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Attachment, Viability and Adipodifferentiation of Pre-adipose Cells on Silk Scaffolds with and Without Co-expressed FGF-2 and VEGF

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Abstract. *Background/Aim: For application of stem cells and progenitor cells in regenerative medicine, scaffolds for carrying the cells play a key role. One promising biomaterial for scaffold generation is silk because of its mechanical strength, good cytocompatibility and low immunogenicity. Furthermore, bioengineering of silk proteins enable co-expression of various growth, differentiation and angiogenic factors on silk fibers, which may promote cell growth, differentiation and angiogenesis. This study aimed to test cytocompatibility and growth/differentiation of pre-adipose cells on scaffolds with and without expressed growth factors fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF). Materials and Methods: Disk-form scaffolds of 15×3 mm (diameter × thickness) were manufactured in two different densities using silk with and without expressed growth factors FGF-2 or VEGF. Pre-adipose cells were prepared from fatty tissues of patients undergoing operation. Cells ($1.6×10^6$) were seeded onto each of the silk-scaffold disks, that were placed into wells of 12-well culturing plates. Adipose-differentiation was induced using differentiation medium containing DMEM/F-12, insulin, pantothenate, biotin, triiodothyronine (T3), transferrin, dexamethasone, isobuthylmethylxanthine and rosiglitazone. Cells on the scaffolds were visualized using a confocal microscope. Viability and adiponectin were measured on days 0, 7 and 14. Expression of adipose-differentiation markers was assessed by means of real-time polymerase chain reaction (RT-PCR). Results: Pre-adipose cells attached well onto the silk fibers. The highest initial viability was measured on the low-density scaffolds with expressed VEGF. Adipose-differentiation was evident in visible oil droplets and significantly increased adiponectin protein levels were seen in ELISA. Furthermore, increased expression of adipose-differentiation genes were measured in RT-PCR. Adipose-differentiation was more profound in cells on high-density scaffolds. In concordance, viability of cells on high-density scaffolds did not increase, while that of cells on low-density scaffolds doubled over the 14-day experimental period. Slightly enhanced adipose-differentiation was observed in cells on scaffolds with expressed FGF-2 or VEGF. Conclusion: Silk scaffolds exhibit excellent cytocompatibility for human pre-adipose cells and have application potential in tissue engineering and regenerative medicine. VEGF and FGF-2 expressed on silk fibers could have a potential positive effect on pre-adipose cells, while the effect of VEGF should be further addressed in vivo.*

Adipose tissue provides a potential biomaterial in plastic reconstructive surgery for filling hereditary or acquired defects of subcutis and other soft tissues. However, transplantation of freshly harvested, unprocessed adipose tissue (1-5) and processed material from liposuctions led to disappointing results (6-8). In most cases, 90-100% of transplanted adipose cells from liposuction will be absorbed (9-14). A major reason for those disappointing results seems to be lack of sufficient neovascularization (15). Without blood supply, adipose cells start to die after 4 days (9, 13, 16, 17).

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One strategy to compensate the loss of adipose cells *in vivo* has been proposed to be the expansion of adipose cells and/or pre-adipose cells *in vitro* on scaffolds prior to implantation (5). Various types of cells, scaffolds and methods have been tested (15, 18-20). Regarding the scaffolds, they should enable sufficient vascularization (21) and facilitate cell expansion and differentiation (5). Furthermore, cytocompatibility and degradation are also crucial (18).

Silk fibroin provides a potential optimal material for scaffold design. It has excellent cytocompatibility and degradation features (22-25). Silk can be easily manufactured into designed forms and density (Figure1A). Furthermore, bioengineering enabled expression of fibroin with covalently bound growth, differentiation or angiogenesis factors, such as the basic fibroblast growth factor (bFGF) and the vascular endothelial growth factor (VEGF). Scaffolds made of silk protein with co-expressed factors may promote expansion and differentiation of cells and may further enhance vascularization *in vivo*.

In the present study, we evaluated silk scaffolds with/without expressed bFGF or VEGF for their biocompatibility and effects in enhancing adipose-differentiation of pre-adipose cells.

Materials and Methods

Human adipose tissue was obtained from subcutis of 25 subjects undergoing plastic surgery in the Department of Oral and Maxillofacial Surgery at the University Medical Centre Hamburg Eppendorf, Hamburg, Germany. The ethical review board of the medical association Hamburg has approved a correspondent study protocol (approval WF-015/12). All patients were informed about the study and have given written consent.

Isolation and cultivation of cells from adipose tissues. The isolation of cells from adipose tissues was performed accordingly to a previous protocol (26). After removing skin, visible blood vessels and connective tissue, the tissue was mechanically dissected and subsequently digested with 1 mg/ml collagenase in Dulbecco's phosphate-buffered saline (DPBS) containing 2% bovine serum albumin (BSA) for 90 minutes at 37˚C under continuous shaking of 180 rpm. The dissociated cells and remaining debris were collected by centrifuging the suspension at 1,200 rpm for 10 min and re-suspended in 9 ml erythrocyte lysis buffer for lysing remaining erythrocytes for 10 min. The suspension was then filtered sequentially through filters of 150 μm, 70 μm and 40 μm pore sizes. Single cells were counted and seeded in Dulbecco's modified Eagle's medium (DMEM)-F12 medium containing 33 μM biotin, 17 μM panthothenate and 10% FBS at 10,000 cells/cm^2 in flasks.

After 16-24 h, the medium was changed to proliferation medium (PM 4) consisting of DMEM-F12, 33 μM biotin, 17 μM panthothenat, 100 ng/ml epithelial growth factor (EGF), 1 ng/ml basic fibroblast growth factor (bFGF), 8.7 μM insulin and 2.5% FBS. Medium was changed in every 3 to 4 days. At subconfluence, cells of one culture flask were split into 2 culture flasks. After 1 or 2 passages, cells were frozen-stored in medium consisting of 80% FBS, 10% dimethyl sulfoxide (DMSO) and 10% DMEM-F12. For each experiment, cells from more than 2 patients were thawed and pooled together, seeded at 20,000 cells/cm^2 in PM-4 and cultured to confluence. All experiments were carried out with cells within the first 2 passages.

Scaffolds. Fibroin with covalently expressed growth factors FGF-2 or VEGF were obtained from transgenic silk worm (27). Scaffolds of 15×3 mm (diameter x thickness) at two densities, 1.5 and 3.0 g/cm^2, were manufactured by Spintec Engineering GmbH (Aachen, Germany).

Cell attachment and differentiation on fibroin scaffolds. Scaffolds were incubated in wells of 24-well plates with basal medium for one day. On the next day, the scaffolds were transferred to new plates with no medium. Cells, 1.5×10^6, in 200 μl medium were added onto each scaffold. Subsequently, 1.5 ml basal medium was added into each well. On the next day, the scaffolds with cells were transferred into wells containing differentiation medium-I consisting of DMEM-F12, 33 μM biotin, 17 μM panthothenat, 0.01 mg/ml transferrrin, 1 nM 3,3',5-Triiodo-L-thyronine sodium salt, 100 nM dexamethason, 450 μM isobuthylmethylxanthin (IBMX), 2 μM rosiglitazone and 66 nM insulin. Three days later, the scaffolds were transferred into wells containing differentiation medium-II, which was identical to differentiation medium I except lacking dexamethasone, IBMX and rosiglitazone. Differentiation medium-II was changed at days 7, 10 and 13. The whole experiment period was 14 days.

Cell viability assay. Cell viability was measured at days 1, 7 and 14 using the non-cytotoxic CellTiter-Fluor Cell Viability Assay (Promega, Mannheim, Germany). To exclude viability from cells attached to the culturing surface, scaffolds with cells were transferred into new wells one day before the assay. For each scaffold, 1 ml of the 1.5 ml medium in the well was removed and 500 μl assay reagent was added. After one hour, the scaffolds were transferred into wells of new plates with fresh medium. The remaining medium in each well was transferred into 3 wells of a 96-plate at 100 μl/well for measuring fluorescence emission; these emission values were normalized using that of 7×10^6 3T3 cells measured under the same condition.

Confocal laser microscopy. Cells were stained with 1% Nile-Red and 5% Fluorescein diacetate (FDA). Confocal laser microscopy was performed with a Nikon A1+ microscope (Nikon, Tokyo, Japan) at excitation of 488 nm and emission of 561 nm.

ELISA. Adiponectin was measured with a DuoSet ELISA kit (R&D Systems, Minneapolis, MN, USA) at days 1, 7 and 14. From each well, 1.5 ml medium was taken and centrifuged. Three hundred microliter cell-free medium from each well were used for ELISA. Values were normalized against viability.

Real-time polymerase chain reaction (RT-PCR). On day 14, at the end of the experiment, medium was removed from the wells. The plates were centrifuged for 2 min at 600 rpm to squeeze out remaining medium from the scaffolds. After completely removing the medium, 1 ml peqGOLD Trifast (peqlab; VWR international GmbH, Erlangen, Germany) was added onto each well to lyse the cells on the scaffold by shaking for 2 min at 300 rpm. The lysates were transferred into tubes. The scaffolds were transferred into 1.5 ml tubes and centrifuged again for 2 min at 13,200 rpm to squeezing out remaining lysates. The total lysates were processed according to the

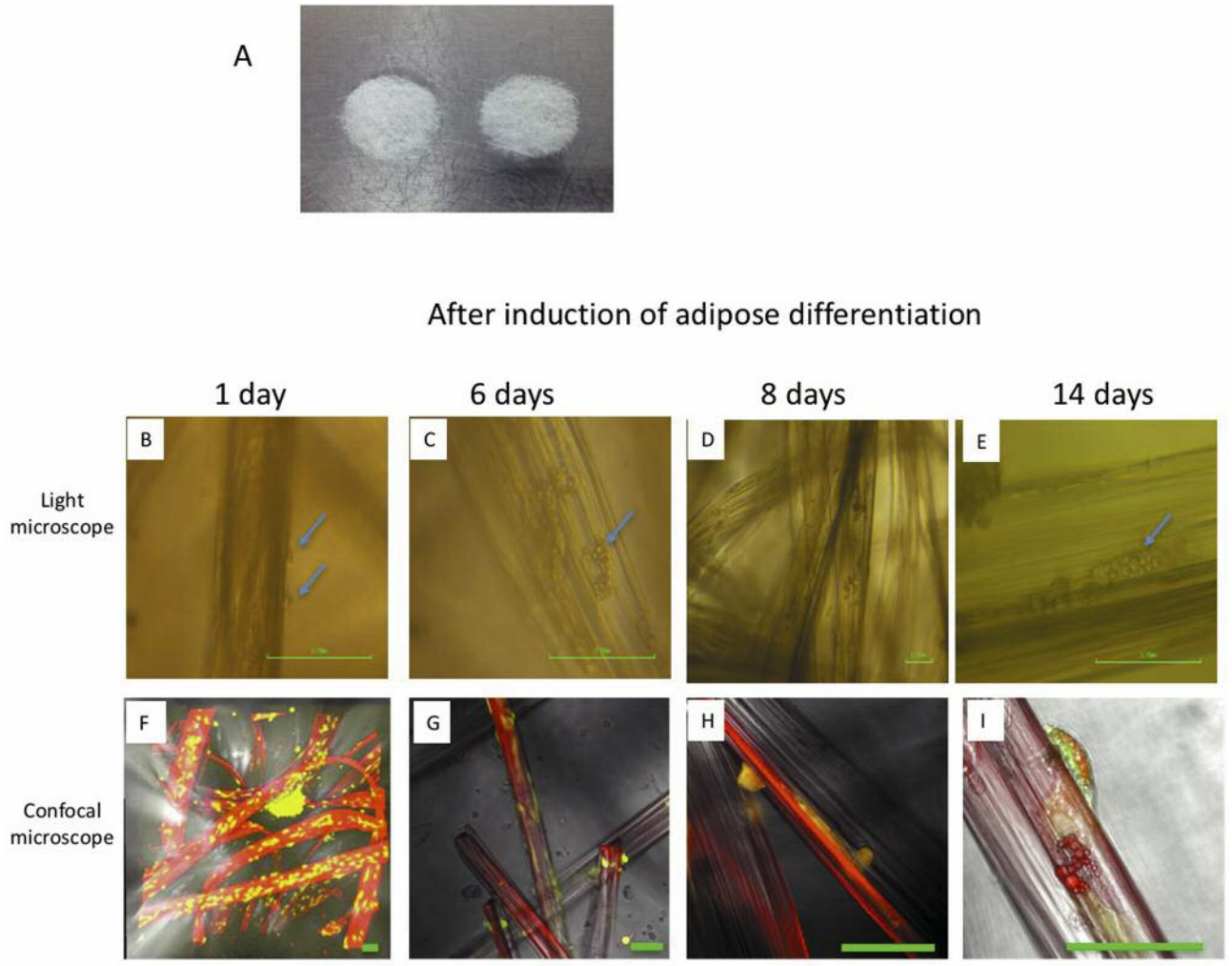

Figure 1. *Silk scaffolds of low- and high-density (A) and pre-adipose cells on the silk fibers under microscope (B-E) and confocal microscope (F-I). Cells were observed on day 1 (B, F), 6 (C, G), 8 (D, H) and 14 (E, I) after start of adipose-differentiation. Scale in confocal images equates to 80 µm.*

manufacturers' instructions. The obtained total RNA was subjected to reverse transcription using a 'high capacity cDNA reverse transcriptions kit' (Life Technologies, Darmstadt, Germany).

RT-PCR was performed using validated TaqMan kits for genes involved in adipodifferention (*hPPARgamma, hCD36, hFABP4, hLPL* and *hSLC2A4*), as well as for the housekeeping gene *hTAF1*. Expression of target genes was normalized to that of the *hTAF1* gene and viability.

Results

Cell attachment, viability and adipose-differentiation. Cells prepared from human adipose tissues attached well to silk fibers in the scaffolds (Figure 1). Six days after induction of adipose-differentiation, oil droplets became visible (Figures 1 C, G). No such oil droplets were visible in cells without induction of adipose-differentiation. These oil droplets continuously increased in number and size, indicating ongoing adipose-differentiation over the entire experiment period of 14 days (Figures 1D, E, H, I). These results also show that the majority of the cells are pre-adipose cells.

Viability of cells on low-density scaffolds increased continuously over the entire differentiation period of 14 days. By contrast, viability of cells on high-density scaffolds remained nearly unchanged (Figure 2A).

In concordance, adipose-differentiation was more profound in cells on high-density scaffolds. This can be seen in oil droplets and measured in the increasing amount of adiponectin (Figure 2B) and in increasing expression of adipo-relevant genes *hPPARgamma, hCD36, hFABP4, hLPl* and *hSLC2A4* (Figures 2C-G). Without induction of adipose-

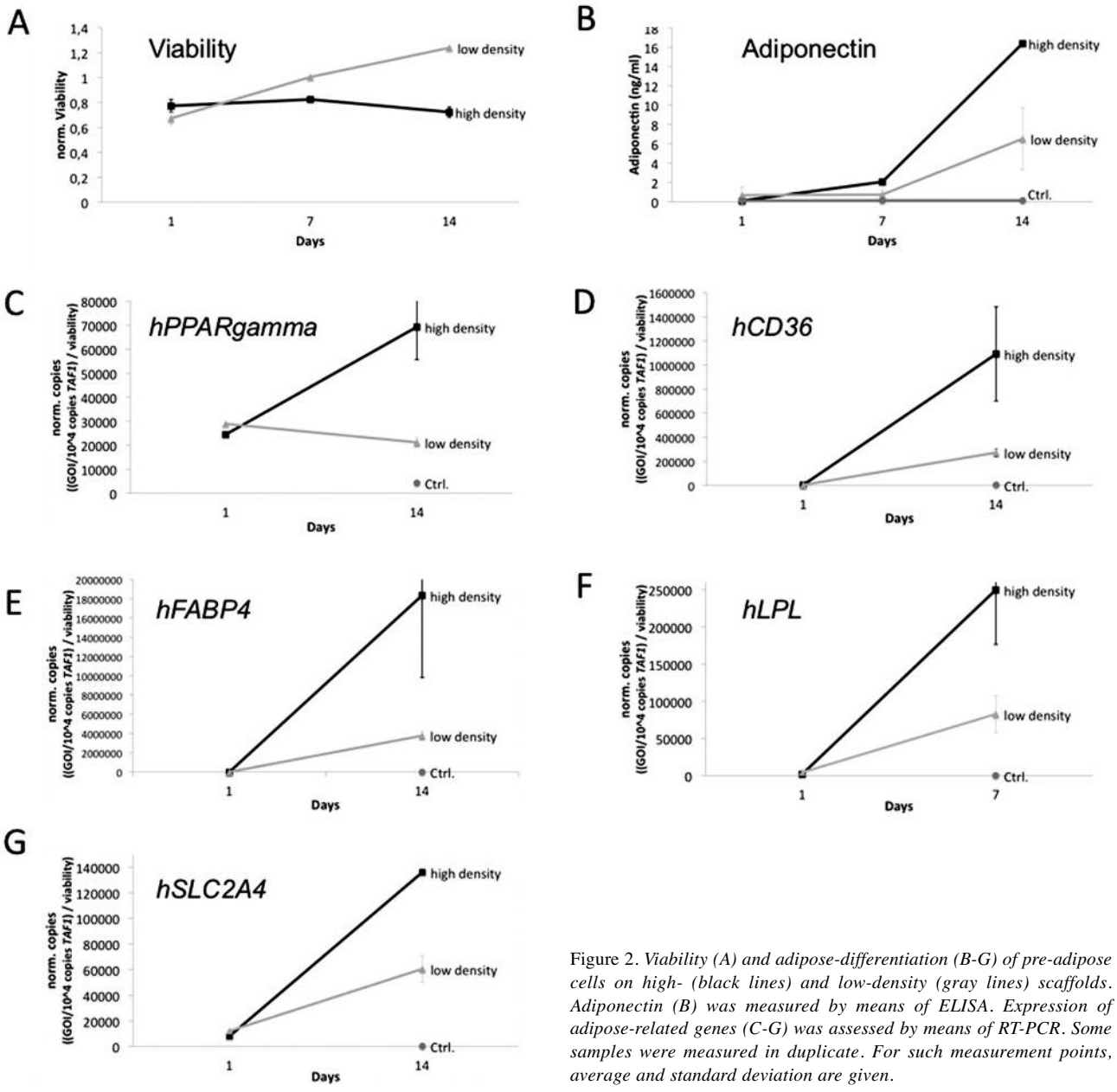

Figure 2. *Viability (A) and adipose-differentiation (B-G) of pre-adipose cells on high- (black lines) and low-density (gray lines) scaffolds. Adiponectin (B) was measured by means of ELISA. Expression of adipose-related genes (C-G) was assessed by means of RT-PCR. Some samples were measured in duplicate. For such measurement points, average and standard deviation are given.*

differentiation, adiponectin remained at basal levels. With induction, adiponectin remained rather low until day 7; it then started to accelerate rapidly (Figure 2B).

Effect of expressed bFGF-2 or VEGF. Both bFGF-2 and VEGF enhanced attachment of cells on low-density scaffolds (Figure 3B). Furthermore, the two factors also enhanced adipose-differentiation of cells on low-density scaffolds (Figure 3D). By contrast, these effects were not seen on cells in high-density scaffolds (Figure 3A, C).

Discussion

We showed that pre-adipose cells can be successfully prepared from all adipose specimens. These cells are unlikely mature adipose cells because they do not generate oil droplets and the level of adiponectin was low. However, the cells have the potential to differentiate into adipose cells since they started to generate oil droplets upon induction of adipose-differentiation. Also, adiponectin increased upon induction. These findings

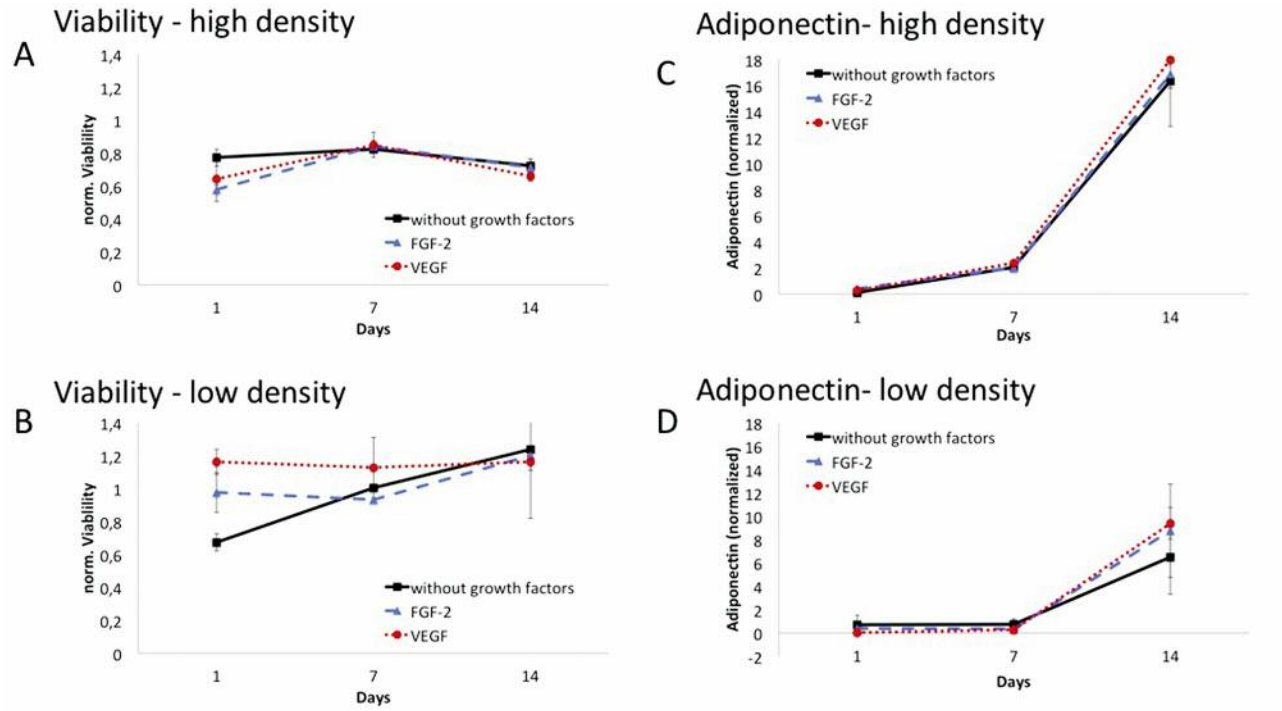

Figure 3. *Effect of expressed FGF-2 or VEGF on cell viability (A, B) and adipose-differentiation (C, D) of the pre-adipose cells. Some samples were measured in duplicate. For such measurement points, average and standard deviation are given.*

suggest that the cells prepared from adipose specimens are pre-adipose cells or they contain large proportion of pre-adipose cells.

In the absence of expressed factors, attachment of cells on high-density and low-density scaffolds was similar. Viability of cells on low-density scaffolds doubled in 14 days, indicating the expansion potential of pre-adipose cells on the silk scaffolds. By contrast, viability in cells in high-density scaffolds did not increase. This can be explained by the more profound adipose-differentiation, which was measured by adiponectin presence and expression of several relevant genes. It is well known that pre-adipose cells cease proliferating when they enter the differentiation phase.

To further address the expansion potential of pre-adipose cells on silk-scaffolds, future studies should maintain the cells for much longer periods of time under culture conditions not inducing adipose-differentiation.

An innovative aspect in our study is the expressed FGF-2 or VEGF in the silk scaffolds, manufactured from transgenic silk worms. Unpublished data (Personal communication with Spintec Engineering GmbH) showed significantly higher proliferation of mouse fibroblasts on silk scaffold with co-expressed platelet-derived growth factor (PDGF), FGF-2 or epidermal growth factor (EGF) than on silk scaffold without such co-expressed factors. Similar proliferating effect on lung epithelial cells was observed for silk scaffold with co-expressed keratinocyte growth factor (KGF). The present study focused on adipose-differentiation, which was not -or only slightly- promoted by the expressed FGF-2 or VEGF.

The limited effect of expressed FGF-2 and VEGF is reasonable since major effects of these factors are expected on proliferation and vascularization. Their effects, especially the effect of VEGF on vascularization, would more likely become detectable when the scaffolds with expanded and differentiated cells are implanted *in vivo*.

Conflicts of Interest

None to declare.

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