ENGINEERING NANO-CELLULOSE SCAFFOLDS TO DIRECT STEM CELL ATTACHMENT, PROLIFERATION AND DIFFERENTIATION

A scientific report for exchange visit grant

INTRODUCTION

Reconstruction of the microtic or damaged auricle represents one of the most challenging problems in reconstructive surgery. Commonly used techniques for ear reconstruction, using an autologous rib graft, have caused large donor site morbidity. Additionally, the complexity of the surgical technique has sometimes provoked an unsatisfactory surgical outcome and is thereby extremely depending on the experience and technical expertise of the surgeon. Tissue engineering techniques could represent an alternative treatment for the reconstruction of damaged or even absent ear cartilage. In regenerative medicine of ear cartilage one aims to prevent the use of rib cartilage with its additional donor site morbidity and tries to simplify the conventional surgical procedure, improving cosmetic outcome.

EAREG, a European multidisciplinary research project, aims to reconstruct the damaged or absent auricle using cartilage regenerative medicine with human cells and a novel nano-cellulose scaffold (figure 1).

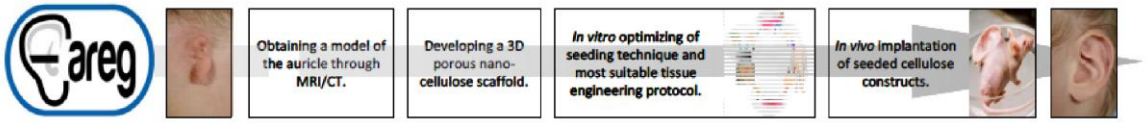

Figure 1. EAREG
Ear tissue regeneration using human cells and novel nano-cellulose scaffolds.

In detail, EAREG is trying to implement a simplified surgical procedure for the reconstruction of ear cartilage using tissue regenerative medicine with novel nano-cellulose scaffolds. Thereby, it wants to improve tissue engineering techniques of ear cartilage to eventually realize regenerative medicine for surgical treatment of ear cartilage defects. Our research group (Erasmus MC), who is participating in EAREG, will mainly work on the choice of the cell source or the combination of cells to tissue engineer cartilage.

Our research partners in Goteborg (Sweden) are involved in the development of bacterial cellulose (BC) scaffolds [1]. This biomaterial will eventually provide a favorable physiological microenvironment as well as a strong and flexible support for our cells. During the development of BC scaffolds, the possibilities to vary the nanostructure of BC scaffolds became clear [2-3]. However, the influence of these engineered BC microenvironments on cell commitment has not yet been studied so far. Cell behavior directed by biomaterial design is on the other hand, a big topic in literature [4-8].

Purpose of the visit

The aim of this visit was to investigate the influence of surface properties of novel engineered BC scaffolds on mesenchymal stem cell fate. Specifically, the ability of cells to attach to the material, proliferate and eventually differentiate into specific lineages (adipogenic, chondrogenic and osteogenic lineage) was examined. These studies concerning the interaction between cells and the BC biomaterial and could provide interesting possibilities for long-term collaboration between the Swedish and Dutch research group.

WORKPLAN

Bacterial Cellulose

Control Bacterial Cellulose (BC) was prepared as described previously [1]. In short, bacterial cultures of *Gluconacetobacter xylinus* were grown in 10 g/L Bactopeptone, 10 g/L yeast extract, 4 mM KH_2PO_4, 6 mM K_2HPO_4 and 20 g/L D-glucose dissolved in deionized water (pH 5.1 - 5.2). The bacteria preculture suspension was added to a glass tube bioreactor (figure 2) and reactors were filled with culture media. Bioreactors were then put into an incubator at 30°C and connected to an oxygen flow of 100 mL/min for seven days.

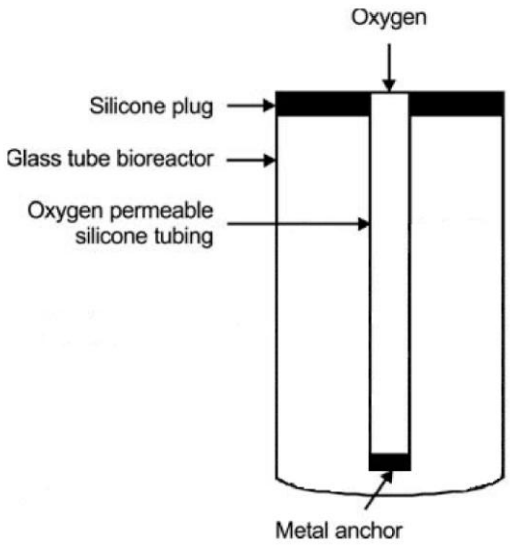

Figure 2. Cultivation of Bacterial Cellulose tubes. A silicone support in the center of a glass bioreactor allows oxygen transport to the bacteria. These will thereby produce cellulose in the presence of the oxygen/media interface.

After incubation, BC was purified, using 4% SDS in deionized water at 70°C for 3 hours followed by 4% NaOH in deionized water at 70°C for 90 minutes. Scaffolds were then rinsed with deionized water and stored at room temperature prior to use.

To examine the influence of biophysical microenvironments on stem cell fate, diverse nano-cellulose structures were engineered by the Gothenburg research group. Finally, the following scaffolds were used:

1. ***(Control scaffold)***
   As described above.

2. ***Porous scaffold A***
   Porous scaffold were fabricated as reported before [2]. The same bacterial preculture suspension was used as for the control BC scaffolds, but instead of using an empty glass bioreactor, this bioreactor was now filled with paraffin micro particles (150-300 µm in diameter). These paraffin micro particles were produced by adding 250 ml 90°C melted paraffin to 350 ml of 90°C 0.5% w/v solution of poly vinyl alcohol in a 2 L beaker agitated by an impeller at 1200 rpm. The emulsion was agitated for 2 min and then rapidly cooled by a hasty addition of cold water. The particles were collected and size sorted in a series of stacked sieves. They were sterilized by immersion in 70% ethanol over night and then frozen to -80°C, lyophilized until completely dry and then stored at room temperature until use.
   After sterilization, paraffin micro particles were put in the glass tube bioreactor, and cellulose was produced around the fused paraffin particles. After incubation, particles

were removed by rinsing the tubes in deionized water. Tubes were then purified as described previously.

3. Porous scaffold B

Porous scaffold A and B were very similarly made except of one step in the preparation process. Specifically, paraffin particles were either powered onto the media surface (porous scaffold A) or mixed through the media solution (porous scaffold B) to create two different porosity morphologies.

4. Stiff scaffold

High cellulose content BC scaffolds were produced by mechanical pressing non-porous normal BC scaffolds (control scaffolds). The pressed pellicles were then placed in an oven at 50°C until they were completely dry and lost 90-95% of their water content. Mechanical characterization was not yet performed.

5. THAMP-treated scaffold

To induce cell attachment, control BC scaffolds were negatively charged.

Cell culture

Bone marrow derived Mesenchymal Stem Cells (bMSCs) were harvested from a femoral shaft biopsy during total hip replacement surgery in our orthopedic department. To isolate stem cells, bone marrow derived heparinized aspirates were seeded at a density of 2-5x10^5 nucleated cells/cm^2 and cultured overnight in expansion medium containing Dulbecco's modified Eagle's medium comprising 1 g/L glucose (low glucose-DMEM [LG-DMEM]) with 10% FCS, 50 µg/mL gentamycin, 0.5 µg/mL Fungizone, 10^{-4} M L-ascorbic acid 2-phosphate and 1 ng/mL basic Fibroblast Growth Factor 2 (bFGF2). After 24 hours, non-adherent cells and cell debris were washed out and adherent bMSCs were further expanded. When cell cultures reached 80% confluence, cells were trypsinized using 0.05% trypsin–EDTA and were further expanded to increase cell number. Second-passage (P2) cells were cryopreserved and send to the Gothenburg research laboratory. In Gothenburg, cells were thawed, and further expanded until they reached subconfluence. Eventually, cells were again detached and seeded on different engineered BC scaffolds.

Cell seeding protocol

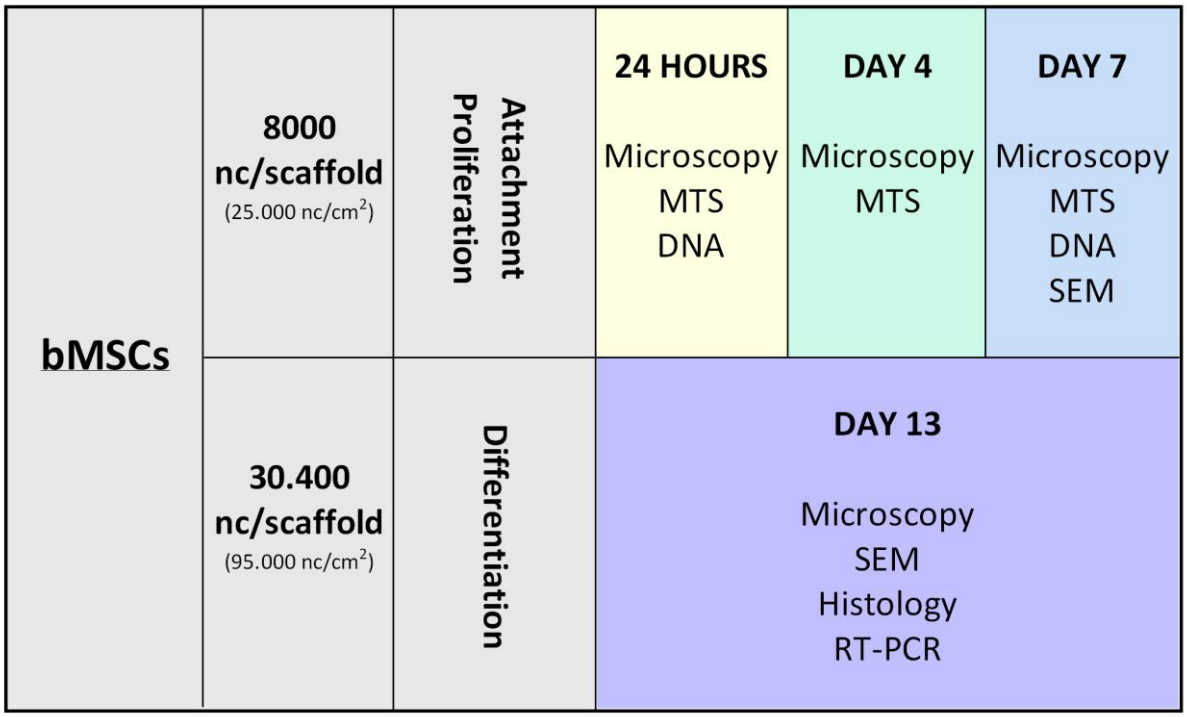

Figure 3. Experimental setup. Five different engineered cellulose scaffold were designed to examine its influence on cell attachment, proliferation and differentiation. (hBMSCs = human bone marrow derived mesenchymal stem cells ; MTS = colorimetric viability assay ; SEM = scanning electron microscopy ; RT-PCR = reverse transcription polymerase chain reaction)

1. Designed scaffolds (0.32 cm^2) were placed in coated-24-well plates. Agarose coating was used to prevent cell attachment on the bottom of the well instead of on the BC scaffolds.
2. To promote cell attachment, scaffolds were immersed in complete culture media (with 10% FCS) for 24 hours prior to cell seeding.
3. Cell seeding. Cells were seeded at a density of 8000 cells (attachment, migration and proliferation) or 30.400 cells (differentiation) per scaffold immersed in a small volume. After an incubation period of 3 hours, expansion medium was added.
4. Culture medium was changed twice a week.

I. To analyse cell attachment and proliferation, expanded cells were cultured for 24h, 4 and 7 days in normal expansion medium (DMEM LG + 10% FCS + Antibiotic/Antimycotica + Ascorbic acid + bFGF). The following analyses were done to examine cell attachment and proliferation on different engineered BC scaffolds (figure 3):

- Microscopy
- Scanning Electron Microscopy (SEM)
- MTS viability analysis
- DNA analysis

II. To analyse stem cell lineage commitment directed by BC biomaterial design, expansion medium was changed in standard differentiation medium after one day of culture (table 1). As a control, cells were also stimulated to differentiate into specific lineages (bone, fat, cartilage), using specific differentiation media (table 1). Furthermore, differentiation of stem cells in monolayer and in a pellet culture system was performed as a control. Analyses were done after a culture period of 13 days. The following tests were done to examine differentiation of stem cells on different engineered BC scaffolds (figure 3):

- Microscopy
- SEM
- Histology
- Gene expression analysis (RT-PCR)

Standard medium	**Chondrogenic medium**	**Adipogenic medium**	**Osteogenic medium**
- DMEM HG - Penicilline/streptavidine - Fungizone - 10% FCS (MSC)	- DMEM HG - Penicilline/streptavidine - Fungizone - Sodium pyruvate - Proline - ITS^{+} ***Add fresh*** - Ascorbic-acid - Dexamethason - TGFβ1	- DMEM HG - Penicilline/streptavidine - Fungizone - 10% FCS (MSC) ***Add fresh*** - Isobutyl-methylxanthin - Dexamethason - Indomethacin - Insuline	- DMEM HG - Penicilline/streptavidine - Fungizone - 10% FCS (MSC) ***Add fresh*** - Ascorbic-acid - Dexamethason - β-Glycerophosphate

Tabel 1. Differentiation media.

RESULTS

Material design

To obtain a clear view of the microenvironments of all five engineered BC scaffolds, Scanning Electron miscopy (SEM) was performed. As can be seen in figure 4, the microstructure of the different BC scaffolds varied. The porous scaffolds (figure 4 [2,3]) showed interconnected pores, but the overall porosity varied to a great extent in different regions of the scaffold. SEM images of stiff BC scaffold (figure 4 [4]) demonstrated a very dense cellulose network. Macroscopically, a flat and compact surface structure was noticed. THAMP-treatment (figure 4 [5]), did not seem to influence the architecture, since THAMP-treated scaffolds looked practical similar to control BC scaffolds (figure 4 [1]).

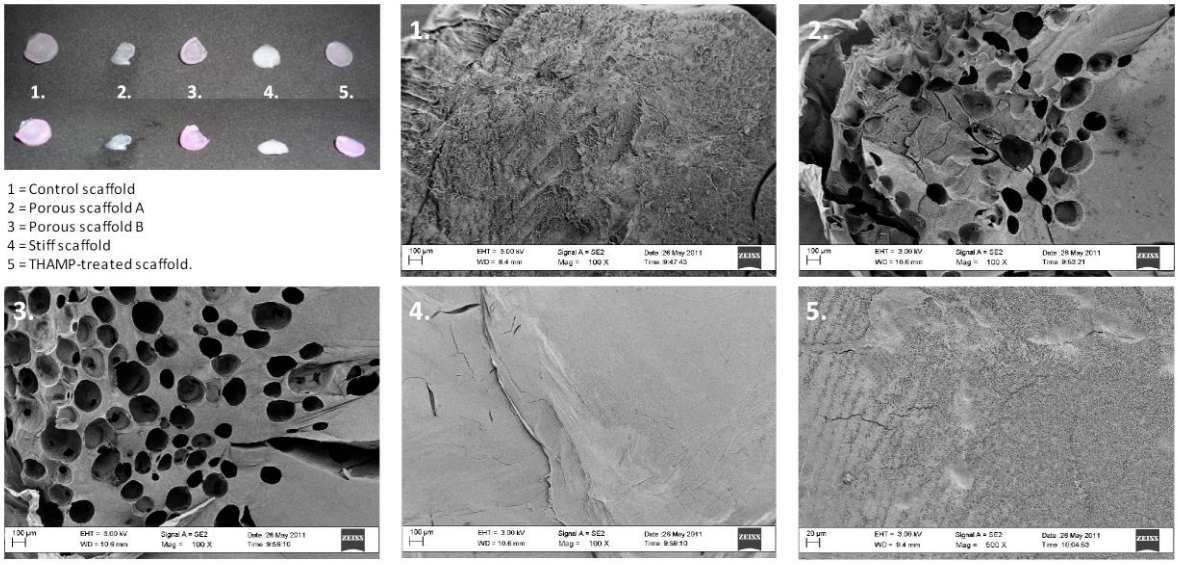

Figure 4. Material design. Note the heterogenecity of the different materials.

Cell culture

Attachment

Microscopic images of stem cell attachment on engineered BC scaffolds are demonstrated in figure 5. Stem cells were able to attach to all engineered BC scaffolds. Nevertheless, THAMP-treatment appeared to improve cell attachment, since more cells seemed to connect to the material (figure 5 [5]). Cells were evenly attached to both porous BC scaffold (figure 5 [2,3]). Some cells were anchored within the pores (figure 5 [3]), although visualization of cells in porous material was not ideal. Unfortunately, visualization of stem cells on stiff BC scaffolds was more or less impossible, due to the dense cellulose network of these scaffolds (figure 5 [4]).

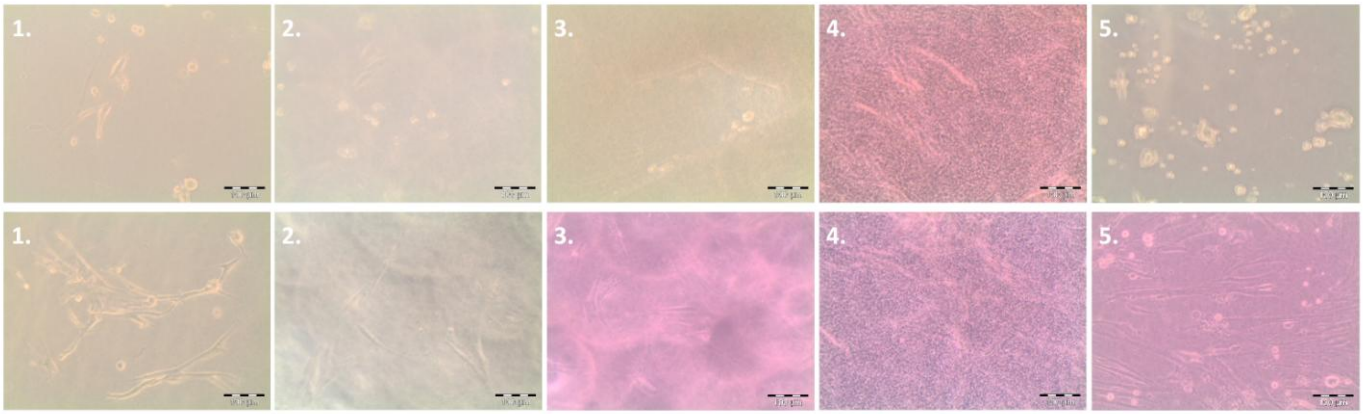

Figure 5. Microscopy . Microscopy after 24 hours and 4 days of culturing. Cells were able to attach to all scaffolds. In time, cells were strechting out and became more fibroblast-like. THAMP-treatment seemed to promote initial cell attachment. Note that a microscopic view was very hard to get from the porous and stiff scaffolds. (1 = Control scaffold ; 2 = Porous scaffold A ; 3 = Porous scaffold B ; 4 = Stiff scaffold ; 5 = THAMP-treated scaffold)

After the initial phase of cell attachment, stem cell phenotype changed towards a more elongated shape in all BC scaffolds. Phenotypic differences between the different BC scaffolds were not noticed.

Proliferation

Viable proliferating cells and cell number as a result of proliferation was examined after 24 hours, 4 days and 7 days of culturing using a MTS viability assay and DNA analysis respectively. Besides proliferation, the viability assay was also able to observe possible cytotoxity of the BC scaffolds.

The MTS viability assay showed an increasing metabolic activity of stem cells in time compared to negative acellular controls (figure 6). Although, a significant difference between the different scaffolds was not observed, stiff BC scaffolds seem to have a higher number of viable cells, indicating a higher proliferation rate. Given the increasing viability in this time frame, cytotoxity of the BC scaffolds was not expected.

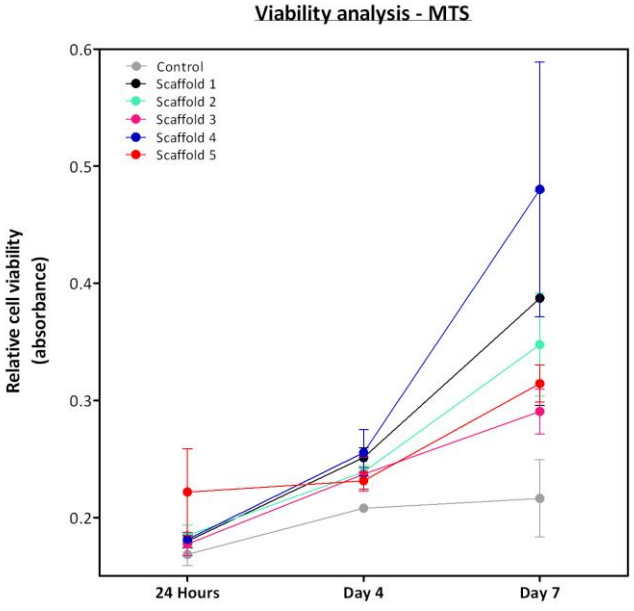

Figure 6. Relative cell viability analysis (MTS). Colorimetric assay for determining the number of viable cells in proliferation. MTS is bioreduced by viable cells into a formazan product and the absorbance of formazan is measured (490nm). The quantity of formazan is directly proportional to the number of living cells in culture (proliferation and cytotoxity). This figure shows an increase of viability in time of cells cultured on five different BC scaffolds. (Control = acellular BC scaffolds ; 1 = Control scaffold ; 2 = Porous scaffold A ; 3 = Porous scaffold B ; 4 = Stiff scaffold ; 5 = THAMP-treated scaffold)

To quantify proliferation, the total amount of DNA was evaluated. After 24 hours and 7 days of culturing, total DNA was determined by Ethidium bromide, using calf thymus DNA as a standard. Samples were further analyzed with a spectrofluorometer, by means of an extinction (340 nm) and emission (590 nm) filter. Afterwards, the total amount of DNA was converted to the total number of cells per scaffold, knowing that one human cell contains around 7.7 pg DNA per cell.

Figure 7 demonstrates the total number of cells on five different engineered BC scaffold after 7 days of culturing. The initial amount of seeded cells is shown as a dotted line. DNA analysis after 24 hours was not possible, due to a very small amount of cells which are not detectable with used DNA detection method (data not shown). Still, after 7 days of culturing, small amounts of DNA were detectable by our DNA assay, making this detection method not sensitive enough for the low amounts of cells on these scaffolds, even after 7 days of culturing. Besides, proliferation could not be quantified, because our 24 hours DNA data was not available. Therefore, increasing cell number could be induced by proliferation, although apoptosis and cell release from the scaffolds were probably also influencing the total amount of cells. However, quantification of proliferation would be nice to illustrate our MTS viability results, but a more sensitive DNA testing technique should be implemented for these kinds of experiments.

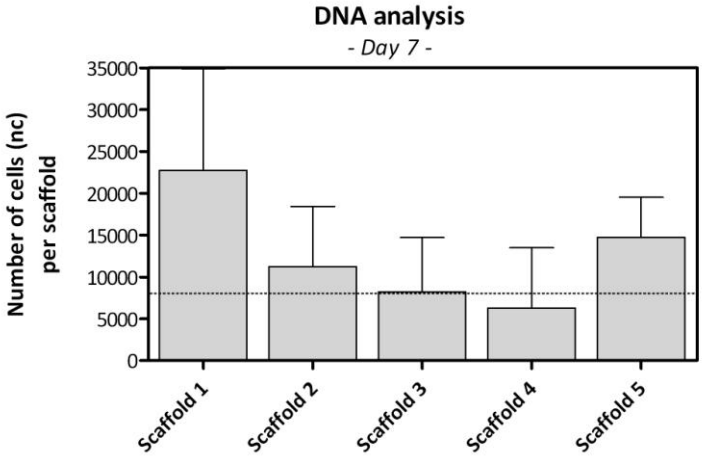

Figure 7. DNA analysis. This figure demonstrates the total number of cells per scaffold after 7 days of culturing. The dotted line indicates the seeding density at T=0. (1 = Control scaffold ; 2 = Porous scaffold A ; 3 = Porous scaffold B ; 4 = Stiff scaffold ; 5 = THAMP-treated scaffold)

Differentiation

The capability of stem cells to differentiate towards a specific lineage influenced by its biophysical microenvironment was examined using Scanning Electron Microscopy (SEM), microscopy, histology and gene expression analysis (RT-PCR).

Visualization of stem cell phenotype with microscopy (data not shown) and SEM (figure 8) after 13 days of culturing in standard differentiation medium, demonstrated nonspecific morphological cell characteristics, distinctive for lineage differentiation (compared with adipogenic, osteogenic and chondrogenic differentiated cells). Lineage commitment of bMSCs after 13 days of culturing was not observed in here.

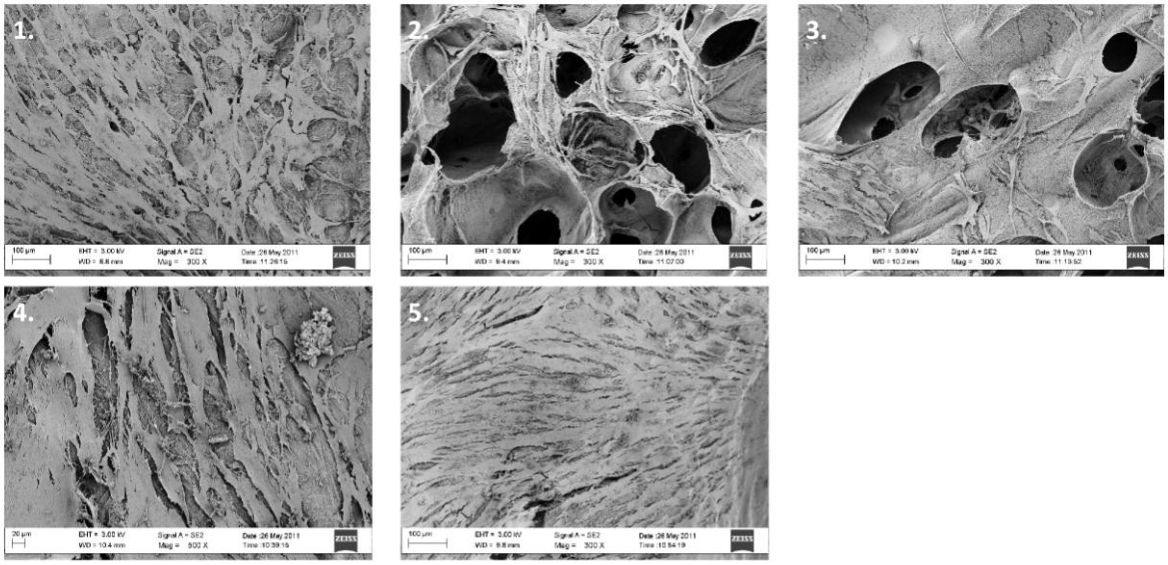

Figure 8. Scanning Electron Microscopy (SEM). Stem cells were cultured for 13 days at standard differentiation medium. Specific morphologic characteristics for lineage differentation were not seen on the different engineered scaffolds. (1 = Control scaffold ; 2 = Porous scaffold A ; 3 = Porous scaffold B ; 4 = Stiff scaffold ; 5 = THAMP-treated scaffold)

To further evaluate lineage differentiation of directed bMSCs into fat, bone and cartilage, samples were analyzed by histological staining of Oil red O, von Kossa and Alcian blue, respectively. Unfortunately, background staining artifacts of the BC scaffolds were too prominent to analyze them (data not shown).

Gene expression analysis (RT-PCR) was used to detect the expression of lineage specific genes. Here fore, RNA was first isolated using 1 mL RNA-Bee™. After extraction, RNA was purified and quantified using the NanoDrop® ND-1000 Spectrophotometer. Because RNA analysis was not yet performed on BC scaffolds before, a pilot evaluation of acellular empty BC scaffolds was done, compared with three cellular stiff BC scaffolds. The total RNA concentration (ng/µL) and an indication of RNA purity are given in table 2. The low ratios of A260/A280 indicated a disappointing purity of our pilot samples. Moreover, the very low A260/A230 ratio (<1) clearly showed low amounts of RNA with an increased risk of sample contamination.

Although the expression level of mRNA varied significantly between the different samples, probably due to low cell number and (bacterial) contamination, gene expression analysis of human glyceraldehyde-phosphate-dehydrogenase (GAPDH) was performed. Figure 9 demonstrates the cycle threshold (Ct) of our samples, representing 3 cellular and 5 acellular empty scaffolds with water as a negative control. Only cellular scaffolds were able to express the gene GAPDH, compared to acellular empty scaffolds and controls. The Ct level nevertheless, indicates a weak RT-PCR reaction, suggestive for minimal amounts of target nucleic acid which could correspond to sample contamination.

In future experiments, cell number must increase. Secondary, contamination of BC scaffolds with (bacterial) DNA/RNA remnants or other contaminants needs to be clarified.

Sample ID	Concentration (ng/µL)	A260	A280	260/280	260/230
Scaffold 4 Cells	77.97	1.949	1.243	1.57	0.17
Scaffold 4 Cells	25.51	0.638	0.435	1.47	0.85
Scaffold 4 Cells	17.34	0.434	0.299	1.45	0.39
Scaffold 1	6.975	0.174	0.130	1.35	0.86
Scaffold 1	40.34	1.008	0.663	1.52	0.14
Scaffold 2	10.42	0.261	0.183	1.42	0.79
Scaffold 2	21.47	0.537	0.371	1.45	0.17
Scaffold 3	21.67	0.542	0.371	1.46	0.23
Scaffold 3	54.94	1.373	0.905	1.52	0.16
Scaffold 4	16.10	0.402	0.284	1.41	0.81
Scaffold 4	40.68	1.017	0.677	1.50	0.18
Scaffold 5	11.26	0.282	0.199	1.41	0.88
Scaffold 5	20.86	0.522	0.349	1.49	0.20

Table 2. Quality evaluation of RNA. To detect possible background noise of BC scaffold, acellular scaffolds were examined and compared with three cellular scaffolds (first three samples). An indication of purity was given using the 260/280 ratio (pure RNA has a 260/280 value of 1.8-2.0). (A260 = absorption at 260 nm; A280 = absorption at 280 nm)

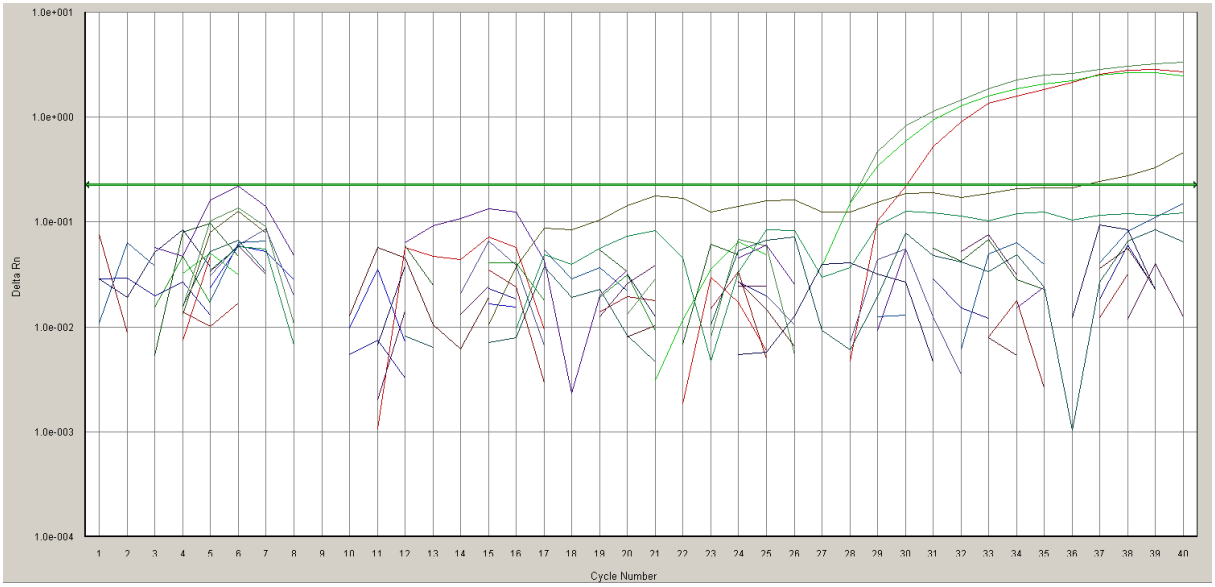

Figure 9. RT-PCR GAPDH. Only cellular scaffolds were able to express GAPDH.

SUMMARY & FUTURE PLANS

Summary

The aim of this visit was to investigate the influence of surface properties of engineered BC scaffolds on mesenchymal stem cell behavior and commitment. For this study five different BC scaffolds were produced by our research partners in Gothenburg, and cell attachment, proliferation and differentiation was analyzed.

Data demonstrated that bMSCs were able to attach to all biomaterials, although THAMP-treatment seemed to improve initial cell attachment. Besides, cells were able to proliferate on all BC biomaterials, without signs of cytotoxity. Although a more sensitive DNA assay would illustrate our data even more. Furthermore, lineage differentiation was not yet seen in these samples after 13 days of culturing, mostly because low amounts of (contaminated) RNA ware extracted from our samples. Illustrated detection methods need be optimized for analyzing BC scaffolds in the future.

Future plans

First attempts to clarify the influence of BC scaffolds on stem cell behavior have made us even more curious. Though cell attachment was seen and proliferation was expected, the influence of BC scaffolds on stem cell differentiation was not yet clarified. For future experiments it would again be interesting to examine cell behavior and specifically lineage differentiation directed by different engineered BC scaffolds.

Increasing cell number

To increase cell number, BC scaffolds need to increase in size. Furthermore, we would not prefer to work with porous scaffold, because these scaffolds have a three dimensional structure, which provides a large surface area and thus more cells per scaffolds are needed in comparison with two dimensional constructs (control stiff and THAMP-treated scaffolds).

Extension of differentiation period

In this pilot study, cells were only differentiated for 13 days. Extension of the differentiation period to 35 days is preferable, because commitment to specific lineages is easier to visualize after a longer time phase.

Optimizing analyzing protocols

Because background staining artifacts of the BC scaffolds were too prominent to analyze them, optimization of the histological staining would consolidate our data. The same goes for our DNA assay, which was not sensitive enough for the low amounts of cells on the scaffolds after 24 hours and 7 days of culturing. A more sensitive DNA analysis would really illustrate our MTS results and would quantify proliferation rate. Last, contamination of BC scaffolds with (bacterial) DNA/RNA remnants or other contaminants needs to be clarified.

Proposal new experiment

Scaffold	Specification		Proliferation	Differentiation	Mechanical tests?
Scaffold 1 **Scaffold 2** **Scaffold 3**	Control Stiff THAMP	Increase scaffold size and differentiation period (35 days)	MTS DNA	RT-PCR Histology	Zurich?

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