actin, calponin and smooth muscle myosin heavy chain (SM MHC) at protein and messenger ribonucleic acid (mRNA) levels. RT-PCR results demonstrate that SOC also expressed smooth muscle protein 22 α (SM22 α), a contractile protein, and extracellular matrix components elastin and matrix Gla protein (MGP), as well as vascular endothelial growth factor (VEGF). After seeded on the SF-PHBHH_x scaffold, the cells showed excellent metabolic activity and proliferation. Conclusion: SPCs isolated from peripheral blood can be differentiated into the SMCs in vitro and have an impressive growth potential in the biodegradable synthesized scaffold. Thus, SPCs may be a promising cell source for constructing TEBVs.

Key words: Smooth muscle progenitor cells (SPCs), Tissue-engineered blood vessels (TEBVs), Silk fibroin (SF), Poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHH_x)

doi:10.1631/jzus.B0820257

Document code: A

CLC number: Q291

INTRODUCTION

Blood vessel replacements are frequently necessary in the treatment of advanced atherosclerosis, vascular trauma, aneurysmal and peripheral vascular diseases. The autologous saphenous veins and

mammary arteries are currently the preferred graft materials. However, the availability of the appropriate dimensional tissues is limited, and donor site morbidity is a significant complication in these procedures. The emerging and interdisciplinary field of tissue engineering has evolved to represent a potential means to construct functional grafts that could be used in vascular replacement procedures where autologous tissue is unavailable and synthetic materials fail (Nugent and Edelman, 2003).

[†] Corresponding author

* Project supported by Shanghai Science Committee Fund for Key Research Project (No. 04JC14012) and Fudan University Med-X Fund, China

To construct tissue engineered blood vessels (TEBVs), the key processes are to obtain seeding cells, culture them, and plant them in scaffolds. Despite the significant progress toward the development of biomaterials and methods to cultivate three-dimensional (3D) vascular constructs, cell sourcing remains a major problem. Blood vessels are mainly composed of endothelial cells (ECs) and smooth muscle cells (SMCs), fibroblasts, and pericytes. SMCs help to maintain the contraction function of blood vessels, adjust blood distribution, and secrete extracellular matrix (ECM) and angiogenic factors (Wu *et al.*, 2004).

SMCs used in TEBVs largely come from mature blood vessels. However, the limited proliferation ability of mature SMCs, traumatic obtaining processes and even deficiency of healthy blood vessels impede the application of SMCs in TEBVs. Therefore, an autologous source of SMCs with high proliferative capacity is necessary for preparation of TEBVs.

Adult stem cells from peripheral blood provide a promising alternative as they are easy to be obtained and they show an extensive in vitro proliferation capacity. Moreover, adult stem cells are not compounded by the ethical considerations of embryonic stem cells and they are readily available for research (Wu *et al.*, 2006). Former researches proved that endothelial progenitor cells (EPCs) have the capacity to resemble those of mature ECs in TEBVs construction (Kaushal *et al.*, 2001; Melero-Martin *et al.*, 2007; Fang *et al.*, 2007). However, there are much fewer reports about the application of smooth muscle progenitor cells (SPCs) in TEBVs. Liu *et al.* (2007) selected a proliferative cell population that displayed morphologic and biochemical characteristics of SMCs from bone marrow by using an SM α -actin promoter and fluorescence activated cell sorting. However, acquiring bone marrow would cause pain and discomfort and the method was quite complicated.

Simper *et al.* (2002) demonstrated smooth muscle outgrowth cells (SOCs) with a specific growth, adhesion, and migration profile from putative SPCs in human peripheral blood. Later research indicated that the adult peripheral blood CD34⁺ cells can be differentiated into cardiomyocytes, mature ECs, and SMCs in vivo (Yeh *et al.*, 2003). Collectively, these studies suggested that there are SPCs in peripheral blood. Therefore, we try to isolate SPCs among

mononuclear cells from rat peripheral blood, allow them to differentiate into SOCs, and test whether SOCs can replace mature SMCs as a new source of TEBVs seeding cells.

The differentiated SOCs were seeded on the synthesized scaffold. Synthetic polymers have been commonly used as the scaffolds to support cell growth and provide the mechanical support necessary for the implantation. Recently, poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHH_x) is applied as a novel biodegradable material for tissue engineering scaffold; it has been demonstrated to be non-thrombogenic, significantly reducing the potential for hyperplasia and thrombosis that have plagued many other TEBVs materials. Our former studies also suggested that surface modification with silk fibroin (SF), a natural protein regenerated from silkworm silk fiber, would be an effective way to enhance the cell adhesion and improve the biocompatibility of synthesized scaffold, facilitating its application in practical tissue engineering (Chen *et al.*, 2004). Thus we apply the SF-modified PHBHH_x (SF-PHBHH_x) scaffold to assess SOCs' potential as seeding cells in TEBVs.

MATERIALS AND METHODS

Preparation and differentiation of peripheral blood mononuclear cells (PBMCs)

PBMCs were isolated from a volume of 5 ml heparinized rat peripheral blood. The blood was diluted 1:2 with sterilized phosphate buffered saline (PBS). PBMCs were then separated by density-gradient centrifugation using HISTOPAQUE-1083 (Sigma, St. Louis, MO, USA). After washed twice in PBS, PBMCs were then resuspended in endothelial growth medium-2-MV (EGM-2-MV) medium (Cambrex-Clonetics, Walkersville, MD, USA) containing 50 ng/ml platelet-derived growth factor-BB (PDGF BB) (R&D Systems Inc., Minneapolis, MN, USA), and then placed on 2 wells of a 6-well plate. After PBMCs were differentiated to SOCs, the cells were cultured in M199 (Gibco-BRL, Grand Island, NY, USA) supplemented with 10% (w/v) fetal bovine serum (Hyclone, USA), 100 U/ml penicillin (Sigma), 100 mg/ml streptomycin (Sigma), 10 ng/ml basic fibroblast growth factor (bFGF) (Sigma) and 4 mmol/L L-glutamine (Sigma). Cultures were

incubated at 37 °C in a humidified atmosphere of 5% (v/v) CO₂ in air. The preparation and differentiation of PBMCs were repeated at least five times using different rats.

Immunofluorescent analysis

The cells on glass cover slips were washed twice with PBS at room temperature, fixed with 4% (w/v) formaldehyde, penetrated with 0.2% (v/v) TritonX-100, and blocked with 10% (v/v) normal goat serum. The cells were incubated with the following antibodies: monoclonal mouse anti smooth muscle (SM) α -actin (1:250 dilution, Sigma), monoclonal mouse antibody to smooth muscle myosin heavy chain (SM MHC) (NeoMarkers, Fremont, CA, USA), calponin (1:250 dilution, NeoMarkers) for 1 h at room temperature. Rhodamine labeled anti-mouse antibodies (Miles Scientific, Naperville, IL, USA) were used as a secondary antibody. Cell nuclei were counterstained with 0.5 μ g/ml 4,6-diamidino-2-phenylindole (DAPI) (Sigma). The immunostained cells were examined under a fluorescent microscope (Olympus BX-50, Japan). PBMCs were used as a negative control for the SMC-specific markers.

Western blot analysis

The cells were washed in PBS and lysed in a buffer containing 50 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.02% (w/v) sodium azide, 0.1% (w/v) sodium dodecyl sulfate (SDS), 100 μ g/ml phenylmethylsulphonyl fluoride (PMSF), and 1 μ g/ml aprotinin. After incubation on ice for 30 min, the lysates were subjected to centrifugation at 12000 $\times$ g for 10 min at 4 °C. The supernatants were collected after the centrifugation. After separated on 10% (w/v) denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes by electroblotting, the

membrane was incubated with 5% (w/v) nonfat dry milk at 4 °C overnight to block nonspecific antibody binding. Then the membrane was immunoblotted using monoclonal antibodies to SM α -actin, calponin, and SM MHC at dilutions of 1:500. Secondary alkaline phosphatase conjugated affinipure goat anti-mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA, USA) at a 1:1000 dilution were used for detection using Luminescent Detection Kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) and X-ray film exposure (Kodak, Rochester, NY, USA). Mature human SMCs (hSMCs) and PBMCs were used as positive and negative control cells for smooth muscle-specific markers, respectively.

Reverse transcription polymerase chain reaction (RT-PCR) analysis

The messenger ribonucleic acid (mRNA) levels of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), rat SM α -actin, rat calponin, rat smooth muscle protein 22 α (SM22 α), rat SM MHC, rat elastin, rat matrix Gla protein (MGP) and rat vascular endothelial growth factor (VEGF) from SOC and PBMCs were analyzed by RT-PCR analysis. Total RNA from PBMCs and SOC was isolated by TriZol (Molecular Research Center, Cincinnati, OH, USA) and RT-PCR was performed using RT-PCR Kit (Promega, Madison, WI, USA) according to the manufacturer's protocol: the complementary DNA (cDNA) was synthesized from 1 μ g of total RNA using oligo(dT)₁₅ as a primer and moloney murine leukemia virus (M-MLV) reverse transcriptase. The sequences of sense and anti-sense primers and the experimental conditions are given in Table 1. The reaction was carried out for 30 cycles with the following steps: denaturation for 30 s at 94 °C, annealing for 30 s and extension for 90 s at 72 °C. Each PCR reaction included a sample in which H₂O was used as template (negative control).

Table 1 Sequences of primers and the experimental conditions for RT-PCR

Gene	Sense primer (5' to 3')	Anti-sense primer (5' to 3')	Annealing temp. (°C)	Expected size (bp)
SM α -actin	GATCACCATCGGGAATGAACGC	CTTAGAAGCATTGCGGTGGAC	60	389
Calponin	TAGAGCTTGAGATGGGGAGCAA	TGGGAAAGCTCCAGGGATGA	60	339
SM22 α	GCCGTGAAGTGCAGTCCAAG	TCCCTCTTATGCTCCTGGGC	62	454
SM MHC	ATCGCCCAGCTGGAGGAGGAG	TCATCCTCCACCTGCAG	60	371
Elastin	AACGAGCTGTTCCAAGCCTA	CTGGAGACCCACCAACTTGT	60	126
MGP	CCTGTGCTATGAATCTCACGA	GCAACGAACACGAATCTGTG	60	351
VEGF	CTGCTCTCTTGGGTGCACTGG	CACCGCCTTGGCTTGTACAT	62	431, 563, 635
GAPDH	ATGGGTGTGAACCACGAGAA	GGCATGGACTGTGGTCATGA	60	144

The PCR products were visualized by ethidium bromide staining following electrophoresis on 1.2% (w/v) agarose gel. Each PCR reaction was conducted at least twice with RNA from two independent experiments.

Seeding of SOC_s on SF-PHBHH_x matrices

The porous PHBHH_x film was prepared by solvent-casting and particle-pulling off as described by Mei *et al.* (2006). The scaffold was made into a round porous membrane (0.4 mm thick and 15 mm in diameter). Then PHBHH_x film was immersed into SF solution for 24 h to form the SF-coated scaffold, freeze-dried, and fixed by methanol. The sheets were sterilized with 75% (v/v) ethanol overnight and then by ultraviolet light for 2 h. The sterilized matrices were pre-incubated in PBS solution to replace the ethanol remaining in the samples and then were transferred to a sterile 24-well cell culture plate. The SOC_s were digested with 0.25% (w/v) trypsin (Sigma) and resuspended to be 1×10^6 cells/ml. Then 100 μ l cell suspension was dripped into each matrix. After the cells were cultured in a humidified incubator (5% (v/v) CO₂, 37 °C) for 1 h, additional 1 ml culture medium was added into each well. The culture medium was replenished every 2 d.

MTT [3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide] assay

Cell numbers of viable SOC_s in the SF-PHBHH_x matrices were quantitatively assessed with MTT (Sigma) at various cultural time courses up to 21 d. The cell-contained matrices were rinsed with the serum free medium to remove the unattached dead cells, transferred to another cell culture plate, and incubated with 100 μ l MTT stock solution (0.5 mg/ml in M199) for 4 h at 37 °C. The formed MTT formazan was then dissolved into 300 μ l/well dimethyl sulfoxide (DMSO). Optical density (OD) of the MTT formazan in DMSO solution was measured with an automatic microplate reader (ELX 800, Bio-Tek, USA) at the wavelength of 565 nm (OD_{565}) referred to the value at 630 nm. Both DMSO and the matrices without cells cultured were assayed as the background. Four parallel samples were measured each time.

Cell morphology

After cells in the SF-PHBHH_x matrices were cultured for different days, the cell-contained

SF-PHBHH_x matrices were washed with PBS three times, and then SOC_s were fixed with 5% (w/v) glutaraldehyde (Sigma) in PBS for 24 h at 4 °C. The matrices were dehydrated sequentially in 50%, 70%, 90%, and 100% (v/v) ethanol, each for 15 min, and then freeze-dried, sputter-coated with gold, and examined by a scanning electron microscope (SEM). To observe SOC_s' distribution in the internal section of the scaffolds, the scaffolds were stained with 0.5 mg/L DAPI for 10 min and the sections were frozen and statically observed by immunostaining.

Statistical analysis

The data represent mean $\pm$ SD of four replicates. Statistical relevance was assessed by using the *t*-test through Excel. $P < 0.05$ was considered significant.

RESULTS

SPCs' differentiation and proliferation

Approximately 30% of the freshly isolated PBMCs adhered to culture plates and parts of them showed "polarity" after 3 d of culture with PDGF BB-containing EGM-2-MV medium (Fig.1a). The cells elongated further toward a spindle-shaped morphology 1 week later (Fig.1b). More and more long-and-narrow cells appeared and trended to form clusters after 2 weeks (Fig.1c). Three weeks after isolation, some outgrowth cells displayed specific morphological characteristics of typical mature SMCs observed through phase contrast microscope (Fig.1d). Some cells showed myofilament (Fig.1e). Hereafter, many colonies with "hill and valley" morphology showed impressive growth potential, i.e., the cells could be sub-cultured repeatedly with no apparent loss of the proliferative potential even after 30 passages (Fig.1f).

Immunophenotyping of smooth muscle outgrowth cells

After three weeks of culture, the SOC_s were characterized by the immunofluorescent staining using antibodies specific to smooth muscle cell markers. More than 95% SOC_s differentiated from PBMCs were stained positively for SM α -actin, calponin and SM MHC, whereas the starting PBMCs population stained negatively for all smooth muscle cell markers (Fig.2).

Fig.1 Representative cell morphology during the differentiation process. (a) Cells began to show “polarity” after 3 d of culture; (b) A spindle shape on the 7th day; (c) Trend to form clusters after 2 weeks; (d) Mature smooth muscle cell morphology after 3 weeks in phase contrast microscope; (e) Typical myofilament; (f) “Hill and valley” morphology at the 30th passage

To further evaluate the phenotype and function of the cultured SOC population, the cell lysates from SOC, hSMC, and PBMCs were examined by Western blot. The results show that the expression pattern of the markers changed toward a more mature SMC phenotype after four-week culture in PDGF BB-containing EGM-2-MV medium as seen by a significant expression of mature SMC-specific markers, including SM α -actin (42 kD), calponin (34 and 29 kD), SM MHC (200 kD). The levels of these three proteins in SOC were parallel to those in mature hSMCs. However, PBMCs did not express SM α -actin, calponin, or SM MHC (Fig.3).

Expression levels of smooth muscle markers and function molecules by mRNA examination

The expression of SM α -actin, calponin, and SM MHC was analyzed by mRNA level as well (Fig.4). In addition, the expression of smooth muscle cell function genes including SM22 α , MGP, elastin, and VEGF was also analyzed. The experiments demonstrated that SOC highly expressed SM α -actin (389 bp), calponin (339 bp), SM MHC (371 bp), and

Fig.2 Immunofluorescent stainings of SOC and PBMCs. The cells were incubated with (a) anti-SM α -actin, (b) anti-calponin, and (c) anti-SM MHC antibodies, respectively, and then reacted with Rhodamine-conjugated secondary antibodies. Cell nuclei were counterstained with DAPI (blue)

Fig.3 Western blot analysis for smooth muscle cell markers. The proteins were reacted with anti-SM α -actin, anti-calponin, and anti-SM MHC antibodies, respectively after blotting. GAPDH was served as an inner reference

SM22 α (454 bp), whose proteins hold contraction function. The SOC also significantly express extracellular matrix components: elastin (126 bp) and MGP (351 bp), as well as VEGF (three major forms: VEGF120, 431 bp; VEGF164, 563 bp; VEGF188, 635 bp), while fresh PBMCs did not express these specific genes (data not shown).

Fig.4 RT-PCR analysis of smooth muscle cell-specific genes. Total RNA was extracted from SOC and PBMCs. The mRNA levels were analyzed by RT-PCR. The amplified cDNA sizes were: lane 1, SM α -actin (389 bp); lane 2, calponin (339 bp); lane 3, SM22 α (454 bp); lane 4, SM MHC (371 bp); lane 5, elastin (126 bp); lane 6, MGP (351 bp); lane 7, VEGF (431, 563, and 635 bp for the three major forms of VEGF120, VEGF164, and VEGF188, respectively). GAPDH (144 bp) was used as an internal control

Cellular proliferation in the scaffolds

Because of the linear correlation between the viable cell number and the optical density of MTT formazan (Zund *et al.*, 1999), the OD_{565} values of MTT formazan in the SF-PHBHH_x hybrid scaffold (Fig.5) indicated that SOC in the SF-PHBHH_x hybrid scaffold kept a good growing from Day 3 to Day 21. The cellular proliferation in SF-PHBHH_x hybrid scaffold was significantly higher than that in PHBHH_x scaffold ($P < 0.05$) (data not shown).

Fig.5 Optical density (OD_{565}) of MTT formazan at different SOC-culturing periods in the SF-PHBHH_x hybrid scaffold

Adhesion and growth of SOC by SEM observation and DAPI staining

Three days after seeded on the SF-PHBHH_x hybrid scaffold, SOC adhered to the surface of the scaffold with filopodia (Fig.6a). The cells attached well to the scaffold and began to aggregate after one-week culture (Fig.6b). Three weeks later, SOC

spread through the pores, formed the confluent lamellar structure and grew into the deeper section of scaffolds (Figs.6c and 6d). DAPI staining showed that 90% of scaffolds were evenly occupied by SOC after one-week culture, which was in accordance with the observation by SEM, and that SOC grew into the deeper pores of the scaffolds (Fig.6e).

Fig.6 Representative SEM images and DAPI staining of cells cultured on SF-PHBHH_x 3D scaffolds. (a) & (b) Cells attached on the SF-PHBHH_x scaffolds after 3 d and 1 week, respectively; (c) Confluent cell layers on the surface; (d) Cells in the pores of 3D scaffolds after 3 weeks; (e) DAPI staining

DISCUSSION

Lack of availability of autologous vascular grafts and the pain and discomfort associated with the donor site necessitate the development of TEBVs for tissue regeneration. Because TEBVs are expected to have the potential to grow, self-repair, and self-remodel, they should contain living donor cells (Campbell and Campbell, 2007). SPCs are autologous and readily available, and exhibit a high proliferative potential in

vitro, share a low potential for tumor genesis, and thus could serve as promising donor cells.

To date, characterization of SMCs often is done by demonstrating the presence of SM α -actin, which is also expressed by myofibroblasts and ECs under certain conditions, and hence not alone to SMCs. SM22 α and calponin are increasingly SMCs restrictive, but transient expression in non-SMCs cannot be ruled out. Expression of SM MHC has been extensively scrutinized by many different laboratories, and seems to be highly restricted to SMCs (Sartore *et al.*, 1994; Regan *et al.*, 2000). Of particular note, Miano *et al.* (1994) carried out very detailed in situ hybridization analyses of expression of SM MHC throughout the development and maturation of whole mouse embryos, and found no evidence for the expression of this marker in cell types other than SMCs. Together, these SMC-restricted genes provide a template for phenotypic characterization (Owens *et al.*, 2004; Liu *et al.*, 2008).

Phenotypically, SMCs differ from cardiac and skeletal muscle cells not only by their expression of specific contractile proteins, but also by their plasticity or ability to reenter the cell cycle and exhibit a "synthetic" phenotype, secreting ECM proteins (Mahoney and Schwartz, 2005; Stegemann *et al.*, 2005). RT-PCR showed that the SOC highly expressed elastin, MGP and VEGF at mRNA level. The outcome proteins of these mRNAs consist of the components of extracellular matrix in blood vessels. A lack of organized extracellular elastin would result in lower compliances of engineered vessels (Dahl *et al.*, 2007; Gao *et al.*, 2008). MGP, a vitamin K-dependent protein and a major calcification inhibitory factor, plays an important role in performing unique vascular wall biomechanical properties (Shanahan *et al.*, 1998; Schurgers *et al.*, 2005). VEGF appears to be a strong stimulator of new vessel growth in adults (Gao *et al.*, 2001).

In our study, SOC displayed morphological and phenotypic properties of SMC as shown by expression of smooth muscle markers—SM α -actin, calponin, SM MHC, SM22 α , elastin, MGP, and VEGF. Notably, SOC displayed contractile and synthetic properties, suggesting that these cells had developed a functional SMC phenotype for constructing blood vessels.

Our study proves that the cells cultured in PDGF

BB-enriched medium caused rapid outgrowth and expansion of SOC to more than 30 population doublings in a 3-month period with no apparent loss of proliferative potential. In contrast to adult hSMCs that became senescent after approximately 10 population doublings, SPCs have a much higher proliferation potential. Another important advantage of using SOC derived from mononuclear cells for TEBVs is that the cells can be obtained from peripheral blood, i.e., from a newborn's own cord blood that may be stored for later use, or from juvenile or adult peripheral blood. The use of cells from peripheral blood eliminates the need to sacrifice a blood vessel or tissue to obtain SMCs (Jevon *et al.*, 2008).

Our study shows that the activity and proliferation of SOC on PHBHH_x scaffold were improved greatly in a 3-week-culture period, greater than those of SMCs in the same experiment condition (data not shown). Meanwhile, we also proved that SF-PHBHH_x scaffold is more suitable for SOC growth compared with PHBHH_x scaffold (data not shown), which is consistent with Mei *et al.* (2006)'s and Zhang *et al.* (2008)'s work.

Although a serious limitation thus far for a therapeutic application of postnatal SPCs is their low number in the human circulation, this should not be an impediment, since the improvements in the cell purification and culture methods have been rapid in recent years. In addition, approaches to overcome this problem also include the mobilization of PBMCs in vivo by use of growth factors. Especially, granulocyte colony-stimulating factor mobilized PBMCs (G-PBMCs) has been widely used in clinical therapy (Bensinger *et al.*, 1993; Pettengell *et al.*, 1993). However, further studies are necessary for understanding the physiologic function of SPCs and the factors that determine their number and turnover rate, as well as the mechanisms that stimulate or inhibit their differentiation in vitro and in vivo.

References

- Bensinger, W., Singer, J., Appelbaum, F., Lilleby, K., Longin, K., Rowley, S., Clarke, E., Clift, R., Hansen, J., Shields, T., *et al.*, 1993. Autologous transplantation with peripheral blood mononuclear cells collected after administration of recombinant granulocyte stimulating factor. *Blood*, **81**:3158-3163.
- Campbell, G.R., Campbell, J.H., 2007. Development of tissue engineered vascular grafts. *Curr. Pharm. Biotechnol.*, **8**(1):43-50. [doi:10.2174/138920107779941426]

- Chen, G., Zhou, P., Mei, N., Chen, X., Shao, Z., Pan, L.F., Wu, C.G., 2004. Silk fibroin modified porous poly(epsilon-caprolactone) scaffold for human fibroblast culture in vitro. *J. Mater. Sci. Mater. Med.*, **15**(6):671-677. [doi:10.1023/B:JMSM.0000030208.89523.2a]
- Dahl, S.L., Rhim, C., Song, Y.C., Niklason, L.E., 2007. Mechanical properties and compositions of tissue engineered and native arteries. *Ann. Biomed. Eng.*, **35**(3):348-355. [doi:10.1007/s10439-006-9226-1]
- Fang, N.T., Xie, S.Z., Wang, S.M., Gao, H.Y., Wu, C.G., Pan, L.F., 2007. Construction of tissue-engineered heart valves by using decellularized scaffolds and endothelial progenitor cells. *Chin. Med. J.*, **120**(8):696-702.
- Gao, G., Li, Y., Zhang, D., Gee, S., Crosson, C., Ma, J., 2001. Unbalanced expression of VEGF and PDGF in ischemia-induced retinal neovascularization. *FEBS Lett.*, **489**(2-3):270-276. [doi:10.1016/S0014-5793(01)02110-X]
- Gao, J., Crapo, P., Nerem, R., Wang, Y., 2008. Co-expression of elastin and collagen leads to highly compliant engineered blood vessels. *J. Biomed. Mater. Res. A*, **85**(4):1120-1128. [doi:10.1002/jbm.a.32028]
- Jevon, M., Dorling, A., Hornick, P.I., 2008. Progenitor cells and vascular disease. *Cell Prolif.*, **41**(Suppl. 1):146-164.
- Kaushal, S., Amiel, G.E., Guleserian, K.J., Shapira, O.M., Perry, T., Sutherland, F.W., Rabkin, E., Moran, A.M., Schoen, F.J., Atala, A., et al., 2001. Functional small-diameter neovessels created using endothelial progenitor cells expanded ex vivo. *Nat. Med.*, **7**(9):1035-1040. [doi:10.1038/nm0901-1035]
- Liu, J.Y., Swartz, D.D., Peng, H.F., Gugino, S.F., Russell, J.A., Andreadis, S.T., 2007. Functional tissue-engineered blood vessels from bone marrow progenitor cells. *Cardiovasc. Res.*, **75**(3):618-628. [doi:10.1016/j.cardiores.2007.04.018]
- Liu, J.Y., Peng, H.F., Andreadis, S.T., 2008. Contractile smooth muscle cells derived from hair-follicle stem cells. *Cardiovasc. Res.*, **79**(1):24-33. [doi:10.1093/cvr/cvn059]
- Mahoney, W.M., Schwartz, S.M., 2005. Defining smooth muscle cells and smooth muscle injury. *J. Clin. Invest.*, **115**(2):221-224. [doi:10.1172/JCI200524272]
- Mei, N., Zhou, P., Pan, L.F., Chen, G., Wu, C.G., Chen, X., Shao, Z.Z., Chen, G.Q., 2006. Biocompatibility of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) modified by silk fibroin. *J. Mater. Sci. Mater. Med.*, **17**(8):749-758. [doi:10.1007/s10856-006-9686-8]
- Melero-Martin, J.M., Khan, Z.A., Picard, A., Wu, X., Paruchuri, S., Bischoff, J., 2007. In vivo vasculogenic potential of human blood-derived endothelial progenitor cells. *Blood*, **109**(11):4761-4768. [doi:10.1182/blood-2006-12-062471]
- Miano, J.M., Cserjesi, P., Ligon, K.L., Periasamy, M., Olson, E.N., 1994. Smooth muscle myosin heavy chain marks exclusively the smooth muscle lineage during mouse embryogenesis. *Circ. Res.*, **75**:803-812.
- Nugent, H.M., Edelman, E.R., 2003. Tissue engineering therapy for cardiovascular disease. *Circ. Res.*, **92**(10):1068-1078. [doi:10.1161/01.RES.0000073844.41372.38]
- Owens, G.K., Kumar, M.S., Wamhoff, B.R., 2004. Molecular regulation of vascular smooth muscle cell differentiation in development and disease. *Physiol. Rev.*, **84**(3):767-801. [doi:10.1152/physrev.00041.2003]
- Pettengell, R., Morgenstern, G.R., Woll, P.J., Chang, J., Rowlands, M., Young, R., Radford, J.A., Scarffe, J.H., Testa, N.G., Crowther, D., 1993. Peripheral blood progenitor cell transplantation in lymphoma and leukemia using a single apheresis. *Blood*, **82**:3770-3777.
- Regan, C.P., Manabe, I., Owens, G.K., 2000. Development of a smooth muscle-targeted cre recombinase mouse reveals novel insights regarding smooth muscle myosin heavy chain promoter regulation. *Circ. Res.*, **87**:363-369.
- Sartore, S., Scatena, M., Chiavegato, A., Faggini, E., Giuriato, L., Pauletto, P., 1994. Myosin isoform expression in smooth muscle cells during physiological and pathological vascular remodeling. *J. Vasc. Res.*, **31**:61-81.
- Schurgers, L.J., Teunissen, K.J., Knapen, M.H., Kwaijtaal, M., van Diest, R., Appels, A., Reutelingsperger C.P., Cleutjens, J.P., Vermeer, C., 2005. Novel conformation-specific antibodies against matrix gamma-carboxyglutamic acid (Gla) protein: undercarboxylated matrix Gla protein as marker for vascular calcification. *Arterioscler. Thromb. Vasc. Biol.*, **25**(8):1629-1633. [doi:10.1161/01.ATV.0000173313.46222.43]
- Shanahan, C.M., Proudfoot, D., Farzaneh-Far, A., Weissberg, P.L., 1998. The role of Gla proteins in vascular calcification. *Crit. Rev. Eukaryot. Gene Expr.*, **8**(3-4):357-375.
- Simper, D., Stalboerger, P.G., Panetta, C.J., Wang, S., Caplice, N.M., 2002. Smooth muscle progenitor cells in human blood. *Circulation*, **106**(10):1199-1204. [doi:10.1161/01.CIR.0000031525.61826.A8]
- Stegemann, J.P., Hong, H., Nerem, R.M., 2005. Mechanical, biochemical, and extracellular matrix effects on vascular smooth muscle cell phenotype. *J. Appl. Physiol.*, **98**(6):2321-2327. [doi:10.1152/jappphysiol.01114.2004]
- Wu, K.H., Liu, Y.L., Zhou, B., Han, Z.C., 2006. Cellular therapy and myocardial tissue engineering: the role of adult stem and progenitor cells. *Eur. J. Cardiothorac. Surg.*, **30**(5):770-781. [doi:10.1016/j.ejcts.2006.08.003]
- Wu, X., Rabkin-Aikawa, E., Guleserian, K.J., Perry, T.E., Masuda, Y., Sutherland, F.W., Schoen, F.J., Mayer, J.E., Bischoff, J., 2004. Tissue-engineered microvessels on three-dimensional biodegradable scaffolds using human endothelial progenitor cells. *Am. J. Physiol. Heart. Circ. Physiol.*, **287**(2):H480-H487. [doi:10.1152/ajpheart.01232.2003]
- Yeh, E.T., Zhang, S., Wu, H.D., Körbling, M., Willerson, J.T., Estrov, Z., 2003. Transdifferentiation of human peripheral blood CD34⁺-enriched cell population into cardiomyocytes, endothelial cells, and smooth muscle cells in vivo. *Circulation*, **108**(17):2070-2073. [doi:10.1161/01.CIR.0000099501.52718.70]
- Zhang, X., Baughman, C.B., Kaplan, D.L., 2008. In vitro evaluation of electrospun silk fibroin scaffolds for vascular cell growth. *Biomaterials*, **29**(14):2217-2227. [doi:10.1016/j.biomaterials.2008.01.022]
- Zund, G., Ye, Q., Hoerstrup, S.P., Schoeberlein, A., Schmid, A.C., Grunenfelder, J., Vogt, P., Turina, M., 1999. Tissue engineering in cardiovascular surgery: MTT, a rapid and reliable quantitative method to assess the optimal human cell seeding on polymeric meshes. *Eur. J. Cardiothorac. Surg.*, **15**(4):519-524. [doi:10.1016/S1010-7940(99)00068-8]