Proliferation and Differentiation of Adipose Stem Cells Towards Smooth Muscle Cells on Poly(trimethylene carbonate) Membranes

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Summary: Multipotent human adipose stem cells (hASCs) are an abundant and potential source of cells for vascular tissue engineering when combined with a suitable biomaterial scaffold. Poly(trimethylene carbonate) (PTMC) has been shown to be a useful biodegradable material for tissue engineered vascular grafts due to its flexibility, excellent biocompatibility and enzymatic degradation by surface erosion in vivo. The purpose of the current study was to evaluate the proliferation and differentiation of hASCs towards smooth muscle cells (SMCs) on gamma-crosslinked and photo-crosslinked PTMC membranes.

PTMC macromers were functionalized with methacrylate end groups and crosslinked by UV initiated radical polymerization. High molecular weight linear PTMC was crosslinked by gamma irradiation. Cell viability, cell numbers and SMC differentiation of hASCs were evaluated on the differently crosslinked PTMC films at 7 and 14 days (d). On the photo-crosslinked membranes, homogenous monolayers of hASC were detected by live/dead assay. Consistently, cells on the photo-crosslinked membranes had significantly higher cell numbers compared to cells on the gamma-crosslinked membranes after 14 d of culture. SMC specific genes were expressed on both membranes at 14 d. Photo-crosslinked membranes showed higher expression of SMC specific proteins at 14 d compared to gamma-crosslinked membranes.

These results suggest that especially the photo-crosslinked PTMC membranes are suitable for vascular tissue engineering applications when combined with hASCs.

Keywords: adipose stem cell differentiation; poly (trimethylene carbonate); smooth muscle cells; tissue engineering

Introduction

There is an increasing need for suitable biodegradable biomaterials for vascular tissue engineering applications. An optimal tissue engineered graft would allow both endothelial cell (EC) and smooth muscle cell (SMC) attachment and preserve their functions. Concurrently the biomaterial should be able to perform as well or similarly as a native blood vessel until absorption and ingrowth of the host tissue takes place.[1,2] Several materials have been tested in order to mimic the mechanical properties of blood vessels. The most studied polymers in vascular tissue engineering involve homopolymers and copolymers of poly glycolic acid (PGA),[3,4] poly lactic acid (PLA),[5,6] poly(ε-caprolactone) (PCL)[7–9] and poly hydroxylalkanoate (PHA).[10] However, the use of these polymers is limited due to the inadequate

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mechanical properties,[11] immunological reactions by acidic degradation,[12,13] narrow processability window, low melting point and degradation rate.[14]

Instead, poly (trimethylene carbonate) (PTMC)[15,16] has excellent properties appropriate for vascular tissue engineering. Diverse cross-linked PTMC networks have been previously shown to be flexible, elastic and biodegradable.[17–19] Moreover, PTMC with molecular weight higher than 10,000 g mol^{-1} has demonstrated to have a higher resistance to tearing than vascular xenografts[20] and a good biocompatibility for SMCs among other cell types.[15,16,21] Creep resistance of PTMC can be achieved by gamma-irradiation crosslinking[15,17,21,22] or by UV-irradiation crosslinking.[18–20] The gamma-irradiation method is rapid, effective and simultaneously crosslinks and sterilizes the material.[1] The UV-irradiation method allows temporal and spatial control of polymerization,[23] high curing rates at room temperature,[18] in situ and in vivo solidification.[24]

Photo-crosslinked PTMC networks and co-polymers have been previously studied with bone marrow mesenchymal stem cells (BMSCs) for vascular tissue engineering.[18,19,25] However, the interaction between PTMC with other types of mesenchymal stem cells such as adipose stem cells (ASCs) has not been previously studied. Adipose tissue is easily extracted as liposuction aspirates or subcutaneous adipose fragments,[26] making it a more easily accessible source of multipotent stem cells compared to bone marrow.[27,28] Furthermore, ASCs can be extracted from adipose tissue with an average yield of 5000 ASCs per gram, which is 100 fold higher than the number of BMSCs obtained in one gram of bone marrow.[29–31] Moreover, ASCs have been previously shown to differentiate towards myogenic lineages when treated with specific factors such as transforming growth factor-β1 (TGF-β1),[32,33] therefore, being a potential cell source for vascular tissue engineering. In the present study, the effects of gamma- and photo-crosslinked PTMC membranes on human ASC (hASC) proliferation and differentiation towards SMCs were compared for the first time.

Materials and Methods

Synthesis of Polymers

Three-armed PTMC oligomers (20,000 g mol^{-1}) were prepared by ring-opening polymerization of polymer grade 1,3-trimethylene carbonate (1,3-dioxan-2-one) (TMC; Foryou Medical Device, Huizhou City, Guangdong China) using trimethylol propane (TMP; Fluka, Buchs Switzerland) as initiator and stannous octoate (stannous 2-ethylhexanoate SnOct2; Sigma-Aldrich, St. Louis, MO, USA) (0.02 wt%) as catalyst. A three necked flask was loaded with TMC monomer and TMP, which were polymerized for 3 days (d) at 130 °C under argon atmosphere. Subsequently, the hydroxyl-terminated oligomers were functionalized by reaction with methacrylic anhydride (Sigma-Aldrich) in dichloromethane (biosolve, Valkenswaard, The Netherlands) solution at room temperature. An excess of triethyl amine (6.24 mmol excess per hydroxyl group) was added to the PTMC oligomer solution. The reaction was continued for 5 d. Purification of the macromers was accomplished by precipitation into cold methanol and drying under vacuum for 3 d.

High molecular weight PTMC (250,000 g mol^{-1}) was polymerized at 150 °C by ring opening polymerization of TMC (Boehringer Ingelheim, Germany) using SnOct2 (Sigma-Aldrich) as catalyst. The polymerizations were performed under a nitrogen blanket for 6 hours. Residual TMC of concentration less than 2% was calculated from NMR spectra, no further purification was required.

Preparation of Films by Photo- and Gamma-Crosslinking

PTMC networks were prepared by photo-crosslinking films of approximately 300 μm made by compression moulding. First, PTMC macromer was dissolved in

dichloromethane containing 1 wt% photo-initiator Irgacure 2959 (Ciba Specialty Chemicals, Switzerland). The solution was precipitated and the remaining solvent was allowed to evaporate overnight. Subsequently, the obtained solution was compression moulded at 70 °C for 2 min with a compression force of 30 kN and 1 min with a compression force of 50 kN. Finally, the obtained PTMC films were photo-crosslinked at room temperature under a nitrogen atmosphere in a crosslinking cabinet (Ultralum, USA) for 5 minutes at 365 nm.

High molecular weight PTMC polymer was used directly for the compression moulding of membranes with a thickness of approximately 300 μm. First, the PTMC polymer was heated to 160 °C for 2 minutes with a compression force of 30 kN and for 1 minute at 350 kN. Additionally, PTMC samples were vacuum-sealed in poly(ethylene)/poly(amide) bags (Hevel Vacuum B.V, Zaandam, The Netherlands) and exposed to 25 kGy gamma-irradiation from a ^{60}Co source (Synergy Health, Ede, The Netherlands). This method simultaneously crosslinked and sterilized the polymer.[22] Finally, photo and gamma-crosslinked membranes of 14 mm diameter were punched out from the irradiated films. Characterization of the two type of membranes have been previously described.[20,22,34]

Human Adipose Stem Cell Isolation and Expansion

Adipose tissue was collected from 3 different donors at the Tampere University Hospital. hASC isolation was conducted in accordance with the Ethics Committee of Pirkanmaa Hospital District, Tampere, Finland. Minced tissue samples were digested with collagenase type I (1.5 mg/ml; Invitrogen, Paisley, UK). Subsequently isolated hASC were expanded in T-75 polystyrene flasks (Nunc, Roskilde, Denmark) in maintenance medium (MM) consisting of Dulbecco's modified Eagle's medium: nutrient mixture F-12 (DMEM/F-12, Gibco, New York, USA) with 1% L-glutamine (GlutaMax I; Invitrogen), 10% human serum (PAA Laboratories GmbH, Pasching, Austria) and 1% antibiotics (100 U/ml penicillin; 100 U/ml streptomycin; Lonza Biowhittaker, Verviers, Belgium).

Surface Marker Expression Analysis by Flow Cytometry

hASCs were assessed for stem cell markers by a fluorescence-activated cell sorter (FACSAria; BD Biosciences, Erembodegem, Belgium) prior to cell seeding on PTMC membranes. Monoclonal antibodies against CD3-PE, CD14-PE-Cy7, CD19-PE-Cy7, CD45RO-APC, CD54-FITC, CD73-PE and CD90-APC (BD Biosciences); CD34-APC and HLA-DR-PE (Immunotools GmbH Friesoythe, Germany); and CD11a-APC, CD80-PE, CD86-PE and CD105-PE (R&D Systems Inc, MN, USA) were used. Analysis was performed on 10,000 cells per sample, and the positive expression was defined as the level of fluorescence greater than 99% of the corresponding unstained cell sample.

Cell Seeding and Smooth Muscle Cell Differentiation

Before cell seeding, membranes were sterilized by immersion in ethanol 70%. Next, membranes were let to dry for 30 min and washed 5 times with Dulbeco's phosphate-buffered saline (DPBS; Gibco). Finally, membranes were incubated in DMEM-F12 and 1% antibiotics for 3 d at 37 °C.

Cells at passage 2–4, were seeded on the crosslinked films at a cell density of 5000 cells cm^{-2} in a volume of 1 ml of MM supplemented with 5 ng/ml recombinant human TGFβ-1 (Sigma-Aldrich).[35–38] Medium was changed 3 times per week during the culture period of 14 d.

Cell Viability

Cell attachment and viability were assessed at d 1, 7 and 14 using the live/dead assay (Molecular Probes, Eugene, OR, USA) in which viable cells (green fluorescence) and dead cells (red fluorescence) can be visualized. The hASCs were incubated for 45 min at room temperature with a mixture of 0.5 μM calcein acetoxymethylester and

2.5 μM ethidium homodimer-1. The cells were observed with the AMG EVOS fl (Thermo Fischer Scientific, Waltham, US) microscope.

Cell Proliferation

The CyQUANT® cell proliferation assay kit (Molecular Probes) was used to asses the number of cells on the two different materials at 1, 7 and 14 d. Membranes were placed on a new 24 well plate, washed with DPBS after which the cells were lysed with 0.1% TritonX-100 (Sigma-Aldrich) buffer solution. Lysed samples were stored at −70 °C and freeze thawed twice to enhance cell lysis before analysis. After completion of the assay, measurements were done on a 96 well plate in triplicates. All samples were analyzed at an excitation of 480 nm and emission of 520 nm with the fluorescence microplate reader (TECAN Safire, Männedorf, Switzerland). Data was calculated against a DNA standard curve, created with dilutions of 100 μg/ml bacteriophage λ DNA (Molecular Probes).

Gene Expression Evaluation by Real Time Quantitative Polymerase Chain Reaction

Gene expression at the mRNA level was evaluated by real time quantitative polymerase chain reaction (RT-qPCR). Total RNA isolation of hASCs on membranes was performed using NucleoSpin® RNA II kit (Macherey-Nagel GmbH, Düren, Germany). RNA quantification and purity was measured with a Nanodrop Spectrophotometer ND-1000 (Thermo Fisher Scientific). Next, cDNA was synthesized in a MJ Mini™ Personal Thermal Cycler (Biorad, Hercules, California, USA) using an iScript™ cDNA synthesis kit (Biorad), according to the manufacturer's instruction. RT-qPCR was performed with the RT-PCR iQ™5 (Biorad) detection system using SensiMix SYBR & Fluorescein kit (Bioline, London, UK). The sequences of the forward and reverse primers used are shown in Table 1. The expression of RPLP0 was used to normalize gene expression levels. The expression levels of all genes at 14 d time point were calculated as relative to the expression level of hASCs on gamma-crosslinked membranes at d 14.

Immunofluorescence Staining

Immunofluorescence was used to determine the presence and organization of SMC marker proteins. Cells were fixed for 10 min in 3.7% paraformaldehyde (Sigma-Aldrich) in DPBS and permeabilized with 0.5% TritonX-100 for 30 min. Cells were blocked with 10% goat serum (Invitrogen, Frederick, MD, USA) for 10 min and incubated with primary antibodies overnight at 4 °C. Primary antibodies for SMC used were mouse anti-smooth muscle Myosin heavy chain 11 antibody (Abcam, Cambridge, UK), anti-α-SMA (Abcam), anti-calponin antibody (Abcam) at a dilution of 1:100. After three washes with 0.05% Tween (Sigma-Aldrich) in DPBS, cells were incubated with secondary Alexa

Table 1.
Primer sequences used for RT-qPCR.

Gene		Sequence (5′-> 3′)	bp	Accesion number
α-SMA	Fwd	CTATGAGGGCTATGCCTTGCC	122	NM_001613.2
	Rev	GCTCAGCAGTAGTAACGAAGGA		
caldesmon	Fwd	TCGACCCAACAATAACAGATGC	123	NM_033157.3
	Rev	TCTCGTATCTTTCTTGGCGACT		
calponin	Fwd	CTGTCAGCCGAGGTTAAGAAC	123	NM_001299.4
	Rev	GAGGCCGTCCATGAAGTTGT		
smoothelin	Fwd	CTCCCAGTCCTAGCGAGAAGA	97	NM_198501.2
	Rev	GGTGGGCTATTGCTGTGTTTG		
SM-MHC	Fwd	CATTATGCTGGGAAGGTGGA	81	NM_002474.2
	Rev	GGAAGTCACGTTGTCATTCAG		
RPLP0	Fwd	AATCTCCAGGGGCACCATT	74	NM_001002.3
	Rev	CGCTGGCTCCCACTTTGT		

(bp) base pair.

Fluor 488 goat anti-mouse IgG (Molecular Probes) and secondary Alexa Fluor 594 (Molecular Probes) goat anti-rabbit IgG for 60 min. Cells were washed 3 times with 0.05% Tween 20 (Sigma-Aldrich) in DPBS and incubated with 4′,6-diamidino-2-phenylindole (DAPI; Molecular Probes) for 10 min. Cells were observed with the BD-Pathway 435 (BD-Biosciences) microscope.

Statistical Analysis

Statistical analyses were performed with SPSS® Statistics version 21 (IBM, Armonk, New York, USA). The effects of gamma and photo cross-linked films on cell numbers were analyzed using a Student's t-test for independent samples. One-way analysis of variance (ANOVA) was performed to evaluate the cell numbers at 1,7 and 14 d. The equal variance assumption was tested by Levene's test. Bonferroni post hoc correction was implemented for ANOVA. Results were considered significant when $p < 0.05$. All experiments were repeated 3 times and a different hASC donor was used in each repeat.

Results

Surface Marker Expression Analysis by Flow Cytometry

The flow cytometric analysis (Table 2) demonstrated that for the hASCs isolated from 3 donors exhibited high expression of CD90 (Thy-1), moderate expression of CD73 (ecto 50 nucleotidase), CD105 (SH-2, endoglin) and CD34 (Sialomucin-like adhesion molecule) and no expression (≤2%) of CD14 (Serum lipopolysaccharide binding protein), CD19 (B lymphocyte lineage differentiation antigen), CD45 (Leukocyte common antigen), CD80 (B7-1), CD86 (B7-2) and HLA- < PREFIX > DR </PREFIX > (HLA class II). For 2 donors, hASCs showed moderate expression of CD54 (intercellular adhesion molecule 1) and no expression of CD3 (T-cell surface antigen) and CD11a (Integrin alpha L). These results are in accordance with the minimal criteria for defining multipotent mesenchymal stem cells proposed by the Mesenchymal and Tissue Stem Cell Committee of the ISCT.[39–41]

Cell Viability

Live/dead assay (Figure 1) showed that the amount of viable cells increased with time on both types of membranes. On photo-crosslinked membranes a monolayer of hASCs started to form at d 7 and at 14 d a homogenous monolayer of hASCs was observed. In contrast, on gamma-cross-linked membranes hASCs were more sparsely spread and no homogenous sheet of hASCs was formed during the 14 d culture period. Qualitatively estimated, a higher number of cells was detected on

Table 2.
Surface marker expression of undifferentiated hASCs by flow cytometry.

Antigen	Surface protein	Mean ± SD
CD 3	T-cell surface antigen	0.20 ± 0.00 [a)]
CD 11a	Integrin alpha L	0.50 ± 0.14 [a)]
CD 14	Serum lipopolysaccharide- binding protein	1.20 ± 0.80
CD 19	B lymphocyte-lineage differentiation antigen	0.43 ± 0.12
CD 34	Sialomucin-like adhesion molecule	21.87 ± 18.39
CD 45	Leukocyte common antigen	1.83 ± 0.78
CD 54	Intercellular adhesion molecule 1	16.35 ± 2.62 [a)]
CD 73	Ecto-50-nucleotidase	78.70 ± 18.99
CD 80	B7-1	0.30 ± 0.10
CD 86	B7-2	0.90 ± 0.61
CD 90	Thy-1 (T cell surface glycoprotein)	99.00 ± 0.56
CD 105	SH-2, endoglin	79.17 ± 30.72
HLA-DR	Major histocompatibility class II antigen	0.93 ± 0.75

The results are displayed as mean percentage of the surface marker expression ($n = 3$ donors) ± SD;
[a)] calculated from two samples ($n = 2$ donors).

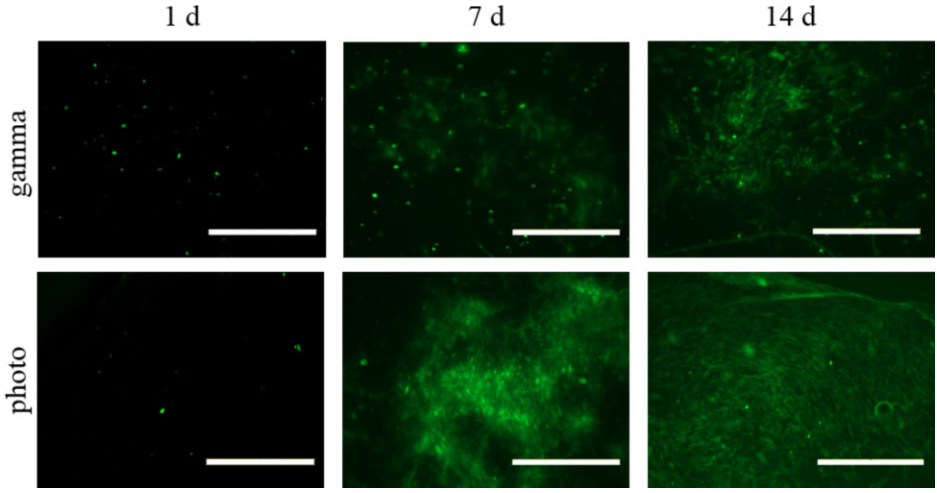

Figure 1.
Live/dead assay of hASCs at 1, 7 and 14 d on photo- and gamma-crosslinked membranes (scale bar 1000 μm).

photo-crosslinked compared to gamma-crosslinked membranes at 7 and 14 d.

Cell Proliferation

The number of hASCs on the membranes was quantitatively analysed at 1, 7 and 14 d (Figure 2). hASC attachment was similar on both membranes and no significant differences were found. Consistent with the live/dead staining, the DNA content on photo-crosslinked membranes was significantly higher ($p < 0.05$) with respect to gamma-crosslinked membranes at 14 d. No significant differences were found at 7 d, although the DNA content was following the similar trend as at 14 d. Cell numbers significantly

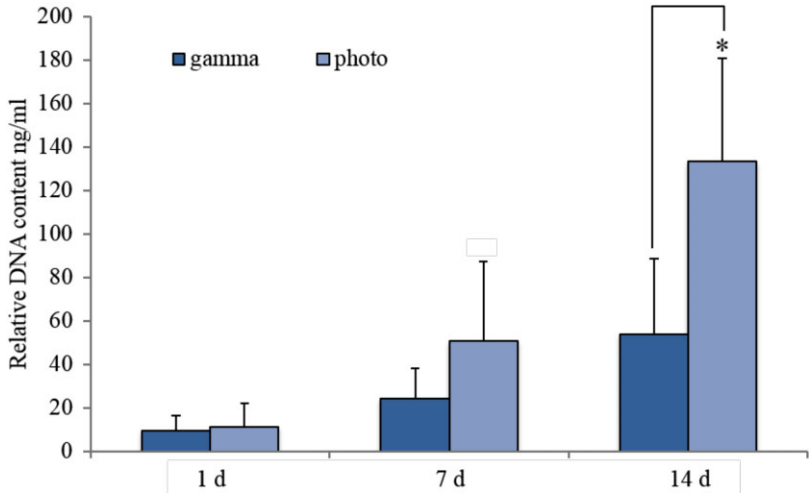

Figure 2.
Relative DNA content of hASCs at 1, 7 and 14 d on gamma- and photo-crosslinked membranes. Cell numbers significantly increased ($p < 0.05$) on both membranes with time (when comparing d 1 to 14 and d 7 to 14). The results are expressed as mean ± SD, $n = 3$. The number of technical samples was 9. $^{*}(p < 0.05)$ with respect to gamma-crosslinked membrane.

increased ($p < 0.05$) on both membranes with time.

Evaluation of Differentiation Towards Smooth Muscle Cells

To determine the SMC differentiation of the hASCs on the membranes, the expression of specific SMC makers was evaluated by immunofluorescence staining and RT-qPCR. On the photo-crosslinked membranes, a notably higher expression of α-SMA and calponin of hASCs from all the 3 donors was detected compared to gamma-crosslinked membranes at 14 d of culture (Figure 3). Also, strong expression of SM-MHC was observed on both membranes at both time points, photo-crosslinked membranes having higher number of cells expressing SM-MHC. Both membranes showed specific SMC markers expression at 7 and 14 d of culture. However, the expression of α-SMA was not detected at d 14 on gamma-crosslinked membranes with any of the 3 donors.

A quantitative detection of the relative SMC specific gene expression was performed for all 3 donors, however, the yield of mRNA from 1 donor was not sufficient affecting the reliability of the results and therefore, data from 2 donors is only shown without statistical analysis of the results. The relative expression of α-SMA, caldesmon, calponin, SM-MHC and smoothelin genes was evaluated (Figure 4). At 14 d, the expression of caldesmon, calponin, SM-MHC and smoothelin was upregulated on photo-crosslinked membranes compared to gamma-crosslinked membranes for donor 1. However, there were no notable changes on SMC specific gene expression of hASCs from donor 2 on the different membranes. Also, α-SMA gene expressions stayed on equal levels on both membranes for both donors.

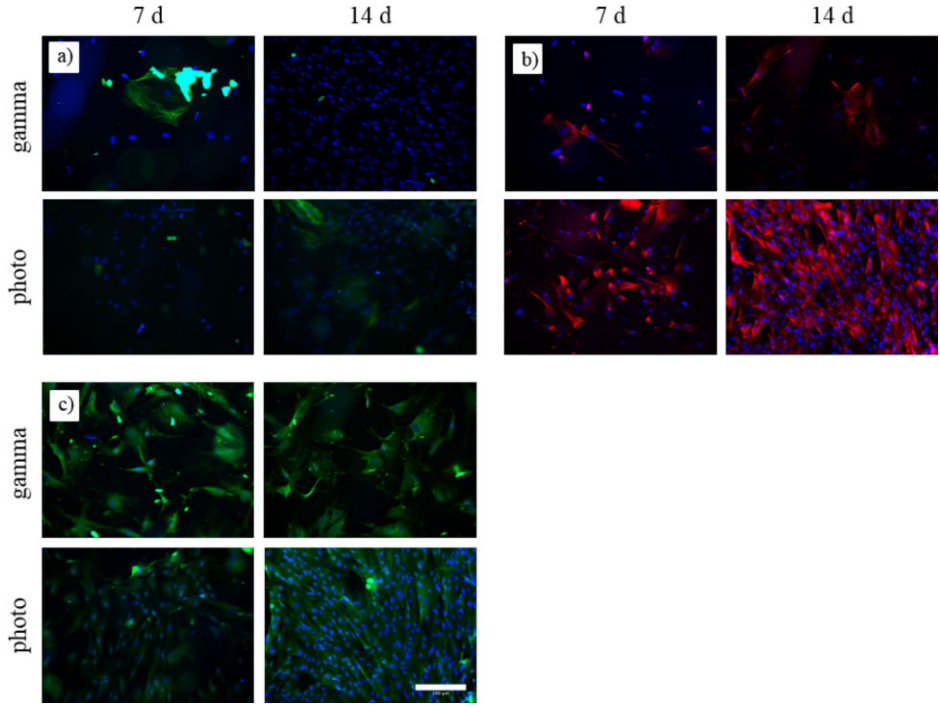

Figure 3.
a) α-SMA (green), b) calponin (red) and c) smooth muscle-myosin heavy chain (SM-MHC) (green) expression at 7 and 14 d by immuno-fluorescence on gamma-crosslinked and photo-crosslinked membranes. Nuclei stained with DAPI (blue) (scale bar 200 μm).

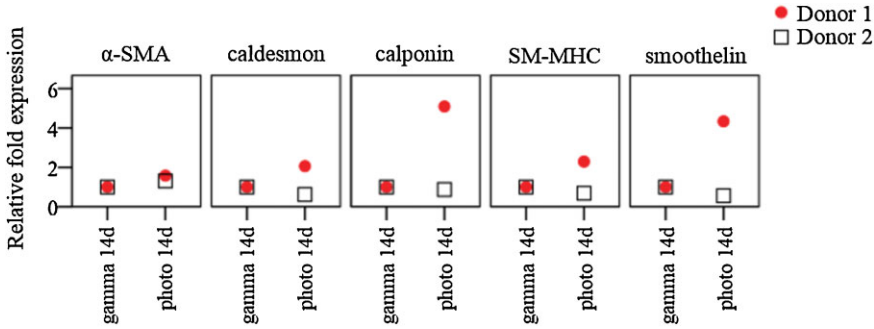

Figure 4.
Gene expression of SMC markers per donor. Data are expressed as relative fold mRNA expression of α-SMA, caldesmon, calponin, SM-MHC and smoothelin at 14 d on gamma and photo-crosslinked membranes.

Discussion

The present study demonstrated that different molecular weight and crosslinking methods of PTMC, directly influenced hASC morphology, proliferation and differentiation towards SMCs. Mesenchymal stem cells respond to the environment they interact with, such as surface topography, biochemical factors, stiffness, mechanical load, neighbouring cells and extracellular matrix.[32,42,43] This work was performed on two-dimensional surfaces, having the advantage of being able to reduce the complexity of such environment and attempting to better understand individually the effect of different variables.

In this work both membranes were compressed using the same polytetrafluoroethylene mold producing the same surface topography for the membranes, indicating that this variable had a negligible effect on the cell culture results. Instead, a possible explanation for the enhanced cell response on photo-crosslinked membranes may be related to the different stiffness of the membranes. Mechanical characterization has been previously performed by our group on photo-crosslinked PTMC networks of 20,000 g mol^{-1} and on gamma-crosslinked PTMC of 250,000 g mol^{-1}, showing a Young's Modulus of 4.3 MPa[20,34] and 6.6 MPa,[22] respectively. These mechanical characterization results indicate that photo-crosslinked membranes are more flexible, hence, allowing a better cell attachment. Similar results were obtained by Li et al.,[44] who showed that rat adipose stem cells attached better on softer substrate than on a stiffer one. Likewise, Evans et al.[45] showed that the proliferation of embryonic stem cells increased on a softer substrate. Interestingly, the stiffness of photo-crosslinked membranes resembles more the human native blood vessels having a Young's modulus range of 1 to 3 MPa,[46] than the mechanical properties of gamma-crosslinked membranes.

The viability and cell number assays indisputably showed that cells grew and spread on PTMC, independently of the crosslinking methods studied here. No signs of cytotoxicity were observed regarding the cell viability as no dead cells were observed. This is in accordance to similar work done with PTMC co-polymers.[25] Although PTMC has been previously shown to support the growth of BMSCs and other cell types,[18,19,25] yet, this work showed for the first time the suitability of PTMC to support hASC attachment and proliferation. Interestingly, hASCs proliferated significantly at a higher rate on photo-crosslinked membranes in comparison to gamma-crosslinked membranes.

Immunofluorescence and RT-qPCR results indicated that hASCs differentiate towards SMC lineage in the presence of TGF β-1, consistently with previous experiments done with hASCs.[2,32] Overall, the

expression of SMC specific proteins was evidently increased on photo-crosslinked membranes. Correspondingly on gene level, donor 1 showed up-regulation of most of the SMC specific genes on photo-crosslinked membranes at 14 d. However, no major enhancement of α-SMA on gene level was observed for both donors on both membranes. The degree of SMC marker specificity has been thoroughly studied,[47,48] demonstrating that SM-MHC is exclusively found only in late contractile SMCs.[49] Smoothelin is also considered as a late SMC marker and it has been shown that in vascular SMCs, the expression is downregulated when SMCs shift to synthetic phenotype.[50] Moreover, calponin is considered as late intermediate marker for contractile phenotype[51] and together with caldesmon they regulate the smooth muscle contractility.[52] α-SMA is the earliest SMC marker that can be detected and it is expressed during aortic development,[53] however, this marker can also be found in cardiac and skeletal lineages during embryogenesis and in adults.[54,55] Therefore, in order to detect SMCs, more than one SMC specific marker should be considered. Furthermore, other methods such as the quantification of protein expression using Western blot or the evaluation of SMC contraction ability in response to stimulation with KCl should be considered in order to verify the full differentiation of hASCs towards SMCs.

The RT-qPCR results indicated a notable variation between donors in response to the two different membranes (Figure 4). Donor variation has been previously observed with mesenchymal stem cells,[56] including hASCs.[57] Insufficient specificity of the used RT-qPCR marker genes could be another reason for differences in the SMC marker expression of the two donors. Nevertheless, the expression of early SMC marker α-SMA and contractile phenotype SMC markers calponin and SM-MHC[51] at both mRNA and protein level indicate that especially PTMC photo-crosslinked membranes support the differentiation of hASCs towards SMC. Detection and up-regulation of smoothelin and caldesmon expression further support this finding. Furthermore, in order to achieve SMCs with fully contractile phenotype, a simulation of the native environment, for example, (dynamic culture) could be implemented on photo-crosslinked scaffolds. A recent study done by Song et al.[15] demonstrated that human SMCs can proliferate on porous gamma-crosslinked PTMC scaffolds, and that proliferation is improved under dynamic culturing conditions. Also this study supports our results, showing that PTMC is suitable for SMCs proliferation.

Conclusion

In summary, our results show that both gamma and photo-crosslinked PTMC membranes allowed hASC proliferation and differentiation towards SMC lineage. Interestingly, photo-crosslinked membranes significantly enhanced hASCs proliferation and better supported the SMC differentiation compared to gamma-crosslinked membranes. hASCs used in combination with photo-crosslinked PTMC membranes show a great potential in future vascular tissue engineering applications.

[1] B. D. Ratner, A. S. Hoffman, F. J. Schoen, J. E. Lemons, *Biomaterials Science*, 3rd edition, Elsevier, **2013**.
[2] L. J. Harris, *et al.*, *The journal of surgical research* **2011**, *168*, 306.
[3] B. S. Kim, J. Nikolovski, J. Bonadio, E. Smiley, D. J. Mooney, *Experimental cell research* **1999**, *251*, 318.
[4] L. E. Niklason, *Science* **1999**, *284*, 489.
[5] C. M. Vaz, S. van Tuijl, C. V. C. Bouten, F. P. T. Baaijens, *Acta biomaterialia* **2005**, *1*, 575.
[6] T. Wang, X. Ji, L. Jin, Z. Feng, J. Wu, *ACS Applied materials & interfaces* **2013**, *5*, 3757.
[7] A. Sarasam, S. V. Madihally, *Biomaterials* **2005**, *26*, 5500.
[8] N. Diban, *et al.*, *Acta biomaterialia* **2013**, *9*, 6450.
[9] M. Zhang, *et al.*, *Journal of materials science. Materials in medicine* **2012**, *23*, 2639.
[10] D. Shum-Tim, *et al.*, *The annals of thoracic surgery* **1999**, *68*, 2298.
[11] L. E. Niklason, *et al.*, *Journal of vascular surgery* **2001**, *33*, 628.

[12] J. Wang, *et al.*, *Acta biomaterialia* **2013**, doi: 10.1016/j.actbio.2013.02.041
[13] X.-H. Qu, Q. Wu, K.-Y. Zhang, G. Q. Chen, *Biomaterials* **2006**, *27*, 3540.
[14] G. R. Saad, Y. J. Lee, H. Seliger, *Journal of applied polymer science* **2002**, *83*, 703.
[15] Y. Song, *et al.*, *Tissue engineering. Part A* **2011**, *17*, 381.
[16] A. A. Poot, D. W. Grijpma, J. Feijen, I. Vermes, *Nederlands Tijdschrift voor Klinische Chemie en Laboratoriumgeneeskunde* **2012**, *37*, 44.
[17] Z. Zhang, R. Kuijer, S. K. Bulstra, D. W. Grijpma, J. Feijen, *Biomaterials* **2006**, *27*, 1741.
[18] Q. Hou, D. W. Grijpma, J. Feijen, *Acta biomaterialia* **2009**, *5*, 1543.
[19] E. Bat, *et al.*, *Biomaterials* **2010**, *31*, 8696.
[20] S. Schüller-Ravoo, J. Feijen, D. W. Grijpma, *Acta biomaterialia* **2012**, *8*, 3576.
[21] B. L. Dargaville, *et al.*, *Acta biomaterialia* **2013**, doi: 10.1016/j.actbio.2013.02.009
[22] A. P. Pêgo, D. W. Grijpma, J. Feijen, *Polymer* **2003**, *44*, 6495.
[23] J. L. Ifkovits, Ja. Burdick, *Tissue engineering* **2007**, *13*, 2369.
[24] A. K. Burkoth, K. S. Anseth, *Biomaterials* **2000**, *21*, 2395.
[25] B. L. Dargaville, *et al.*, *Biomacromolecules* **2011**, *12*, 3856.
[26] B. Lindroos, R. Suuronen, S. Miettinen, *Stem cell reviews* **2011**, *7*, 269.
[27] P. A. Zuk, *et al.*, *Tissue engineering* **2001**, *7*, 211.
[28] B. Puissant, *et al.*, *British journal of haematology* **2005**, *129*, 118.
[29] A. Casadei, *et al.*, *Journal of biomedicine & biotechnology* **2012**, *2012*, 462543.
[30] H. Eto, *et al.*, *Plastic and reconstructive surgery* **2009**, *124*, 1087.
[31] P. A. Zuk, *et al.*, *Molecular biology of the cell* **2002**, *13*, 4279.
[32] C. Wang, *et al.*, *Biomaterials* **2010**, *31*, 621.
[33] Y. M. Kim, *et al.*, *The international journal of biochemistry & cell biology* **2008**, *40*, 2482.
[34] S. Schüller-Ravoo, J. Feijen, D. W. Grijpma, *Macromolecular bioscience* **2011**, *11*, 1662.
[35] N. Shi, W.-B. Xie, S.-Y. Chen, *The Journal of biological chemistry* **2012**, *287*, 6860.
[36] J. Y. Liu, H. F. Peng, S. Gopinath, J. Tian, S. T. Andreadis, *Tissue engineering. Part A* **2010**, *16*, 2553.
[37] J. S. Park, *et al.*, *Biomaterials* **2011**, *32*, 3921.
[38] K. Kurpinski, et al., *Stem cells (Dayton, Ohio)* **2010**, *28*, 734.
[39] M. Dominici, *et al.*, *Cytotherapy* **2006**, *8*, 315.
[40] R. H. Lee, *et al.*, *Cellular Physiology and Biochemistry* **2004**, *14*, 311.
[41] B. M. Strem, *et al.*, *The Keio journal of medicine* **2005**, *54*, 132.
[42] J. Liu, *et al.*, *Biomaterials* **2012**, *33*, 5036.
[43] K. Kolind, K. Leong, F. Besenbacher, M. Foss, *Biomaterials* **2012**, *33*, 6626.
[44] X. Li, *et al.*, *Journal of biomedical materials research. Part A* **2013**, doi: 10.1002/jbm.a.34774
[45] N. D. Evans, *et al.*, *European cells & materials* **2009**, *18*, 1.
[46] T. Uchida, *et al.*, *Journal of biotechnology* **2008**, *133*, 213.
[47] C. S. Madsen, *et al.*, *Circulation Research* **1998**, *82*, 908.
[48] C. P. Regan, I. Manabe, G. K. Owens, *Circulation Research* **2000**, *87*, 363.
[49] S. S. M. Rensen, P. A. F. M. Doevendans, G. J. J. M. van Eys, *Netherlands heart journal: monthly journal of the Netherlands Society of Cardiology and the Netherlands Heart Foundation* **2007**, *15*, 100.
[50] T. Christen, *et al.*, *Circulation Research* **1999**, *85*, 99.
[51] L. V. Thomas, P. D. Nair, *BioResearch open access* **2013**, *2*, 227.
[52] S. J. Winder, M. P. Walsh, *The Journal of biological chemistry* **1990**, *265*, 10148.
[53] M. G. Frid, B. V. Shekhonin, V. E. Koteliansky, M. A. Glukhova, *Developmental Biology* **1992**, *153*, 185.
[54] E. N. Olson, *Journal of Biological Chemistry* **1996**, *271*, 7095.
[55] M. C. Ruiter, R. Poelmann, L. Iperen, A. C. Groot, *Anatomy and Embryology* **1990**, *181*, 341.
[56] K. J. Portalska, *et al.*, *Tissue engineering. Part A* **2013**, doi: 10.1089/ten.TEA.2012.0646
[57] F. Guilak, *et al.*, *Journal of cellular physiology* **2006**, *206*, 229.

