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Effects of Scaffold Material Used in Cardiovascular Surgery on Mesenchymal Stem Cells and Cardiac Progenitor Cells

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Abstract

Background—Polytetrafluoroethylene (PTFE) and porcine small intestinal submucosa (pSIS) are patch materials used in congenital heart surgery. Porcine SIS is an extracellular-matrix scaffold that may interact with stem or progenitor cells. To evaluate this, we determined the in vitro effects of pSIS and PTFE on human bone marrow mesenchymal stromal cells (MSCs) and cardiac progenitor cells (CPCs) in 3 areas; cell proliferation, angiogenic growth-factor production, and differentiation.

Methods—Human MSCs and CPCs were seeded onto pSIS and PTFE patches. Cell-seeded patches were cultured and then assessed for cell viability and proliferation and supernatant vascular endothelial growth factor A (VEGFA) levels. Cell proliferation was quantified by MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide). Quantitative real-time polymerase chain reaction was performedon cell-seeded scaffolds to determine relative changes in gene expression related to angiogenesis and cardiogenesis.

Results—The MSCs and CPCs were able to attach and proliferate on pSIS and PTFE. The proliferation rate of each cell type was similar on pSIS. Total RNA isolation was only possible from the cell-seeded pSIS patches. The MSC VEGFA production was increased by pSIS. Porcine SIS promoted an angiogenic gene profile in MSCs and an early cardiogenic profile in CPCs.

Conclusions—Both PTFE and pSIS allow for varying degrees of cell proliferation. Porcine SIS elicits different phenotypical responses in MSCs as compared with CPCs, which indicates that pSIS may be a bioactive scaffold that modulates stem cell activation and proliferation. These findings highlight the differences in scaffold material strategies and suggest potential advantages of bioactive approaches.

Reconstruction of congenital cardiac defects often requires synthetic materials such as polytetrafluoroethylene (PTFE) when native tissue is not adequate. Although PTFE is durable and hemostatic, it does not grow and incites the formation of intimal hyperplasia.

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This may lead to reoperation for replacement and therefore increases the patient's risk of morbidity and mortality from repeat cardiac operations [1].

Decellularized porcine small intestinal submucosa (pSIS) patches has been advocated as an alternative to synthetic materials. Porcine SIS has been described in cardiac and vascular reconstructions, abdominal wall reconstruction, tendon repair, dural grafting, and lower urinary tract reconstruction [2–8]. Porcine SIS has been shown in animal studies to allow ingrowth of native tissue, as well as improved vascularization with minimal calcification or scarring [9, 10]. Porcine SIS is biodegradable and is replaced by native tissue or scar. It is unclear as to what mechanisms promote tissue ingrowth and neoformation versus scar formation. We hypothesized that patch materials may have varying effects on resident and remote progenitor or stem cells that populate the scaffold after implantation.

Mesenchymal stem cells (MSC) are being evaluated as a therapeutic agent after myocardial infarction in preclinical and clinical trials [11]. While it remains unclear the extent to which MSCs are able to differentiate into cardiomyocytes in vivo [12], numerous studies have shown that MSCs improve myocardial function through paracrine effects that improve compliance, increase angiogenesis, alter remodeling, increase cellular migration, and increase cardiomyocyte contractility [13–15]. The clinical effectiveness of MSC therapy in infarcted myocardial tissue has been limited as immediate cell retention after injection is minimal. This is believed to be due to a suboptimal delivery of cells as well as the inhospitable post-infarcted environment [16]. Research into the effectiveness of MSC seeded scaffolds as delivery agents is currently ongoing.

It was previously believed that cardiac tissue was terminally differentiated and cardiac regeneration in the mammalian heart was not possible from local cell populations. In 2003, Beltrami and colleagues [17] described adult cardiac progenitor cells (CPCs) that were self-renewing as well as multipotent, with the ability to differentiate into cardiomyocytes as well as smooth muscle and endothelial cells. This discovery has fueled intensive research efforts into the application of CPCs in infarcts with both significant and negligible results [18, 19]. Delivery of CPCs on scaffolds has not been investigated, and the effects of scaffold material on CPCs have to be first delineated.

In our study, we sought to compare the effect of pSIS on the proliferation, gene expression profile, and activation in MSCs and CPCs to determine which material would provide a superior niche for stem cell populations and provide further insight into their potential for cardiac regeneration.

Material and Methods

Scaffolds

Sterile PTFE (0.4 mm thickness; W. L. Gore & Associates, Inc, Newark, DE) and pSIS (CorMatrix Cardiovascular, Inc, Alpharetta, GA) were used in this study. Patch materials were soaked in phosphate buffered saline (PBS) for 10 minutes. For the pSIS patches, the 4-ply material was separated into single layers to allow for microscopic visualization of seeded

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cells and facilitation of RNA isolation for quantitative real time polymerase chain reaction (qPCR) analysis.

Cell Culture

Bone marrow MSCs were purchased from Lonza (Basel, Switzerland). The MSCs were cultured in MSC medium (Dulbecco's modified Eagle medium, with high-glucose concentration, GLUTAMAX I, 10% heat inactivated adult bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, all from Life Technologies, Carlsbad, CA), incubated at 37°C and 5% carbon dioxide (CO_2), and allowed to achieve 80% confluence prior to use.

The CPCs were isolated from neonates undergoing open heart surgery and characterized as described previously under an approved Institutional Review Board protocol from the Children's Memorial Hospital [20, 21]. Briefly, right atrial tissue were minced and partially digested with 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA) and collagenase type II (Worthington Biomedical Corp, Lakewood, NJ). Explants were plated on fibronectin in CPC medium (Iscove modified Dulbecco medium with 20% fetal bovine serum). When there appeared to be an adequate number of phase-bright cells (usually 2 weeks after plating), they were removed and plated at low density (3×10^4 cells/mL) in cardiosphere-growing medium. Cardiospheres were removed and plated on fibronectin in human cardiac stem cell expansion medium (HCSCEM; Celprogen, San Pedro, CA). Phase-bright cells were harvested from individual explant cultures every 3 to 5 days up to 4 consecutive times. The CPCs were then expanded on fibronectin-coated plates with CPC media. Detailed characterization of these CPCs has been provided elsewhere [20]. Media was changed every 2 days for all cell types. All experiments were performed with cells from passages 3 to 8.

Scaffold Seeding and Stem Cell Proliferation

Single-layer pSIS and PTFE were cut into 1×1 cm^2 patches and placed in the wells of a 24-well (flat bottom) plate such that the scaffolds covered the bottom surface of the wells. Preliminary experiments indicated that MSC and CPC proliferation on scaffolds were significantly reduced as compared with that obtained on tissue culture treated polystyrene (control surface). Therefore control wells were seeded with 5×10^4 MSCs or CPCs while scaffolds were seeded with 1.5×10^5 MSCs or CPCs in 100 μL of MSC media or CPC media, respectively, on the surface of the scaffolds. Cells were allowed to adhere to scaffolds for 2 hours prior to the addition of more media.

Relative cell proliferation was assessed by MTT assay as previously described after a 7 day culture period [22]. Briefly, a stock solution of 3-(4,5-Dimethylthiazo1-2-y)-2,5-diphenyltetrazolium bromide (MTT, Life Technologies) was diluted with PBS as per manufacturer directions added to each well with patch material and cells and agitated for 3 hours. Dimethyl sulfoxide was then added to each well to lyse the adherent cells; the scaffolds were then removed and absorbance of each solution was measured at 560 nm.

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Effects of Scaffold Material on Gene Expression

The effects of scaffold material on CPC and MSC gene expression were determined. We focused on human genes related to angiogenesis, cell proliferation, and cardiogenesis (Table 1). Cells were plated in 100 μL onto tissue culture treated polystyrene (control), PTFE, or pSIS in a 24-well plate as described above. After 2 hours, 400 μL of CPC medium was added. Media was changed daily until harvest for nucleic acid isolation after 14 days of incubation.

Nucleic Acid Isolation and Amplification

The RNA was isolated using Trizol-Chloroform separation (Life Technologies) followed by the Qiagen RNeasy minikit (Qiagen, Inc, Valencia, CA) according to the manufacturer's protocol. Control CPCs and MSCs were incubated with Trizol for 5 minutes and collected with a cell scraper. The CPCs and MSCs plated on scaffolds were submerged in 500 μL Trizol and vortexed for 1 minute. Phenol:Chloroform:Isoamyl-alcohol was mixed into the samples at a 1:5 dilution, then centrifuged at 13,400 RPM at 4°C for 15 minutes. Samples were then processed through the standard Qiagen RNeasy protocol using the aqueous layer of the centrifugation product in place of RLT buffer (Qiagen). Isolated products were quantified using a Nanodrop2000 (Nanodrop, Wilmington, DE). Complementary (c)DNA was generated from 0.5 μg of RNA in 20 μL reactions according to the protocol for Invitrogen High-Capacity cDNA Reverse Transcription kit (Life Technologies).

Real-Time Polymerase Chain Reaction

Quantitative polymerase chain reaction (PCR) was performed for the genes listed in Table 1 using CPCs from the same passage number plated onto tissue culture treated polystyrene as a control. Beta-actin was used as a housekeeping gene for baseline expression. Real-time expression was assayed with iTaq Universal SYBR Green Supermix (BioRad, Hercules, CA) and 0.25 μL of cDNA per 10 μL reaction according to product protocol on a Step One Real-Time PCR System (Life Technologies), and analysis was completed with Microsoft Excel using the CT calculation method, with standard deviations displayed for triplicate biologic samples within each group.

Vascular Endothelial Growth Factor A (VEGFA) Production

The effect of scaffold material on promoting the production of VEGFA was evaluated for MSCs and CPCs. As described above for the proliferation assay, cells were seeded onto scaffolds and cultured for 7 days. Supernatants were harvested and frozen at −80°C until the time of analysis. Supernatant VEGFA levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (Life Technologies) according to the manufacturer's directions.

Statistical Models and Formulas

All experiments were performed in duplicate (proliferation and VEGFA analysis) or triplicate (gene expression studies). For cell proliferation experiments, p values were calculated using a Student t test. For VEGFA ELISA results and quantitative gene expression, statistical differences were calculated using the Student t test with Bonferroni

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correction for differences in average expression of biologic replicates. For gene expression analysis, all groups were compared with CPCs plated on plastic as a control; expression values of MSCs plated on pSIS were also tested for significant differences relative to control MSCs. An initial significance threshold of 0.05 adjusted using the Bonferroni correction was employed for both analyses.

Results

Cell Seeding and Proliferation

Due to its opaque nature, it was not possible to confirm cell seeding onto the surface of PTFE using bright-field microscopy. The MSCs and CPCs adhered to the surface of 1-ply pSIS. The cells did not attach to the surface of pSIS in a homogeneous fashion; rather, both MSCs and CPCs appeared to cluster along the fibers of pSIS (Figs 1A, 1B).

Cell viability and proliferation was quantified using the MTT assay. In preliminary experiments both cell types proliferated much faster on the control surface (tissue culture treated polystyrene) and a higher seeding density was needed on the scaffolds to achieve a signal from the subsequent MTT assay which would have been too high for the control surface to allow for further proliferation. Therefore, subsequent proliferation experiments utilized a higher seeding density for the scaffold groups and comparisons were only made between scaffold groups. For MSCs, proliferation was significantly increased in the pSIS group as compared with the PTFE group ($p < 0.001$, Fig 2). For CPCs, pSIS also provided increased proliferation as compared with PTFE ($p < 0.001$, Fig 2).

Relative Gene Expression

We were unable to isolate sufficient high-quality RNA from cell-seeded PTFE and thus could not perform gene-expression analysis in these groups. Porcine SIS seeded at a high cell density ($1.5 \times 10^5/cm^2$) was able to support a level of cellular adherence and maintenance to provide sufficient RNA for quantitative expression analysis. After 7 days, RNA isolated from both cell types seeded onto pSIS or polystyrene was used to synthesize cDNA. Real-time PCR was performed for 8 different cardiac and angiogenesis-related genes. As shown in Figure 3, CPCs seeded on pSIS demonstrated significantly increased expression of GATA4 (Fig 3C), MYC (Fig 3A), and SLIT3 (Fig 3D), as well as a significant decrease in ACTC1 (Fig 3H) and FLT1 (Fig 3G) (VEGF receptor) expression ($p < 0.05$). The MSCs on pSIS demonstrated significantly increased expression of PDGF (Fig 3E), MYC, VEGFA (Fig 3F), and SLIT3 compared with control MSCs ($p < 0.05$). When compared with control CPCs, control MSCs showed significantly increased expression in SLIT3, VEGFA, and MYC, and a decrease in GATA4 and FLT1. No significant difference was shown for MEF2C (Fig 3B), a cardiac transcription factor for both MSCs and CPCs.

VEGFA Production

Supernatants were collected after a 7 day culture period to assess VEGFA production by ELISA analysis. When the supernatant VEGFA levels were normalized to cell number (absorbance from the MTT assay), a significant difference in VEGFA production was noted between pSIS and PTFE groups for both MSCs and CPCs (Fig 4). The MSCs had greater

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VEGFA production on pSIS versus control surface and PTFE. On the other hand, CDCs manifested greater VEGFA production on the scaffolds as compared with the control group, with the greatest VEGFA levels seen on PTFE. Negative controls (media only) had no VEGFA content (data not shown).

Comment

Environmental and extracellular factors affect stem cell behavior. The elastic modulus, hardness, and permeability of plating materials alone can influence the lineage of otherwise identically cultured stem cells [23]. Direct contact with endoderm-like cells or cardiac myocytes in a matrix containing cardiac ECM guides the phenotype of cardiac progenitor cells in the direction of cardiac myocytes [24, 25]. Therefore, in order to provide the most effective intervention to recreate native-like environments for patients in need of cardiac tissue regeneration, strategies must consider not only the durability of materials, but also their effect on the local stem-cell populations by which they will eventually be populated.

Scaffold materials used in cardiac intervention procedures are required to fulfill a wide range of physical requirements. Any implanted material must tolerate the wall stress of multiple cardiac cycles, of which PTFE has a proven track record. In addition, scaffold materials should not prompt any untoward immune response, fibrosis, or calcification, any of which would inhibit the aim of returning the surrounding tissue to a native phenotype and normal function. Regarding physical presence within the heart tissue, an ideal material would also be easily integrated by the stem cell population of interest (whether it be MSCs, CPCs, or an as-yet-unspecified undifferentiated cell type) and resorbed within the tissue once the implanted cells had successfully incorporated into the target organ or tissue and synthesized its own extracellular matrix.

We were unable to perform gene expression analysis of MSCs and CPCs seeded on PTFE; however, we were able to obtain insight into the effects of pSIS on the gene expression in MSCs and CPCs. We studied gene expression 7 days after cell seeding to allow for adherence and proliferation; differentiation at such an early time point would be difficult to anticipate. The increased expression of MYC as a result of culturing MSCs and CPCs on pSIS indicates that this scaffold material encourages proliferation, which agrees with the results of the proliferation assay. Interestingly pSIS increased the CPC gene expression of GATA4, a gene involved in cardiac myogenesis, suggesting that delivery of CPCs on pSIS may prime these cells for cardiac regeneration. The ACTC1 was downregulated in CPCs seeded on pSIS, which indicates a mature cardiac phenotype was not promoted. Certainly, other environmental factors such as cyclic stress and electrical stimulation may be needed to promote the expression of ACTC1 in CPCs. The MSCs are also known to express cardiac alpha actin [26]. The bone marrow MSC line that we utilized in this study had a high baseline expression of ACTC1 and was downregulated upon culturing on pSIS.

Porcine SIS induced an angiogenic profile in MSCs; VEGFA, SLIT3, and PDGF were all significantly upregulated in the pSIS group. Recently, SLIT3 expression was demonstrated to influence the ability of MSCs to promote angiogenesis, and we provide the first demonstration that pSIS has the ability to increase SLIT3 expression in MSCs and CPCs

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[27]. The angiogenic profile for CPCs on pSIS did not provide a consistent and unified angiogenic signature as PDGF and VEGFA were low or unchanged, yet the VEGFA levels measured by ELISA demonstrated increased production by CPCs cultured on pSIS. The reason for this discrepancy is unknown but may be explained by a lag in protein translation versus mRNA expression of VEGFA in CPCs at the time of analysis. The decrease in FLT1 expression seen in both MSCs and CPCs cultured on pSIS may thus be a regulatory response to the activation of angiogenic gene networks.

The mechanisms by which pSIS influenced the gene expression in human CPCs and MSCs were not investigated in this study. The biomechanical properties, extracellular matrix, and growth factors of pSIS may all influence cellular gene expression [28]. Understanding the multitude of pSIS characteristics that can influence stem cell gene expression will be important to further tailoring scaffolds to maximize the therapeutic and regenerative effects of seeded stem cells.

Epicardially applied scaffolds of various compositions have been proposed as a delivery vehicle for stem cells that permits cellular retention and alignment [29, 30]. Our results suggest that pSIS could also be used as a delivery system for stem cells. Current methods used for the delivery of stem cells into the disease myocardium include epicardial, endocardial, or intracoronary injection. Injected MSCs have shown some improvement in function and reduction of fibrosis [13]; however, the efficiency of these protocols is extremely limited because so few of the cells are retained in the target areas. The current advantage of pSIS over other biodegradable scaffolds described in the literature is that it is U S Food and Drug Administration (FDA) approved for cardiac patching; there is ample clinical experience with this material and meets the biomechanical requirements needed to withstand hemodynamic stresses present within the heart. These characteristics make clinical translation of stem cell-seeded pSIS less complicated than other synthetic or natural biodegradable scaffolds, of which most are not FDA approved for cardiac patching.

Porcine SIS seeded with rabbit bone marrow MSCs has been evaluated in a coronary ligation model in rabbits [31]. In this study, pSIS seeded with MSCs applied to the epicardial surface of the infarct in rabbits provided the most preservation of systolic and diastolic function, prevention of dilation, and MSC retention, and the highest capillary density as compared with MSC intramyocardial injection. In contrast to our study, pSIS was prepared by these investigators and rabbit bone marrow MSCs were used to seed the pSIS. Nevertheless, the results of the above in vivo study and of our in vitro study provide the rationale of advanced preclinical testing of MSC seeded pSIS. Our results also provide the rationale for in vivo evaluation of CPC seeded pSIS. Finally, the combination of CPCs and MSCs injected intramyocardially has been demonstrated to provide additional recovery in systolic and diastolic function and reduction in infarct size as compared with the monotherapy groups [32]. Future studies will evaluate pSIS as a delivery vehicle for the combination of human CPCs and MSCs.

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DISCUSSION

DR TODD K. ROSENGART (Houston, TX) So you were looking at GATA4, MEF2C. I don't know if you were looking at TBX5. These obviously have been implicated now in cardiac differentiation and cellular reprogramming of fibroblasts. Can you give us any additional information about those findings?

DR WITT In our study we were looking at GATA4, MEF2C, FLT1, MYH7, ACTA, and PDGF. We were not able to find a statistically significant difference between the Gore-Tex or the CorMatrix in the groups outside of FLT-1 however there was a trend towards greater transcription in the CorMatrix group. I think if we take our experiments out further in time we can tease out the differences.

DR ROSENGART Were you expecting that they were somehow related to your end results? Why were you looking at those as early transcription factors, not markers of cardiac differentiation?

DR WITT We did examine the presence of MHC-ß as a marker for cardiac differentiation. The examination of the early transcription factors was primarily looking possible different gene regulation between the scaffolds.

DR ALYCE LINTHURST JONES (Virginia Beach, VA) I was wondering if you were looking to use any other biological scaffolds? It almost seems like comparing a biological scaffold to ePTFE, the results are kind of a foregone conclusion. So I was wondering if you were going to look at other biological scaffolds perhaps?

DR WITT Yes. That is planned to look down the line because, as you mentioned, they're very different scaffolds; biologic versus synthetic. At our institution, they are both used very commonly, and that was the main reason we chose those two.

Acknowledgments

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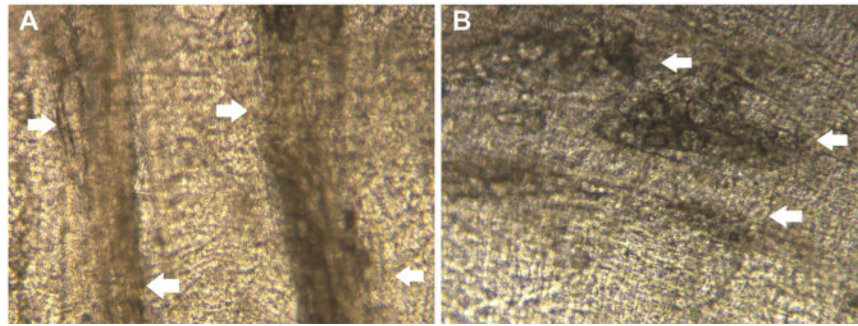

Fig 1.

Cell seeding of pSIS. Heterogeneous aggregation of human mesenchymal stem cells (A) and cardiac progenitor cells (B) on porcine small intestinal submucosa under bright field microscopy (×10 magnification). Cell aggregation indicated by white arrows.

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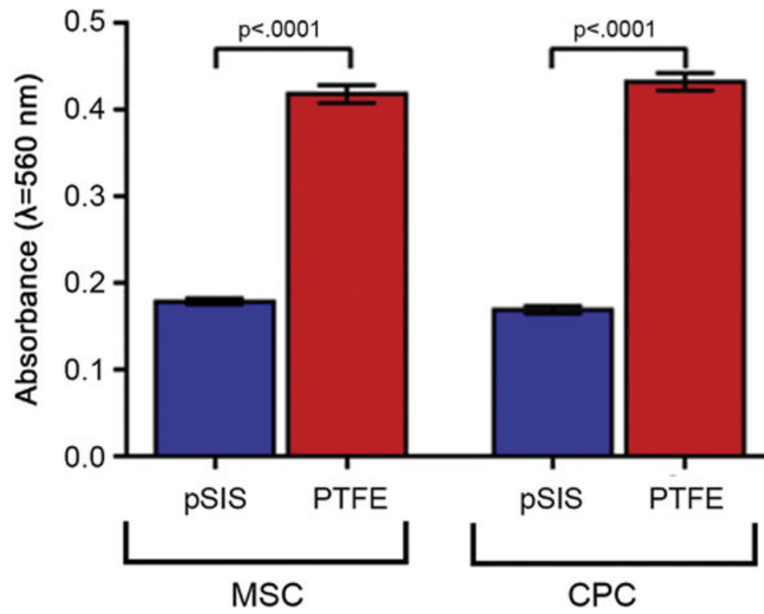

Fig 2.

Effects of scaffold material on mesenchymal stem cells (MSC) and cardiac progenitor cells proliferation (CPC). Polytetrafluoroethylene (PTFE) and porcine small intestinal submucosa (pSIS) had different effects on MSC and CPC proliferation. Porcine SIS significantly promoted both MSC and CPC proliferation as compared with PTFE.

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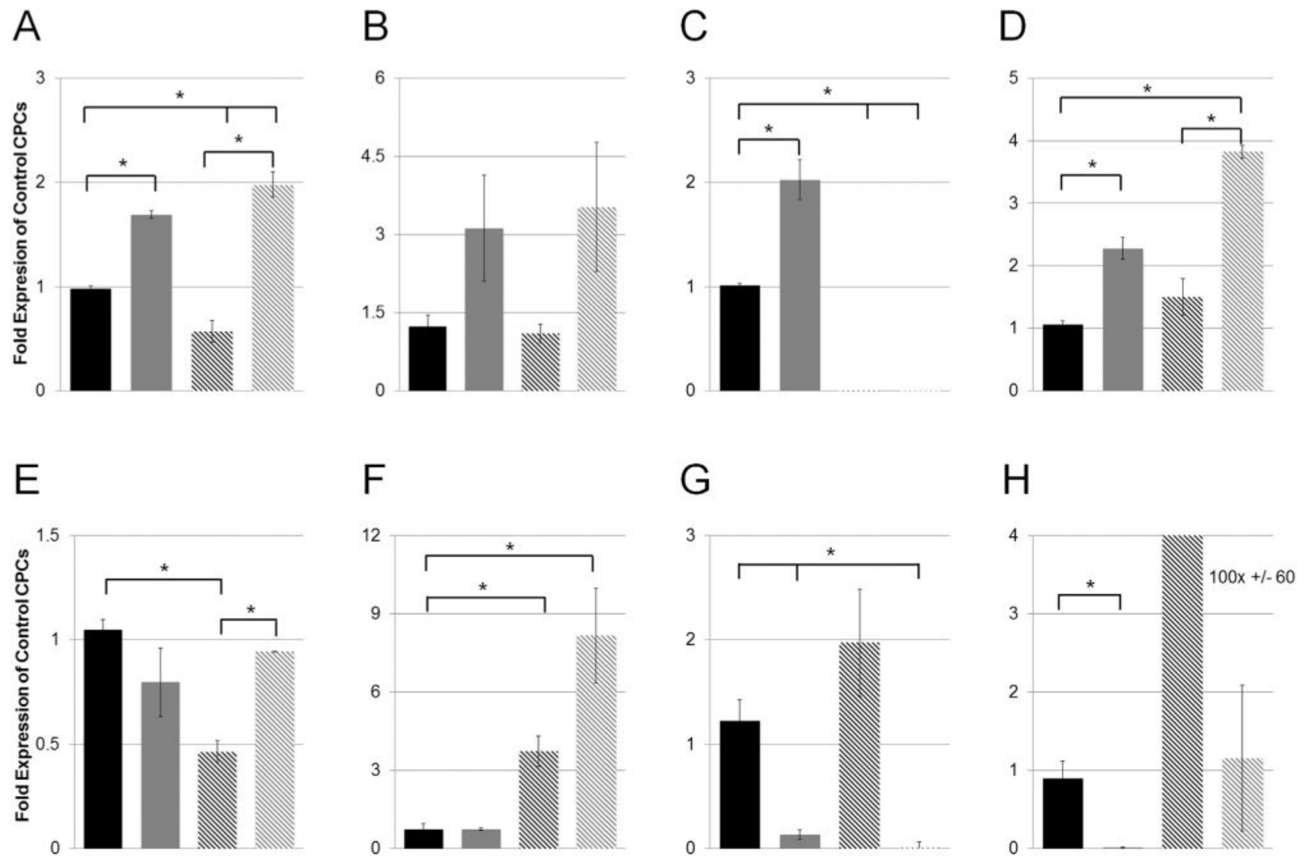

Fig 3.

The RNA expression of mesenchymal stem cells (MSCs) and cardiac progenitor cells (CPCs) seeded on porcine small intestinal submucosa (pSIS). Relative quantitative expression of (A) MYC, (B) MEF2C, (C) GATA4, (D) SLIT3, (E) PDGF, (F) VEGFA, (G) FLT1, and (H) ACTC1 was analyzed 7 days post seeding on pSIS. (Black bars represent control CPCs; grey, CPCs on pSIS; black stripes, control MSCs; and grey stripes, MSCs on pSIS; in a t test of differential expression, *p < 0.01.)

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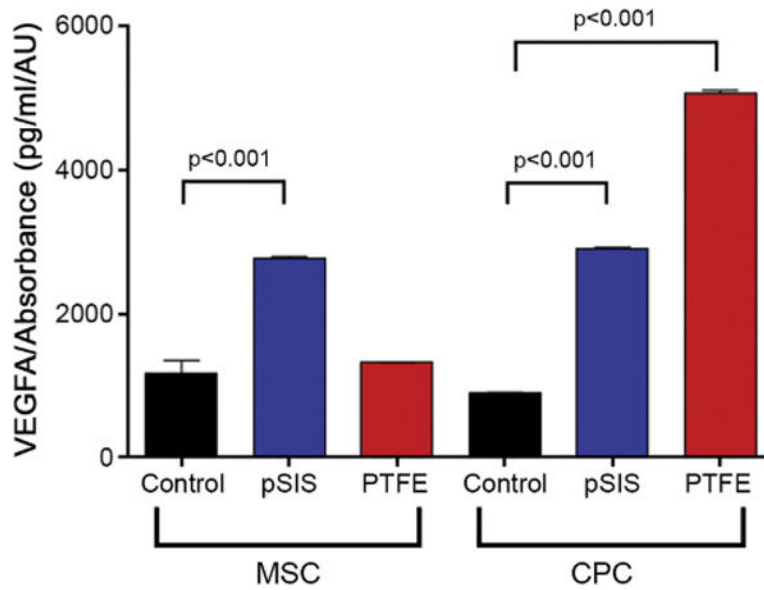

Fig 4.

Cardiac progenitor cells (CPC) and mesenchymal stem cells (MSC) vascular endothelial growth factor A (VEGFA) production is increased by scaffolds. The MSC VEGFA supernatant levels were increased by porcine small intestinal submucosa (pSIS) but not polytetrafluoroethylene (PTFE). The CPC VEGFA supernatant levels were significantly increased by both pSIS and PTFE.

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Table 1

Angiogenic and Cardiogenesis Related Genes Investigated in Cardiac Progenitor Cells and Mesenchymal Stem Cells Seeded on Scaffolds

Gene	Name	Forward & Reverse Primers	Protein Function
MYC	c-myc	AAGACTCCAGCGCCTTCTCTCACCTCTTGAGGACCAGTGGG	Transcription factor affecting cell cycle progression, apoptosis, and proliferation/mitogenesis
ACTC1	Actin, alpha cardiac muscle 1	AAGAGAAGCTGTGCTATGTCGACATTGTTGGCATACAGGTCC	Cardiac-specific contractility
MEF2C	Myocyte enhancer factor 2	TCAGTCAGTCATTGGCTACCCTATGTAGGTGTTGCTGTTGCC	Cis- and trans-activating cardiac transcription factor, cardiomyogenesis
SLIT3	Slit homolog 3	TGATGGCAACGAGGAGAGTAACGGCTGTTAGGTGGTTTCC	Secreted protein affecting cell migration, angiogenesis
GATA4	GATA binding protein 4	TTCCAGCAACTCCAGCAACGGAGACGCATAGCCTTGTGGG	Zinc-finger cardiac transcription factor, cardiomyogenesis
PDGFA	Platelet-derived growth factor A	TACGAGATTCCTCGGAGTCAGGTTCCCGATAATCCGGATTCAGG	Mesenchymal cell-specific mitogenic factor
VEGFA	Vascular endothelial growth factor A	ATGGCAGAAGGAGGAGGGCAGATCGCATCAGGGGCACACAGG	Angiogenesis, vasculogenesis, cellular migration
FLT1	fms-related tyrosine kinase 1	TGGACTGCTGGCACAGAGACCCTTCCTGAATTAAACTTCGGAGC	VEGF Receptor, regulates angiogenesis and vasculogenesis