Tissue engineered uterine tissue supports pregnancy in a rat model

Degree project thesis in Medicine

Student: Sara Bandstein

Supervisor: Mats Hellström

Laboratory for Transplantation and Regenerative Medicine, Department of Obstetrics and Gynecology

UNIVERSITY OF GOTHENBURG

Programme in Medicine

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Abstract

Background: Absolute uterine factor infertility affects about 1:500 women in fertile age. This can now be cured by uterus transplantation, a procedure that involves risky donor surgery and side effects of immunosuppression. The creation of a tissue-engineered uterus/uterine tissue using the patient’s own stem cells would circumvent these issues.

Objective: To create a tissue engineered uterine patch for repair of a partially defect uterus.

Methods: Three different decellularized uterine scaffolds were recellularized in vitro with primary uterine cells and mesenchymal stem cells (MSCs). The patches were transplanted in vivo to investigate their tissue adaptation and supporting capacity during pregnancy. Recellularization efficiency and graft quality were analyzed morphologically, immunohistochemically, and by real-time quantitative polymerase chain reaction (qPCR). The location and number of fetuses were documented during embryonic day 16–20.

Results: Pregnancy and fetal development were normal in groups P1 and P2, with fetal development over patched areas. Group P3 showed significant reduction of fetal numbers, and embryos were not seen in the grafted area. No placentation was formed over the patch areas in any of the experiment groups. qPCR and immunohistochemistry revealed uterus-like tissue in the patches that had been reconstructed by infiltrating host cells after transplantation. No mesenchymal stem cells remained in the grafts.

Conclusions: Primary uterine cells and MSCs can be used to reconstruct decellularized uterine tissue, and the tissue engineered uterine patch can be used to support pregnancy in a partially defect uterus. The bioengineered patches made from triton-X100+DMSO-generate scaffolds were supportive during pregnancy. These protocols should be explored further to develop suitable grafting material to repair a partially defect uteri and possibly to create a whole bioengineered uterus for a uterus transplantation.

Key words: Decellularization, recellularization, tissue engineering, uterus
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Introduction

Multiple diseases and traumatic injuries could result in chronic impairment of tissue function and end-state organ failure. Since the human body has limited regenerative capacity, allogeneic transplantation is often the only definitive medical treatment when this has occurred (1, 2). However, there are a number of limitations related to this procedure such as the risk of rejection by the recipient's immune system and the adverse effects related to the use of immunosuppression. In cases where a live donor is used, there are also surgical risks for the donor, who in general is a healthy individual. For these reasons, alternative sources for organ and tissue development are needed (2, 3).

Novel and promising concepts for functional organ or tissue replacement have emerged within the field of tissue engineering (1, 3-5). Tissue engineering involves several steps, from the development of a template, or a scaffold, to the reconstruction of the needed tissue using various cell sources. The scaffold could be synthetic or biologically derived and should serve to provide structural support for the added cells and aid cell proliferation and differentiation into an appropriate tissue specific cell faith. A normal organ may contain hundreds of millions, or even billions of cells, thus the required cells need to be expanded to vast numbers and the engineered constructs need to be kept in advanced perfusion bioreactors or grown ectopically in vivo to be finalized prior to the clinical application (3).

Decellularization and recellularization

Scaffold generation has received much attention in the past years, in particular biological scaffolds since they to a greater extent mimic the native organ mechanically, geometrically and biologically (4). A biological scaffold can be obtained by decellularization, a process where cells are removed from a normal donated organ, which leaves a framework of tissue specific three-dimensional extra cellular matrix (ECM). The ECM provides an organ-specific tissue architecture with preserved vascular conduits. It also contains molecules, mainly type I collagen, glycosaminoglycans, fibronectin, laminin and a diverse variety of growth factors with tissue specific composition. These molecules provide signals for cell aggregation, migration, proliferation and differentiation for that specific tissue (4-7).

Decellularization can be achieved by flushing the organ with detergent solutions and/or by physical methods such as freeze thawing (4, 5). However, many of these detergents are non-selective and can damage ECM elements, particularly collagen, glycosaminoglycans and growth factors (8). It is important to find a balance between an
aggressive enough decellularization process while maintaining the microenvironment intact. It also matters how the detergents are delivered to the tissues (5, 9).

When an acellular scaffold has been created, it could be implanted directly in vivo to recruit repopulating endogenous cells from the host or (more commonly) cells can be integrated in the scaffold prior to transplantation. One of the biggest challenges in tissue engineering is to find an appropriate cell source for repopulation. For whole-organ engineering, an ideal cell type is one that can proliferate as needed and give rise to all cell types necessary for the particular organ to be regenerated, including the parenchyma, the vasculature and all supporting structures. For these reasons, many stem cells and progenitor cells have been evaluated (4).

To date, embryonic and mesenchymal stem cells are the most prevalent cell types used for recellularization (2). However, it is not only the choice of cells that matters for tissue engineering results. Recellularization requires optimal culture- and cell delivery methods. There are two main methods for cell delivery, perfusion of cells through the vasculature or the injection of cells into the scaffold using a syringe. Perfusion would be the choice in order to reach the vasculature, whereas injection of cells targets the parenchyma more directly.

Progress in tissue engineering

The field of organ tissue engineering is still in its infancy and many challenges remain before the development of complex parenchymal organs has been established. Modest steps have been made in small animal organs where rudimentary in vivo function and maintained patency have been achieved for limited time (10-14). For example, tissue engineered rat livers have shown maintained hepatocyte viability and metabolic function as well as hepatocytes that to some extent produced liver specific proteins (9, 12). In vitro results of macroscopic contractions and pump function of a decellularized rat heart have also been established, describing that cardiovascular progenitor muscle cells could migrate, proliferate and differentiate into heart cells (11). Human umbilical vein endothelial cells have been used to recellularize rat kidney scaffolds that developed into podocytes and initiated the formation of foot processes (14).

The creation of tissue parts is less complicated and has in some areas been applied clinically. One such example is in vitro engineered skin grafts that has been a clinical success (15). In recent years, researchers have clinically tested tissue engineered constructs of
relatively simple hollow structures such as urogenital tissues (16), blood vessels (17, 18) and trachea (19, 20), some with very questionable outcomes.¹

**Uterus transplantation and uterus tissue engineering**

Absolute uterine factor infertility (AUFI) due to dysfunction or absence of the uterus was until recently an untreatable condition affecting about 1:500 women in fertile age (22). In 2014, the first successful human uterus transplantation (UTx) was performed using a live donor (23). Later from the same clinical trial the world’s first child was born from a UTx patient (24) providing a final cure to AUFI. However, uterus transplantation involves the same problems as transplantation of other organs such as risky donor surgery and side effects of immune suppression. The creation of a tissue engineered uterus, using the patient’s own stem cells would circumvent these issues. Tissue engineering uterine tissue is not a novel idea, and earlier reports include the creation of uterine tissue scaffolds derived from collagen (25-27), collagen-silk (28), boiled blood cloths (29) or from biodegradable polymer scaffolds (16, 30), including decellularized uterine tissue segments (31, 32).

Although important and instructive, neither of these published protocols was made to replace a donor in a UTx setting. Instead, they were created to repair a partial uterine defect caused for example by resection of placental tumors or adenomyomectomy. A tissue engineered uterine patch may in these cases be used to increase the strength of the uterine wall in a situation of pregnancy. However, recently the creation of whole uterus scaffolds by decellularization was reported using the rat as an experimental animal (33, 34). These studies provide a platform on to which novel whole uterus tissue engineering experiments can be developed.

**Objective and study outline**

As a step towards the development of a whole tissue engineered uterus, the objective of this study was to create a tissue engineered uterine patch for repair of a partially defect uterus.² Specifically, the aim of the study was to investigate whether the tissue engineered uterine tissue derived from three different decellularization protocols could be transplanted, and if

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¹ For several years it was believed that a bioengineered trachea, made from a cell-seeded semi-synthetic scaffold, was successfully transplanted in human patients by Macchiarini and his colleagues (19, 21). Recently, the results of these studies have been questioned and some of the authors are currently under investigation. Berg et al (20) published a study of a tissue engineered human trachea that was transplanted to cure tracheal stenosis. Due to irregularities regarding some of the published images, the study was recently withdrawn.
² Since the study was carried out by a research group, this master thesis only covers some parts of the overall study. The parts carried out by other group members are clearly noted in the text.
any of the constructs could support a weakened uterus wall during pregnancy in the rat. The study focused on some key aspects:

- The recellularization success of three different decellularized scaffolds
- The functionality of the three recellularized scaffolds *in vivo*
- The faith of the transplanted mesenchymal stem cells

Fig 1. *Study outline.* The current study builds on previous work published by Hellström et al (34), which reported the production of three decellularized uterine scaffolds using three different protocols, P1-P3. These protocols were used to generate the scaffolds used for the current study (A). Isolated primary uterine cells and commercially obtained green fluorescent protein labeled bone marrow derived mesenchymal stem cells (GFP-MSCs) were expanded *in vitro* (B) and then used to recellularize scaffold patches cut out from the uterine scaffolds (C). The patches, together with the cells, were cultured *in vitro* for three days (D) and were then transplanted *in vivo* to repair a defect uterus wall (E). The tissue adaptation and supporting capacity during pregnancy were investigated.
Methods and materials

Animals
Whole-uterus decellularized scaffolds were obtained from female Lewis rats (n=9; 140-180 g, Charles River, Sulzfelt, Germany) using three different protocols (n=3 per protocol) (34). Female Sprague Dawley (SD) rats (140-180 g, Janvier Labs, Janvier, France) were used for isolation of primary uterus cells (n=10) and as recipients of the recellularized patches (n=30). Male SD rats (n=12; 250g - 300g, Janvier Labs, Janvier, France) were used for mating. The study was approved by the Animal Ethics Committee in Gothenburg, Sweden.

Uterus isolation and scaffold decellularization
Hellström et al 2014 (34) describe in detail the isolation and decellularization processes of the uteri. In the current study, decellularization of the uteri was performed according to the three protocols (P1-3) but with the modification that sodium azide was omitted in all solutions. Sodium azide was removed due to its cytotoxic effects that potentially could reduce the recellularization efficiency of the scaffolds. Group P1 and group P2 were decellularized by sequential uterine perfusions for 4h with dimethyl sulfoxide (DMSO; 4%) and then for 4h with Triton-X100 (1%). This was followed by 16h of washing in PBS (group P1) or in dH2O (group P2). For group P3, perfusion was performed using a 2% sodium deoxycholate solution (SDC) for 6h, followed by 18h of washing in dH2O. For group P3, perfusion was performed using a 2% sodium deoxycholate solution (SDS) for 6h, followed by 18h washing in dH2O. These cycles were repeated five times, and on the fifth day, a sterilization process was carried out using per-acetic acid (0.1%) for 30min. After several washes (PBS), the decellularized uterus was frozen (-80°C) for long-term storage. DNA quantification was performed on the decellularized rat uteri.

Uterus primary cell isolation and cell culturing
Two different sources of cells were used for recellularization: uterus primary cells and green fluorescent protein labeled mesenchymal stem cells (GFP-MSCs). To isolate uterus primary cells, 10 SD rats were sedated with 5% inhaled isoflurane, shaved and sprayed with 70% ethanol before they underwent hysterectomy. The excised uteri were placed in PBS on ice before each horn was opened longitudinally and placed in a culture dish containing digestion solution (collagenase, 2mg/ml; DNase I, 0.5μl/ml; 10mM HEPES; 1xAnti-AntiTM; in DMEM; Life Technologies, Stockholm, Sweden) for 1h at 37°C. The

3 These steps were carried out by Hellström and Akouri
endometrium was scraped off the myometrium and put in EM medium (DMEM+10mM HEPES+1xAnti-Anti+10% FCS; Life Technologies). The endometrium cell suspension/tissue was centrifuged and the resulting pellet resuspended in fresh EM medium and then pelleted again by centrifugation. New digestion solution was added to the pellet (10ml/g endometrium tissue), which was resuspended and incubated in a shaker at 37°C for 90 min. The myometrium tissue was minced into pieces of less than 1mm³ and centrifuged, and then the pellet was weighed and resuspended in a digestion solution (10ml/g tissue). The suspension was incubated for 4h on a shaker (37°C). After the enzymatic digestion, endometrial and myometrial cells and cell clusters were filtered through a sterile 40μm cell strainer. The cells and smaller aggregates passed through the strainer and the larger fragments were further dissociated by pipetting them in a solution containing 0,25% Trypsin+25μg/ml DNase I. The single cell mixture was then centrifuged and resuspended in EM medium and plated on collagen-I coated 6-well plates (Life Technologies, Stockholm, Sweden). The cells were incubated in a humidified chamber at 37°C and 5% CO₂ for 6 days, and fed twice with EM medium.

The GFP-MSCs were commercially purchased and originated from SD-rats (Cyagene Bioscience, CA, USA). These cells were cultured according to manufacturer’s protocol (Cat.No. RASMX-01101). Briefly, 1 vial (500,000 cells) of GFP-MSCs were quickly thawed in a water bath (37°C) before the cells were transferred to a Falcon conical tube (15ml) together with 9ml pre-heated (37°C) OriCell™ MSC Growth Medium. These were centrifuged at 250 g for 5 minutes and the cell pellet resuspended with fresh medium, before transferred to a Falcon T75 culture flask with 10ml growth medium. The flasks were incubated at 37°C inside a 5% CO₂ humified incubator and fed every 3-4 days with growth medium. When cells were 80-90% confluent, they were dissociated with pre-heated (37°C) Trypsin-EDTA solution (0.25% Trypsin-0.04% EDTA), and when visibly detached, serum containing growth medium (6ml/flask) was added to neutralize the trypsinization. The dissociated cells were transferred to a conical tube (15ml) and centrifuged at 250 x g for 5 minutes. The supernatant was removed and the cells were resuspended in 5 ml fresh growth medium before split at 1:5 in new T75 flasks. More growth medium was added to each culture flask and the cells were incubated at 37°C in a humidified incubator with 5% CO₂. These steps were repeated to expand the total cell numbers and cells with a passage number below 8 were used for the experiments to prevent cells from losing their true phenotype (that may happen after an extended time in vitro).
Recellularization of patches
Three uteri from each decellularization protocol were thawed and washed. Thereafter, six rectangles per protocol were cut out from the scaffolds (5x20mm) and used for the recellularization and transplantation studies. The remaining scaffold pieces were analyzed. The two cell sources described above were used for the recellularization of the scaffold rectangles. SD-rat primary uterus cells (5x10⁴) were mixed with SD-rat GFP-MSCs (7.25x10⁶), and a total of 7.3x10⁶ cells were added to each patch (5x20mm) by multiple injections using a 27G syringe. Due to a low primary uterus cell yield, the cell ratio used for the recellularization was 1:150 of primary uterine cells to GFP-MSCs. About 14x10⁶ cells were not injected to the scaffolds, but were instead collected in RNALater (Qiagen, Sollentuna, Sweden) and used for qPCR analysis. After recellularization, the patches were cultured for 3 days in a 5% CO₂ humidified incubator and fed every day with EM medium. Each patch was then cut in half; one part was used for pre-transplantation analysis (whole tissue mounts for confocal microscopy, histology and qPCR) while the remaining half (5x10mm) was used for in vivo transplantation studies.

Patch transplantation, mating and graft retrieval⁴
The transplantation and mating processes have been described in detail in Hellström et al (35). In brief: for each individual rat that received a bioengineered construct (n=18), a 5x10mm segment of the uterus horn was surgically removed in order to create a uterus defect. A recellularized bioengineered patch was then used to repair the injury. As control groups, six animals underwent a similar segment removal but the uterine tissue was sutured back again (n=6; auto-transplantation) and six animals were not operated at all and were used as mating controls. In total, 30 animals were used (18 patch transplanted, 6 auto-transplanted and 6 non-operated).

All animals were mated six weeks after transplantation. 16-20 days after mating and just before full-term; the experiment was terminated to assess graft condition, the number of fetuses and their location. Grafts were located in all transplanted animals, except in one animal of group P1, which therefore was excluded from further analysis. In all other animals, the graft was isolated free from surrounding uterine tissue. A biopsy was taken from each graft and placed in RNALater for gene expression analysis. The remaining piece was placed in a preservative (Histocon®; Histolab, Gothenburg, Sweden) for 12-18h before frozen in optimal cutting temperature (OCT) media and used for immunocytochemistry. One longer uterus segment containing a fetus/degenerated fetus and the whole graft was

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⁴ These steps were carried out by Hellström and Moreno-Moya
isolated for one animal per group and placed in 4% formaldehyde for 24h, then further processed for paraffin embedment and histological analysis.

**Histology and immunocytochemistry**
For the histological analyses, the longer uterus-graft-fetus segments from each group were fixed in formaldehyde, and then dehydrated in increasing concentrations of ethanol followed by xylene, and then embedded in paraffin blocks. Sections were then cut at 3µm in a microtome and processed for haematoxylin and eosin (H&E) staining as following: Sections were de-waxed in xylene baths x2 and rehydrated in ethanol baths (100%-70%), before put in Haematoxylin (Mayer’s) for 15 min. After 15 min of washing with H₂O, 3 drops of concentrated ammonia was added to 225mls of water and the slides were dipped in this solution to increase the staining (“blueing”). The slides were then washed for another 5 min in water and then put in an Eosin bath for 20 sec. Finally, the sections were washed rapidly 2 times in dH₂O, then dehydrated in several ethanol steps (70%-100%) and then finally in xylene. They were then cover slipped using DPX mounting media.

For immunohistochemistry, cryosections (7-11µm) of acellular scaffolds, pre-transplanted recellularized patches and isolated transplanted patches were prepared and stored (at -20°C). After removal from the freezer, slides were air-dried for 15 min at room temperature and rehydrated for 3x5 min in PBS. Sections were then fixed for 10 min with 4% formaldehyde before blocking buffer (0.2% Triton X-100 and 10% normal goat serum in PBS) was added for 60 min. Selected slides were stained with primary rabbit or mouse anti-GFP antibody for 60 min at room temperature as follows: mouse anti-e-cadherin (epithelial cells, 1:200; ab76055; Abcam, Cambridge, England), mouse anti-vimentin (stromal cells, 1:200; MA1-19168; Thermo Scientific, Stockholm, Sweden), rabbit anti-SMA (smooth muscle cells, 1:300; ab5694; Abcam), Ki67 (proliferation marker, 1:100; ab16667; Abcam) and cleaved caspase-3 (apoptosis marker, 1:300; 9661S; Cell Signaling, Stockholm, Sweden). Thereafter sections were washed in PBS for 3x5 min and incubated for 1h with an anti-mouse or anti-rabbit Cy3-conjugated secondary antibody (1:300; A10520/A10521; Life Technologies). The sections were then washed for 3x5 min in PBS and DAPI stained (1 min, 25µg/ml DAPI in PBS). Finally they were cover slipped with Dako fluorescent mounting media.

**RNA/DNA quantification and gene expression analysis (qPCR)**
The RNA and DNA were isolated from tissue samples (8,9mg -30,3mg) at various steps along the experimental process using two different kits from Qiagen. All tissue samples
were homogenized individually in 2 ml micro centrifuge tubes containing 1 stainless steel bead and 350 μl Buffer RLT mixed with 10 μl β-Mercaptoethanol/1 ml Buffer RLT (Qiagen) using a TissueLyser II (Qiagen) for 5 min at 30 Hz. The homogenate was transferred to new tubes and centrifuged for 2 min at full speed (15000 x g). The supernatant was transferred to an AllPrep DNA spin column (Qiagen) that was placed in a 2 ml collection tube and then centrifuged for 30 s at 8000 x g. The spin column was put in new collection tube and used for DNA purification and isolation according to manufacturer’s instructions (AllPrep DNA/RNA Mini Handbook, steps 14-17). The flow through was used for RNA purification following the RNeasy®Micro Kit step 2-9 (Qiagen). DNA and mRNA content was measured (NanoDrop) and used for cDNA conversion (high capacity cDNA reverse transcriptase kit; Applied Biosystems, Thermo Scientific).

Quantitative PCR (qPCR) reactions were performed using Applied Biosystems 7500 Fast Real-Time PCR System and Taqman probes. 10 μl sample and 10 μl PCR mix (2 μl RT Buffer, 0.8 μl dNTP Mix (100 nM), 2 μl RT random primers, 1 μl reverse transcriptase, 4.2 μl Nuclease free H2O) were mixed. Two reference genes were tested: GAPDH (Rn01775763_g1) and ACTB (Rn00667869_m1). GADPH was selected for all analyses after confirmation that ACTB gave similar expression patterns. Expression levels were measured for the following genes: CDH1 (E-cadherin; epithelial cells; Rn00580109_m1), Vimentin (stromal cells, Rn00667825_m1), ACTA2 (alpha smooth muscle actin; myometrium cells; Rn01759928_g1), vWF (von Willebrand Factor; epithelial cells; Rn01492158_m1), COL1A1 (collagen 1a1; Rn01463848_m1), BCL2 (B-cell lymphoma 2; proliferation marker; Rn99999125_m1), HOXA11 (uterine tissue development; Rn01410200_m1), ESR1 (estrogen receptor 1; Rn01640372_m1), PGR (progesterone receptor; Rn01448227_m1) and eGFP (cells used for recellularization; Mr04097229_mr). Expression levels were quantitatively analyzed using the 2ΔΔCt method and were relativized to control pregnant rat uteri.⁵

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⁵ Calculations were made by Moreno-Moya
Results

Decellularization
DNA quantification was performed on the decellularized uteri, which revealed that the DNA content of the scaffolds in groups P2 and P3 was below detection level, whereas 18% of the original DNA remained in scaffolds from group P1.

Recellularization
Three days after recellularization with uterus primary cells and GFP-MSC cells, the cell density of the patches was limited (fig. 2A-C). In all three groups, cells were mainly located on the surface or in isolated cell clusters within the scaffolds (fig. 2A-B). In particular on the surfaces of the recellularized patches, it was obvious that there were great differences between the scaffolds’ ability to support cell growth.\(^6\) With the aid of confocal z-scans and automated software, the GFP positive cell coverage was established on the various scaffolds. There was significantly larger cell coverage on the perimetral side of P1-generated scaffolds compared to that of groups P2 and P3, and there was a similar trend (but not significant) on the luminal side (fig. 2C).

Immunocytochemistry using antibodies for the proliferation marker Ki67 and for the apoptotic marker cleaved caspase-3 showed very few positive cells (data not shown), preventing further analysis on proliferation and apoptosis patterns.

Fig 2: Recellularization of scaffolds in vitro pre-transplantation. (A) Cross section and (B) surface view showed a limited cell distribution of the scaffolds in vitro. Most cells were located on the scaffold surface area or in isolated cell aggregates (A). Maximum projection images from the luminal and perimetrial surface of the scaffold (B) were used to analyze cell density (C). As the graph (C) shows, the cell density of group P1-scaffolds was significantly higher on the perimetrium side compared to groups P2-P3-scaffolds. There was a similar trend on the luminal side (*P<0.05).

\(^6\) Due to time-limitations of this master thesis project, the quantification supporting these observations was performed by my supervisor and can be viewed in figure 2C.
Transplantation and pregnancy results

Results showed that the transplanted patches from all groups were well integrated with the host uterine tissue and macroscopically showed no obvious sign of tissue degeneration. The total number of pregnant animals was similar in all groups, as shown in the table below. Of the operated rats, 4-6 animals in each group became pregnant; a result comparable to non-operated rats of which 5 became pregnant. However, the number of fetuses at the transplantation sites differed between groups. In group P3, no fetuses were found over the patch areas, whereas fetal development occurred in the uterine area including the graft and adjacent host uterine tissue in two animals in each of the other groups (autografts, P1 and P2). For group P3, only signs of degenerated fetal tissue was found in the grafted area.

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of pregnant rats</th>
<th>Fetuses at transplantation site</th>
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<tbody>
<tr>
<td>P1</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>P2</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>P3</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Autograft</td>
<td>4</td>
<td>2</td>
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Table 1: Number of pregnant rats in total in each group (P1-P3, autograft) and number of fetuses at transplantation site in each group.

Histological analyses

H&E-staining showed that graft morphology to some extent resembled normal pregnant rat uterus with myometrium- and epithelial-like structures (fig 4B-C, G-H). However, no placentation was formed directly over the patched area (Fig. 4A, E) and some areas of the grafts contained large amounts of infiltrating cells (fig 4E), most likely lymphocytes or other cells of the immune system (fig. 4B). Some areas of the P2-derived grafts showed signs of angiogenesis and infiltrating immune cells (4G).
Immunohistochemistry

Immunohistochemistry showed that the grafts contained a large amount of cells, although with less density and organization compared to cells in normal pregnant uterus tissue (figure 3). Constructs from group P1- and P2 generated constructs appeared more homogenous and organized than P3 generated constructs (Fig. 3B-D, F-H). No GFP labeled cells remained in the grafts, which suggests that the majority of the present cells were infiltrating host uterine cells. At the same time however, uterine specific cell markers for smooth muscle cells, SMA, and epithelial cells, E-cadherin, were positive. Smooth muscle cells were present in all experimental groups (B-D) but less organized in P3 generated scaffolds compared to the other two constructs. Recellularization of epithelial cells occurred in all three constructs but showed more prominent glandular structures in P3-dervied constructs, but again less organized compared to normal uterus (E-H).

Figure 4: Immunohistochemistry on normal uterine tissue (A, E) and on the bioengineered grafts (B-D, F-H). No GFP-labeled cells (green) remained in the grafts. Smooth muscle cell (SMA) specific antibody (red) showed presence of positive cells in all experimental groups (A-D) and an antibody against E-cadherin (red) revealed host cell recellularization of epithelial cells, with more prominent glandular structures in group P3-constructs, but less organized compared to normal uterus (E-H). Scale bars = 100μm.
Gene expression analysis

PCR data from patches before transplantation showed a typical gene expression profile for GFP-MSCs in vitro, which after transplantation changed to a typical expression profile for pregnant uterine tissue in vivo (Fig. 5). No GFP expression was detected in the tissue engineered constructs at the termination of the experiment (after 8–9 weeks in vivo).

Fig. 5 Gene expression analysis from tissues obtained at different stages during the construction of the bioengineered uterine patches (median indicated with the interquartile range and 95% error bars; star/circle indicates outliers). In general, there was a change from a GFP-labeled MSC-like gene expression profile in vitro to a pregnant uterus-like expression profile in vivo. Abbreviations and text explanations: MSCs and uterine cells (MSC/uterus cells) used for recellularization, autografted patches (autografted), uterine tissue from non-pregnant control animals (normal uterus), and uterine tissue from pregnant control animals (normal pregnant uterus).
Discussion

The present study demonstrates that a bioengineered uterine patch from decellularized uterine tissue can be used to give structural support to a partially defect uterus during pregnancy. We further showed that normal pregnant uterus-like tissue can be created using a combination of uterine cells and MSCs for the recellularization process. Furthermore, the results consistently showed that the buffered or non-buffered Triton-X100+DMSO-based protocols (P1 and P2) were superior to the SDC-based protocol (P3). The P1- and P2-based scaffold patches were better at supporting pregnancy and showed higher recellularization efficiency in vitro and more organized tissue structure in vivo, compared to P3-derived scaffolds.

Before transplantation, the decellularized uterus patches were mainly recellularized with allogeneic MSCs, and only a small percentage (0.7%) were primary uterine cells. The initial aim was to use a higher proportion of primary cells but we had difficulties in harvesting and expanding these cells in culture. The MSCs were labeled with green fluorescent protein (GFP), which facilitated the analysis of the recellularization efficiency pre-transplantation and the evaluation of cell faith post-transplantation.

Recellularization efficiency in vitro was limited and mainly placed cells on the scaffold surfaces and in isolated cell clusters within the scaffolds. The reason for these results is not fully understood, but may be due to poor circulation, insufficient cell delivery technique or toxic residues of decellularization detergents. Limited scaffold repopulation was shown in un-vascularized silk scaffolds repopulated with human cervical cells (28) and similar results were reported for other tissues and organs (12, 36). Since a functional vascular network is essential to promote oxygen transfer, to deliver nutrients and the disposal of metabolic waste products, the lack of vascularization may explain the incomplete recellularization.

Furthermore, an inadequate cell retention has been noted when cells are delivered by injection into the tissue parenchyme (37). In a study based on recellularized heart scaffolds, a high cell density was achieved at the site of injection, but cell number declined in remote areas (11). In addition, the injection procedure itself may damage the tissue and compromise the recellularization success (37). The development of optimal cell delivery and retention techniques remains a challenge, but is crucial for future success in tissue engineering. Constructs based on decellularized whole uterus tissue with a preserved
vascular system (33, 34), and the use of perfusion bioreactors may be required to improve recellularization results, even for smaller patches.

Poor graft vascularization may also explain why placentation did not occur directly over the patches in any of the groups in vivo. Based on gross morphology and fetus numbers, the uterine constructs functioned well and gave support during pregnancy. However, the low cell density and the lack of cell organization in the grafts indicate a sub-optimal tissue structure, which may have compromised successful placentation over the grafted constructs. Furthermore, the observed infiltrating immune cells and angiogenesis could be signs of an ongoing inflammatory process that also may have affected placentation. The different protocols used to generate the scaffolds for the current study may lead to different levels of inflammatory response and this should be investigated further.

Hellstrom et al. (34) showed that the scaffolds differed between groups in regards to protein content and mechanical properties. Scaffolds generated from protocol P1 contained higher amounts of intracellular proteins compared to scaffolds of groups P2 and P3. P1-based scaffolds also contained more glycosaminoglycans, which may explain the superiority of these scaffolds in promoting adherence and cell coverage. It was further shown that scaffolds of P1 and P2-protocols had a more compact ECM structure and P3-based scaffolds were more porous (34). Further studies are needed in order to determine whether this could explain the less organized cell structure in P3-grafts.

By using immunohistochemistry and qPCR, we discovered that no GFP-labeled MSCs remained in the transplanted constructs after 8-9 weeks in vivo. Other studies have shown that MSCs are able to differentiate into a number of cell types such as adipocytes and chondrocytes, but also myocytes and vascular endothelial precursors with a MSC-origin have been detected (2, 3). Some studies have shown that MSCs are able to differentiate into various endometrial cell (38-40) and therefore we decided to use MSCs for uterine scaffold recellularization.

The lack of differentiation into uterine cells is not uncommon and previous studies have shown that MSCs are able to migrate into damaged tissue, but that only a few survive for longer periods (41). Instead, the main role of MSCs is likely to act as immune modulators in tissue repair and as host cell homing stimulators through paracrine actions (41). These mechanisms was also noticed when MSCs were transplanted to repair uterine tissue in a mouse model of Asherman Syndrome (42) These beneficial effects may have contributed to a successful repopulation of host uterine cells that eventually replaced the GFP-MSCs in our study.
For whole organ tissue engineering, the immune modulating and homing roles of the MSCs may not be sufficient. It is likely that the parenchyme, vasculature, and support components must be reestablished prior to implantation and MSCs cannot give rise to complex tissue in vitro (2). Thus, the ideal cell type for repopulating whole-uterus scaffolds has yet to be identified (8). Induced pluripotent stem cells (iPSCs) or embryonic stem cells (ESCs) are promising candidates due to their great capacity for cell renewal and differentiation. However, due to their pluripotency, the correct signals for differentiation are required (43, 44). The role of organ scaffolds in differentiation is still unclear but their ability to provide all necessary signals for ESC differentiation into uterine cells is likely limited (2). However, neonatal mouse uterine mesenchyme has been shown to induce differentiation of ESCs into epithelial cells of the female reproductive tract (45). Nonetheless, the propensity of ESCs and iPSCs to give rise to teratomas, the ethical constraint and risk for adverse immune reactions of ESCs and the epigenetic modifications of DNA in iPSCs, limit their clinical relevance (43, 46).

Other possible cell sources for uterine recellularization are endometrial and myometrial stem cells (47, 48), which have been isolated and shown to be able to proliferate and differentiate into mature uterine cells that exhibit phenotypic and functional characteristics of uterine tissue. These cells have the capacity of self renewal and can form endometrium-like tissue in vivo (48-51). The feasibility of these cells in uterine tissue engineering needs to be explored further.

Better recellularization procedures are crucial in order to determine the most appropriate cells for seeding. Ideally, the cells should be of autologous origin, easy to expand and of predictable fate in their new environment. No such cell type exists today and it is likely that a heterogeneous cell mix and a large number of cell types are necessary to adequately reseed the decellularized uterine scaffold in vitro before transplantation in vivo.

Conclusions and Implications
In summary, this study provides a foundation for the development of a tissue engineered uterine patch that can be used to repair uterine defects and give support during pregnancy. Further studies are required to determine whether the techniques used in this study can be scaled up for humans and developed towards clinical use. As a step toward the development of a tissue engineered whole uterus, this study has provided important insights regarding decellularization strategies and recellularization techniques and cell sources. However, many challenges remain before a tissue engineered whole uterus can be used for transplantation to cure absolute uterine factor infertility.
**Sammanfattning**

Infertilitet hos kvinnor kan ha många orsaker, men hos ungefär 1 av 500 beror det på en skada på livmodern eller på att kvinnan helt saknar livmoder. Det enda botemedlet som finns för dessa kvinnor idag är livmodertransplantation, en metod som har flera negativa effekter såsom biverkningar från immunreglerande läkemedel och risker med kirurgin för donatorn. Forskning inom ramen för “tissue engineering” syftar bland annat till att skapa nya organ med hjälp av patientens egna stamceller; och om den lyckas skulle den här metoden lösa många av dagens problem. I dagsläget befinner sig forskning om organodling i ett tidigt skede och att återskapa ett helt nytt organ på det här sättet kommer ta mycket lång tid att utveckla. Däremot har man kommit längre i utvecklingen av att återskapa vävnad eller delar av ett organ vilket är mindre komplicerat.

Syftet med den här studien var att skapa en bit vävnad som kan användas för att reparera en delvis skadad livmoder. Vi ville testa om den tillverkade vävnaden kunde fungera vid en graviditet och vad som hände med de stamceller som användes vid experimenten.


Resultaten från studien visade att graviditeterna var normala i grupp P1 och P2 och i dessa grupper skedde fosterutveckling i transplanteringsområdet. I grupp P3 var antalet foster signifikant lägre och det fanns inga embryo i området där vävnadsbiten opererats in. Inte i någon av experimentgrupperna växte moderkakan in i den inopererade vävnadsbiten. Analyserna visade också att livmoderliknande vävnad hade bildats i de transplanterade...
vävnadsbitarna genom att omkringliggande celler hade migrerat in. Däremot fanns inga stamceller kvar i den transplanterade vävnaden vid experimentets slut.

References


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