## Bioengineered uterine tissue supports pregnancy in a rat model

**Running title:** Bioengineered uterus

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**Capsule**

We investigated the functionality of different bioengineered patches used for partial uterus repair and conclude that uterine cells and mesenchymal stem cells could reconstruct decellularized uterine tissue and support pregnancy.

## Abstract

**Objective:** To create a bioengineered uterine patch for uterine-repair of a partially defect uterus.

**Design:** Three different decellularized uterine scaffolds were recellularized *in vitro* with primary uterine cells and GPF-labelled bone marrow derived mesenchymal stem cells (GFP-MSCs). The patches were transplanted *in vivo* to investigate their tissue adaptation and supporting capacity during pregnancy.

**Setting:** Research laboratory and animal facility at university department.

**Animals:** Rat.

**Intervention(s):** Decellularization was achieved by whole-uterus perfusion with buffered or non-buffered Triton-X100 and DMSO (Group P1/P2) or with sodium deoxycholate (Group P3). Primary uterine cells and GFP-MSCs were used to develop uterine tissue constructs, which were grafted to uteri with partial tissue defects.

**Main Outcome Measures:** Recellularization efficiency and graft quality was analyzed morphologically, immunohistochemically and by real-time qPCR. The location and number of fetuses were documented during pregnancy 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. QuantitativePCR and immunohistochemistry revealed uterus-like tissue in the patches, which had been further reconstructed by infiltrating host cells after transplantation.

**Conclusion:** Primary uterine cells and mesenchymal stem cells can be used to reconstruct decellularized uterine tissue. The bioengineered patches made from triton-X100+DMSO-generated scaffolds was 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 complete bioengineered uterus.

**Keywords:** decellularization, *in vivo,* recellularization, tissue engineering, uterus

## Introduction

Absolute uterine factor infertility (AUFI), due to absence of the uterus or presence of a nonfunctional uterus, affects about 1:500 women of fertile age (1). Through a translational research approach that involved extensive animal research, we developed uterus transplantation (UTx) procedures in several animal models (2-6). We recently reported the first live birth after human UTx (7), and AUFI is now a treatable condition.

As with other organ transplantation procedures, UTx is associated with several risks for the patient. In the case of a live donor concept, as in our clinical trial in Sweden (8), the donor is subjected to lengthy surgery time (10-12h), which naturally is associated with complications. Furthermore, the recipient will after UTx be exposed to the side-effects of immunosuppressive drugs. Another important factor in the future clinical field of UTx is the possible organ shortage which is a common problem in all fields of transplantation surgery (9).

A proposed solution to these problems is the creation of a bioengineered organ/tissue by a scaffold made of a synthetic mold or from a decellularized organ/tissue, which then can be repopulated by the patient´s own cells. A wide range of bioengineered organs have been clinically investigated with this concept to replace a donor, e.g. bladder (10), trachea (11), blood vessel (12) and vagina (13). A great number of pre-clinical studies indicate the substantial interest in surpassing transplantation hurdles using bioengineering solutions (14, 15).

The use of bioengineered uterine tissue may also be applied to clinical situations with uterine defects or scarring, as after resections of persistent placental tumors, extensive myomectomy or adenomyomectomy. A bioengineered uterine tissue patch may be used to cover any myometrial defect and thereby increase the strength of the uterine wall in a situation of pregnancy. For these

reasons, uterine scaffolds made either of collagen/matrigel (16-19), silk/collagen (20), boiled blood clots (21) or biodegradable polymer scaffolds (22, 23) have been assessed in various *in vitro* models or *in vivo* in small animals. Novel tissue engineering protocols using decellularized tissue as scaffolds to reconstruct organs are now widely used in the bioengineering field, and a small number of recent reports exist using this principle on uterine tissue (24-26). Due to protocol differences in scaffold-generation, the physical properties of these uterine scaffolds vary significantly from one another and these differences may potentially impact their functionality.

In this study we explored the creation of bioengineered uterine patches derived from three different decellularization protocols. These scaffolds were repopulated with primary uterine cells and green fluorescent protein labelled bone marrow derived mesenchymal stem cells (GFP-MSCs). We evaluated the *in vitro* recellularization efficiency and the *in vivo* regeneration of the patches and their ability to support pregnancy in a rat model.

## Materials and Methods

### Animals and experimental groups

Female rats (140g –180g) were used as follow: Lewis rats (n=9; Charles River, Sulzfelt, Germany) as donors to generate whole-uterus scaffolds using three different protocols (n=3 per protocol); Sprague Dawley (SD) rats (n=40; Janvier Labs, Janvier, France) for primary uterus cell isolation procedures (n=10) and for transplantation/pregnancy studies (n=30). Male SD rats (n=12; 250g - 300g) were used for mating. The study was approved by the Animal Ethics Committee in Gothenburg, Sweden.

### Uterus isolation and decellularization

The uterus was isolated free to create a complete uterus segment with intact vasculature as previously described in detail (24). The decellularization process was performed according to three protocols (P1-3). Briefly, for specimens of Group P1 and Group P2, decellularization was achieved by sequential uterine perfusions through the aorta (flow speed=2ml/min) for 4h with each of dimethyl sulfoxide (DMSO; 4%) and Triton-X100 (1%) followed by 16h of washing in PBS (Group P1) or $dH_2O$ (Group P2). For Group P3, perfusion was performed using a 2% sodium deoxycholate solution (SDC) for 6h, followed by 18h washing ($dH_2O$). These cycles were repeated five times, and on the fifth day (Group P1-3), a sterilization process was carried out using per-acetic acid (0.1%) for 30min. After several washes (PBS), the decellularized uterus was frozen (-80ºC) for long-term storage. These protocols were based on an earlier study (24), but with the modification that sodium azide was omitted in all solutions to avoid toxicity risks of leaving remnants in the scaffold that potentially could reduce the recellularization efficiency.

**Uterus cell isolation**

SD rats underwent hysterectomy and the excess tissue was removed from the isolated uteri. Each horn was placed in cold PBS, then opened longitudinally and placed in a Petridish containing Digestion solution (collagenase, 2mg/ml; DNase I, 0.5µl/ml; 10mM HEPES; 1xAnti-Anti™; in DMEM; Life Technologies, Stockholm, Sweden) for 1h at 37ºC. The endometrium was scraped off the myometrium and was collected in EM media (DMEM+10mM HEPES+1xAnti-Anti+10% FCS; Life Technologies). The endometrium cell/tissue suspension was centrifuged, the pellet was resuspended in fresh EM media, 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 37ºC for 90 min.

The myometrium tissue was minced in PBS and then centrifuged. Digestion solution was used to resuspend the pellet (10ml/g tissue) and the suspension was incubated for 4h on a shaker at 37ºC.

Dislodged endometrial/myometrial cells and cell clusters were filtered through a 40µm cell strainer. The larger fragments were dissociated further using a trypsin solution (0,25% Trypsin+25µg/ml DNase I). Cells were then centrifuged and resuspended in EM media and plated on collagen-I coated 6-well plates (Life Technologies, Stockholm, Sweden) as a heterogeneous uterus cell mix. The primary cells were maintained in a humidified chamber at 37ºC and 5% $CO_2$ for 6 days, and were fed twice with EM media.

### *In vitro* work and scaffold recellularization

Three decellularized uteri from each protocol were thawed and washed by $dH_2O$ perfusion (2ml/min) for 3 days, followed by PBS for 3 days, and DMEM for 1 day. Six rectangles (20x5mm) per protocol were cut out from the scaffolds which were used for recellularization. The remaining pieces were analyzed.

A graphic illustration of the methods can be viewed in supplementary figure 1. Recellularization was achieved using two cell sources; the SD-rat uterus primary cells described above, and commercially purchased SD-rat GFP-MSCs (passage number <8; Cyagene Bioscience, Cat.No.RASMX-01101, CA, USA). Due to a low primary uterus cell yield during cell isolation, the cell ratio used for the recellularization was 1 primary uterine cell per 150 GFP-MSCs. A total of $7.3x10^6$ cells per patch (patch size = 5x20mm) were injected into the scaffolds by multiple injections using a 27G 1ml syringe. The recellularized constructs were then cultured for 3 days in standard culturing conditions and fed every day with EM media. 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. About $14x10^6$ cells (from the same batch used for the recellularization) were collected in RNALater (Qiagen, Sollentuna, Sweden) and used for qPCR analysis.

### Patch transplantation

Each uterine patch was transplanted to an individual rat (n=6 per group) under isoflurane anesthesia. Animals were given sc injections of analgesic- and antibiotic agents (buprenorphine, 0.05mg/kg; carprofen, 5mg/kg; trimethoprim, 24 mg/kg; sulfadiazine, 120mg/kg; Apoteket,

Sweden), and a laparotomy was performed to expose the uterine horn. A 5x10mm large full thickness segment of the uterus horn was surgically removed to create a uterine defect. In a pre-study we transplanted an acellular scaffold patch (from groups P1, P2, P3; n=6 per group) to replace the excised segment (using 10 stitches of running suture) with 8.0 non-absorbable polypropylene, and evaluated the grafts after three months. At the termination of the experiment, only sutures remained and the acellular scaffolds had degraded in all animals (data not shown). With this background knowledge, we then transplanted a recellularized bioengineered patch in a similar way. As a control group, six animals underwent a comparable segment removal but the uterine patch was immediately sutured back again (auto-transplantation).

**Mating and pregnancy assessment**

The animals with uterine patch transplantation (n=18), auto-transplanted (n=6) and non-operated control animals (n=6) were mated six weeks after transplantation and mating was confirmed with presence of vaginal plug. The experiment was terminated 16-20 days later to assess graft condition, the number of fetuses and their location. Grafts were located in all animals by the suture lines, except for one animal in Group P1, where the sutures could not be localized. This animal was excluded from further analysis. Grafts were isolated free from surrounding uterine tissue and sutures were removed. A biopsy from the graft was taken and placed in RNALater for gene expression analysis and the remaining piece was placed in a preservative (Histocon®; Histolab, Gothenburg, Sweden) for 12-18h before frozen in OCT. For one animal per group, where a fetus/degenerated fetus was located in the graft-area, a larger uterine segment that

included the graft, graft junction and the fetus was isolated and placed in 4% formaldehyde for 24h. These segments were processed for histological analysis.

**Fluorescent measurements, histology and immunocytochemistry**

To quantify the recellularization efficiency of the scaffolds prior to transplantation, a small piece of each recellularized scaffold was fixed (4% paraformaldehyde), washed and mounted on a slide. GFP fluorescence was measured based on maximum intensity projected confocal z-stack images (Carl Zeiss, LLSM 710 NLO) from three surface areas per patch (total area investigated = $0.542mm^2$), from the luminal side (n=3) and from the perimetrial side (n=3). The GFP-MSC-covered area was measured using Volocity6.3.1 software (intensity thresholds = 100-4095; detected objects $<50\mu m^2$ was excluded). The proportion of GFP-labelled cell area and total image area (scaffold) was calculated with an adjustment for the z-stack thickness using the formula $X^2 + Y^2 = Z^2$, where Z = the adjusted side length for each image.

Histology and immunocytochemistry were completed using standard procedures. Briefly, cryosections of acellular scaffolds, recellularized pre-transplantation constructs, and isolated transplanted patches were cut, air-dried and rehydrated in PBS. Sections were fixed (10 min, 4% paraformaldehyde) and washed before adding blocking buffer (0.2% Triton X-100, 10% normal goat serum; NGS, PBS) for 60 min. Sections were then stained with primary mouse or rabbit antibody diluted in blocking buffer as follow; mouse anti-e-cadherin (1:200; ab76055; Abcam, Cambridge, England), mouse anti-vimentin (1:200; MA1-19168; Thermo Scientific, Stockholm, Sweden), rabbit anti-SMA (1:300; ab5694; Abcam), Ki67 (1:100; ab16667; Abcam) and cleaved caspase-3 (1:300; 9661S; Cell Signaling, Stockholm, Sweden) for 1h at room temperature (RT).

Sections were washed and incubated for 1h at RT with an anti-mouse or anti-rabbit Cy3-conjugated secondary antibody (1:300; A10520/A10521; Life Technologies). Sections were then washed, DAPI stained (1min, 25µg/ml DAPI in PBS) and washed again before cover slipped with Dako fluorescent mounting media. The formalin fixed uterus/fetus segments taken from each group were paraffin embedded, cut at 3µm thickness and processed for hematoxylin and eosin (H&E) staining using standard methods.

**DNA/RNA quantification and qPCR**

Tissue samples kept in RNALater were weighed before homogenization (5 min at 30Hz, TissueLyser II, Qiagen). The homogenate was transferred to an AllPrep DNA spin column (Qiagen) and centrifuged. The column was used for DNA purification, and the flow through was used for RNA isolation and purification using an RNeasy Micro Kit (Qiagen). MessengerRNA 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. Two reference genes were tested: GAPDH (Rn01775763_g1) and ACTB (Rn00667869_m1). GADPH was selected after confirmation that similar results were obtained from ACTB (data not shown). 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; endothelial 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 (progesteron 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.

**Statistics**

Two-tailed, paired student t-test was used to compare the number of fetuses in the operated vs. the non-operated horn. Kruskal-Wallis non-parametric analysis for multiple groups was used to calculate significance levels between groups in all other analysis.

## Results

### Decellularization and recellularization

The DNA content after decellularization in Group P1-generated scaffolds was 18% compared to normal uterus tissue, and was below detection levels for scaffolds of Groups P2 and P3. Cryosections of recellularized scaffolds revealed a limited cell distribution. Cells were mainly located on the surface or in isolated cell clusters within the scaffold (Fig. 1A-B). There were only a few cells in each section immunohistochemically positive for Ki67 and cleaved caspase-3 (data not shown). There was significantly larger cell coverage on the perimetrial side of P1-generated scaffolds compared to that of Groups P2 and P3 (Fig. 1C).

### Pregnancy results

The *in vivo* results after patch transplantation (Fig. 1D-E) showed well integrated grafts and no obvious sign of tissue degeneration (Fig. 1. F-G). The transplantation procedure did not affect pregnancy rates and 4-6 animals in each experimental group became pregnant (Fig. 1H), as compared to 5/6 of non-operated rats (not shown). Similarly, the mean number of fetuses per uterus was comparable for all groups (Fig. 1I). Fetus numbers were similar in the patched horn and the non-operated contralateral horn for all groups, except for Group P3 which had a lower number of fetuses in the operated horn (Fig. 1H). The uterine area including the graft and nearby host uterine tissue had fetal development occurring in two animals in each of Groups P1 and P2, but for Group P3 there were only signs of degenerated fetal tissue.

**Histology and immunocytochemistry**

No placentation was formed directly over the patched area (Fig. 2A-B). However, the graft morphology resembled normal pregnant rat uterus with organized well-structured myometrial- and epithelial-like structures (Fig. 2C-D, F-H). Some areas of the Group P1-grafts had a large amount of infiltrating cells of unknown origin (Fig. 2E) and signs of granulation was seen in Group P2-grafted animals (Fig. 2G). Immunohistochemistry showed a large amount of GFP-negative cells in the grafts which were less organized compared to cells in normal pregnant uterus tissue (Fig. 3A-H). Uterine specific cell markers SMA and E-cadherin confirmed that the majority of the present cells were host uterine cells. The Group P1- and P2-constructs appeared more homogenous and organized than Group P3-constructs (Fig. 3B-D, F-H).

**mRNA levels and gene expression analysis**

The total RNA content of the constructs increased throughout the experimental period and was not different to normal- or auto-grafted uterine tissue at the termination day (Fig. 4A). Quantitative PCR data from patches prior to and after transplantation showed a typical gene expression profile for GFP-MSCs *in vitro* prior to transplantation (Fig. 4B), which changed to a typical expression profile for pregnant uterine tissue *in vivo*. No GFP-expression was detected at the termination of the experiment.

## Discussion

The results of the present study demonstrate that the creation of a uterine patch from decellularized uterine tissue is a feasible strategy that can be used to support uterine function, including pregnancy. This strategy should be developed further towards clinical use. Importantly, the results show that uterine-like tissue with supporting properties can be created using a combination of uterine cells and mesenchymal stem cells. The study design was based on our original report, where three different protocols (P1-P3) for rat uterus decellularization were compared and showed slight differences in the extent and specificity of the decellularization process (24). The results of the present study consistently showed that the buffered or non-buffered Triton-X100+DMSO-based protocols (P1 and P2) were superior to the sodium deoxycholate-based protocol (P3). Cell adherence *in vitro* and the *in vivo* remodulation of the constructs by repopulating host cells were better in these groups. Furthermore, both these types of patches were able to support pregnancies in the grafted region of the uterus. In contrast, P3-derived scaffolds had poor recellularization abilities, and cell organization was unparalleled to Groups P1 and P2 *in vivo*. The suitability of scaffolds based on P1- or P2 protocols should thus be evaluated further in rodent models and then in large animal models, including nonhuman primates.

Different protocols for the decellularization of uterine tissue have been explored in the rat (24-26) and in a recent study in that species it was reported that the detergent Triton-X100 was an ineffective decellularization reagent for uterine tissue (26). However, results from the present study of Groups P1 and P2, and in our previous study in the rat (24), consistently showed that this relatively mild decellularization treatment worked well when the solutions were administered through perfusion and in combination with 4% DMSO. Sodium dodecyl sulfate (SDS) is an

effective and common reagent that has been used to decellularize many organs, including the uterus (14, 25, 26). However, this strong detergent has been shown to also compromise extracellular matrix (ECM) components and consequently may negatively affect the recellularization properties of a uterine scaffold (14). Hence, Triton-X100 and DMSO should be explored further as important reagents for uterus scaffold construction. Indeed, the protocols tested herein resulted in higher total number of fetuses in the operated horns (3.0-4.4), compared to SDS-based uterus constructs previously evaluated *in vivo* (0.8-1.3) (25, 26). However, results may not be directly comparable since our grafts were 25% smaller and that it also may exist other technical differences in the protocols of the two studies. For future studies, we found it crucial to obtain sufficient amount of tissue material to also analyze the recellularization efficiency before transplantation. The evident consequence was that this approach reduced the size of the patches used for our *in vivo* experimentation. Furthermore, it is noteworthy that placentation did not occur directly over the patches in any protocol of the present study. The reason for this placentation deficiency of the bioengineered patches is not clear but it is an indication of a suboptimal tissue structure and/or poor revascularization.

A high initial recellularization efficiency of appropriate cells in the scaffolds will be beneficial for further cell repopulation *in vivo* and may possibly enhance implantation rates and allow for placentation over the graft. An *in vitro* based study using human cervical cells reported limited scaffold repopulation in un-vascularized scaffolds (20). These results are similar to those obtained concerning other organ systems (27, 28). Therefore, bioengineered constructs based on decellularized whole uterine tissue including the vasculature conduits (24, 25), and the use of sophisticated perfusion bioreactors may be required to improve recellularization results, even for smaller patches.

We used labelled MSCs to permit an unbiased interpretation and quantification of the recellularization efficiency of the scaffolds. Furthermore, the faith of recellularized cells could be determined *in vivo*, a fundamental detail in order to find the best suitable cell source for the construction of bioengineered organs. We predominantly used GFP-MSCs, and only 0.7% was non-labelled primary uterine cells. Our initial aim was to use a higher proportion of uterine specific cells in the cell mix to aid in MSC differentiation, but we had difficulties expanding these in culture. Donor MSCs have been found in recipient endometrial stroma- and epithelial compartments, which suggests that endometrial cells can originate from MSCs (29-31). Endometrial endothelial cells have also been found to have a MSC origin (32), and we therefore considered MSCs suitable to use for our study. However, by using immunohistochemistry and qPCR, we discovered that there were no labelled MSCs remaining in the transplanted constructs after 8-9 weeks *in vivo*. We cannot rule out a possible immunological elimination of the transplanted cells due to the host-foreign GFP expression. However, previous successful *in vivo* transplantation studies were able to detect GFP-MSCs after a similar time period after cell transplantation to the injured spinal cord in the rat (33), and allogenicity between SD rats is also unlikely (34). It is not uncommon for transplanted MSCs to be removed after transplantation, and others have reported that these cells predominantly are immune modulating and act as host cell homing stimulators through paracrine actions (35). This was also noticed when MSCs were transplanted to repair uterine tissue in a mouse model of Ashermann's syndrome (36). These beneficial effects may have contributed to a successful repopulation of host uterus cells that eventually replaced the GFP-MSCs in our study.

Based on morphology and fetus numbers, our constructs functioned well and gave proper support during pregnancy. For larger uterine constructs however, recellularization with MSCs alone may not be sufficient enough since it has been shown that donor MSCs were unable to replenish the highly regenerative endometrial side population of the recipient, which is thought to be the main source of stem cells in the endometrium (30). Important findings regarding the existence of uterus stem cells have been documented, both from the endometrium and myometrium (37-40). The isolated side population is a promising cell source for uterine bioengineering applications since these cells show capacity of self-renewal, can differentiate into uterus specific cells and can form endometrium-like tissue when transplanted (40-44). Thus, it is likely that a heterogeneous cell mix of at least some of the cell types mentioned above, or that specific homogenous cell populations added in sequence will improve the outcomes.

The principle limitations of the study are the relatively small sample size that the recellularization efficiency was based upon, the pregnancies were not induced be embryo transfer with a set number of embryos, and that relatively small patches were used instead of larger complete segments of the uterus.

In conclusion, this study indicate the principles for creating bioengineered uterine patches that can be used to repair uterine defects and give support during pregnancy. These applications should be developed further towards clinical use, both for uterine tissue repair and for the development of a whole bioengineered uterus with a functional vasculature for uterus transplantation purposes.

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## Figure legends

**Fig 1.** Results of recellularization, gross morphology and pregnancy rates of Groups P1-P3, with comparison to pregnancy rate of auto-grafted control animals. Cross section (A) and surface view (B) of the recellularized scaffolds showed a limited cell distribution of the scaffolds *in vitro*. Most cells were located in isolated cell aggregates or on the scaffold surface areas (A). Maximum projection images from z-scanned confocal images from the luminal- and the perimetrial surface of the scaffolds were used to analyze the cell density (B-C). Scaffolds of Group P1 showed a significantly higher cell confluency on the perimetrium side compared to the scaffolds of Groups P2-3, with a similar trend on the luminal side (C; ***P<0.001; *P<0.05). An excised uterus segment was replaced with a bioengineered construct (D-E; arrow indicate bioengineered construct). Patches had integrated well with the host uterine tissue and pregnancies were observed (F-G; arrows indicate graft). The total numbers of pregnant animals per group and fetuses at transplantation sites were similar in all groups, except in Group P3, with no fetuses found over the patch areas (H). The number of fetuses in the operated horns was not significantly different from each other (I), or to normal non-operated rats (not shown). When compared with the contralateral non-operated horn from respective group, a difference was seen in the Group P3 animals (*p<0.05).

**Fig 2.** Micrographs of a cross section in the fetal sagittal plane of a pregnant animal grafted with a Group P1-construct (A), and a pregnant animal grafted with a Group P2-construct cross sectioned in the fetal transverse plane (B). Graft location is identified by the location of the suture lines (dotted lines; A-B). Placentation was not found over the grafts. The graft morphology was similarly organized to that of normal pregnant uterus with myometrium- and epithelial-like structures (C-D, F-H). Some areas of the graft had a large amount of infiltrating cells (E) and in some areas granulation was seen (G).

**Fig 3.** Immunohistochemistry of normal pregnant uterus (A and E), and of grafted bioengineered patches of pregnant animals of Group P1 (B, F), Group P2 (C, G) and Group P3 (D, H). No GFP-labelled cells remained in the grafts. Immunohistochemistry with smooth muscle cell actin specific antibody (SMA; red) showed presence of positive cells in all experimental groups, but with less density and organization in Groups P1-P3, as compared to the normal uterus (A-D). The Group P3 patch had clearly lower numbers of SMA-positive cells as compared to Groups P1 and P2. Immunohistochemistry with antibody against the epithelial cell protein E-cadherin (E-cadherin; E-Cad; red) revealed host cell recellularization of epithelial cells, with more prominent glandular structures seen in Group P3-constructs, but again less organized compared to normal uterus (E-H). Scale bars = 100μm.

**Fig 4.** A, total amounts of mRNA/mg wet tissue in native uterus and autografted patch (left panel) as compared to that of scaffolds, *in vitro* recellularized scaffolds (Recell) and patches 8-9 weeks after transplantation (Tx patch) of Groups P1, P2 and P3. The total mRNA content increased during recelluarization and after the *in vivo* conditions in all experimental groups. The bioengineered uterine constructs *in vivo* had similar levels of mRNA compared to normal- or autografted uterine tissue (***P<0.001). B, 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 indicate outliers). In general, there was a change from a green fluorescent protein (GFP) labelled mesenchymal stem cell-like gene expression profile *in vitro,* to a pregnant uterus-like expression profile *in vivo*. No GFP expression was detected in the bioengineered constructs after 8-9 weeks *in vivo*. CDH1 (E-cadherin; epithelial cells), Vimentin (stromal cells), ACTA2 (alpha smooth muscle actin; myometrial cells), VWF (von Willebrand Factor; endothelial cells), COL1A1 (collagen 1a1; extra cellular matrix component), BCL2 (B-cell lymphoma 2; anti-apoptotic marker), HOXA11 (uterine tissue development), ESR1 (estrogen receptor 1), PGR (progesteron receptor) and eGFP (cells used for recellularization). Mesenchymal stem cells 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).

**Sup. Fig. 1**

A, whole uterus was isolated with intact vasculature to facilitate decellularization by perfusion of detergents and ionic solutions according to protocol 1, -2 and -3. The decellularized uterus was then cut so that a rectangular segment (patch) was created which acted as a uterine tissue scaffold for the experiment. B, purchased mesenchymal stem cells genetically labelled with green fluorescent protein were expanded *in vitro* (passage number <8) and then mixed with isolated primary uterus cells which had been isolated and cultured *in vitro* for six days. C, recellularization was performed by multiple injections into the scaffold using a 27G syringe. D, the recellularized constructs were then cultured for 3 days *in vitro*, then each patch was cut in half, one part was used for pre-transplantation analysis and, E, the remaining patch was used for *in vivo* transplantation studies. Pregnancy was induced by normal mating 6 weeks after transplantation, and the experiment was terminated 16-20 days later to assess the condition and functionality of the grafts. Histology, immunohistochemistry and qPCR were performed on samples taken at several steps in the process (A, B, D and E).

## References


1. Milliez J. Uterine transplantation FIGO Committee for the Ethical Aspects of Human Reproduction and Women's Health. International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics 2009;106:270.
2. Johannesson L, Enskog A, Dahm-Kahler P, Hanafy A, Chai DC, Mwenda JM *et al.* Uterus transplantation in a non-human primate: long-term follow-up after autologous transplantation. Human reproduction 2012;27:1640-8.
3. Racho El-Akouri R, Kurlberg G, Brannstrom M. Successful uterine transplantation in the mouse: pregnancy and post-natal development of offspring. Human reproduction 2003;18:2018-23.
4. Wranning CA, Akhi SN, Diaz-Garcia C, Brannstrom M. Pregnancy after syngeneic uterus transplantation and spontaneous mating in the rat. Human reproduction 2011;26:553-8.
5. Wranning CA, El-Akouri RR, Lundmark C, Dahm-Kahler P, Molne J, Enskog A *et al.* Auto-transplantation of the uterus in the domestic pig (Sus scrofa): Surgical technique and early reperfusion events. The journal of obstetrics and gynaecology research 2006;32:358-67.
6. Diaz-Garcia C, Akhi SN, Wallin A, Pellicer A, Brannstrom M. First report on fertility after allogeneic uterus transplantation. Acta obstetricia et gynecologica Scandinavica 2010;89:1491-4.
7. Brännström M, Johannesson L, Bokstrom H, Kvarnström N, Molne J, Dahm-Kahler P *et al.* Livebirth after uterus transplantation. Lancet 2015;385:607-16.
8. Brännström M, Johannesson L, Dahm-Kahler P, Enskog A, Mölne J, Kvarnström N *et al.* The first clinical uterus transplantation trial: a six-month report. Fertil Steril 2014.
9. Lesieur O, Leloup M, Gonzalez F, Mamzer MF, Eligibility for organ donation following end-of-life decisions: a study performed in 43 French intensive care units. Intensive Care Med 2014; 40:1323-1331
10. Atala A, Bauer SB, Soker S, Yoo JJ, Retik AB. Tissue-engineered autologous bladders for patients needing cystoplasty. Lancet 2006;367:1241-6.
11. Berg M, Ejnell H, Kovacs A, Nayakawde N, Patil PB, Joshi M *et al.* Replacement of a tracheal stenosis with a tissue-engineered human trachea using autologous stem cells: a case report. Tissue Eng Part A 2014;20:389-97.
12. Olausson M, Patil PB, Kuna VK, Chougule P, Hernandez N, Methe K *et al.* Transplantation of an allogeneic vein bioengineered with autologous stem cells: a proof-of-concept study. Lancet 2012;380:230-7.
13. Raya-Rivera AM, Esquiliano D, Fierro-Pastrana R, Lopez-Bayghen E, Valencia P, Ordorica-Flores R *et al.* Tissue-engineered autologous vaginal organs in patients: a pilot cohort study. Lancet 2014;384:329-36.
14. Crapo PM, Gilbert TW, Badylak SF. An overview of tissue and whole organ decellularization processes. Biomaterials 2011;32:3233-43.
15. Keane TJ, Swinehart IT, Badylak SF. Methods of tissue decellularization used for preparation of biologic scaffolds and in vivo relevance. Methods 2015;84:25-34.
16. Lu SH, Wang HB, Liu H, Wang HP, Lin QX, Li DX *et al.* Reconstruction of engineered uterine tissues containing smooth muscle layer in collagen/matrigel scaffold in vitro. Tissue Eng Part A 2009;15:1611-8.
17. Schutte SC, Taylor RN. A tissue-engineered human endometrial stroma that responds to cues for secretory differentiation, decidualization, and menstruation. Fertil Steril 2012;97:997-1003.
18. Wang HB, Lu SH, Lin QX, Feng LX, Li DX, Duan CM *et al.* Reconstruction of endometrium in vitro via rabbit uterine endometrial cells expanded by sex steroid. Fertil Steril 2010;93:2385-95.
19. Ding L, Li X, Sun H, Su J, Lin N, Peault B *et al.* Transplantation of bone marrow mesenchymal stem cells on collagen scaffolds for the functional regeneration of injured rat uterus. Biomaterials 2014;35:4888-900.
20. House M, Sanchez CC, Rice WL, Socrate S, Kaplan DL. Cervical tissue engineering using silk scaffolds and human cervical cells. Tissue Eng Part A 2010;16:2101-12.

21. Campbell GR, Turnbull G, Xiang L, Haines M, Armstrong S, Rolfe BE *et al.* The peritoneal cavity as a bioreactor for tissue engineering visceral organs: bladder, uterus and vas deferens. J Tissue Eng Regen Med 2008;2:50-60.
22. Sadri-Ardekani H, Atala A. Regenerative medicine for the treatment of reproductive system disorders: current and potential options. Advanced drug delivery reviews 2015;82-83:145-52.
23. Atala A. Tissue engineering of reproductive tissues and organs. Fertil Steril 2012;98:21-9.
24. Hellström M, El-Akouri RR, Sihlbom C, Olsson BM, Lengqvist J, Bäckdahl H *et al.* Towards the development of a bioengineered uterus: comparison of different protocols for rat uterus decellularization. Acta Biomater 2014;10:5034-42.
25. Miyazaki K, Maruyama T. Partial regeneration and reconstruction of the rat uterus through recellularization of a decellularized uterine matrix. Biomaterials 2014;35:8791-800.
26. Santoso EG, Yoshida K, Hirota Y, Aizawa M, Yoshino O, Kishida A *et al.* Application of detergents or high hydrostatic pressure as decellularization processes in uterine tissues and their subsequent effects on in vivo uterine regeneration in murine models. PloS one 2014;9:e103201.
27. Jain RK, Au P, Tam J, Duda DG, Fukumura D. Engineering vascularized tissue. Nature biotechnology 2005;23:821-3.
28. Zhao F, Pathi P, Grayson W, Xing Q, Locke BR, Ma T. Effects of oxygen transport on 3-d human mesenchymal stem cell metabolic activity in perfusion and static cultures: experiments and mathematical model. Biotechnology progress 2005;21:1269-80.
29. Taylor HS. Endometrial cells derived from donor stem cells in bone marrow transplant recipients. JAMA 2004;292:81-5.
30. Cervello I, Gil-Sanchis C, Mas A, Faus A, Sanz J, Moscardo F *et al.* Bone marrow-derived cells from male donors do not contribute to the endometrial side population of the recipient. PloS one 2012;7:e30260.
31. Ikoma T, Kyo S, Maida Y, Ozaki S, Takakura M, Nakao S *et al.* Bone marrow-derived cells from male donors can compose endometrial glands in female transplant recipients. American journal of obstetrics and gynecology 2009;201:608 e1-8.
32. Mints M, Jansson M, Sadeghi B, Westgren M, Uzunel M, Hassan M et al. Endometrial endothelial cells are derived from donor stem cells in a bone marrow transplant recipient. Human reproduction 2008;23:139-43.
33. Sandner B, Ciatipis M, Motsch M, Soljanik I, Weidner N, Blesch A. Limited functional effects of subacute syngeneic bone marrow stromal cell transplantation after rat spinal cord contusion injury. Cell Transplant 2015.
34. Fukushima S, Coppen SR, Lee J, Yamahara K, Felkin LE, Terraccian CMN et al. Choice of Cell-Delivery Route for Skeletal Myoblast Transplantation for Treating Post-Infarction Chronic Heart Failure in Rat. PLoS one 2008;3(8)e3071.
35. Wu T, Liu Y, Wang B, Li G. The roles of mesenchymal stem cells in tissue repair and disease modification. Current stem cell research & therapy 2014;9:424-31.
36. Cervello I, Santamaria X, Miyazaki K, Maruyama T, Simon C. Cell Therapy and Tissue Engineering from and toward the Uterus. Seminars in reproductive medicine 2015;33:366-72.
37. Ono M, Maruyama T, Masuda H, Kajitani T, Nagashima T, Arase T et al. Side population in human uterine myometrium displays phenotypic and functional characteristics of myometrial stem cells. Proceedings of the National Academy of Sciences of the United States of America 2007;104:18700-5.
38. Schwab KE, Gargett CE. Co-expression of two perivascular cell markers isolates mesenchymal stem-like cells from human endometrium. Human reproduction 2007;22:2903-11.
39. Ono M, Bulun SE, Maruyama T. Tissue-specific stem cells in the myometrium and tumor-initiating cells in leiomyoma. Biology of reproduction 2014;91:149.
40. Masuda H, Matsuzaki Y, Hiratsu E, Ono M, Nagashima T, Kajitani T *et al.* Stem cell-like properties of the endometrial side population: implication in endometrial regeneration. PloS one 2010;5:e10387.

41. Cervello I, Gil-Sanchis C, Mas A, Delgado-Rosas F, Martinez-Conejero JA, Galan A *et al.* Human endometrial side population cells exhibit genotypic, phenotypic and functional features of somatic stem cells. PloS one 2010;5:e10964.
42. Masuda H, Maruyama T, Hiratsu E, Yamane J, Iwanami A, Nagashima T *et al.* Noninvasive and real-time assessment of reconstructed functional human endometrium in NOD/SCID/gamma c(null) immunodeficient mice. Proceedings of the National Academy of Sciences of the United States of America 2007;104:1925-30.
43. Cervello I, Mas A, Gil-Sanchis C, Peris L, Faus A, Saunders PT *et al.* Reconstruction of endometrium from human endometrial side population cell lines. PloS one 2011;6:e21221.
44. Miyazaki K, Maruyama T, Masuda H, Yamasaki A, Uchida S, Oda H *et al.* Stem cell-like differentiation potentials of endometrial side population cells as revealed by a newly developed in vivo endometrial stem cell assay. PloS one 2012;7:e50749.

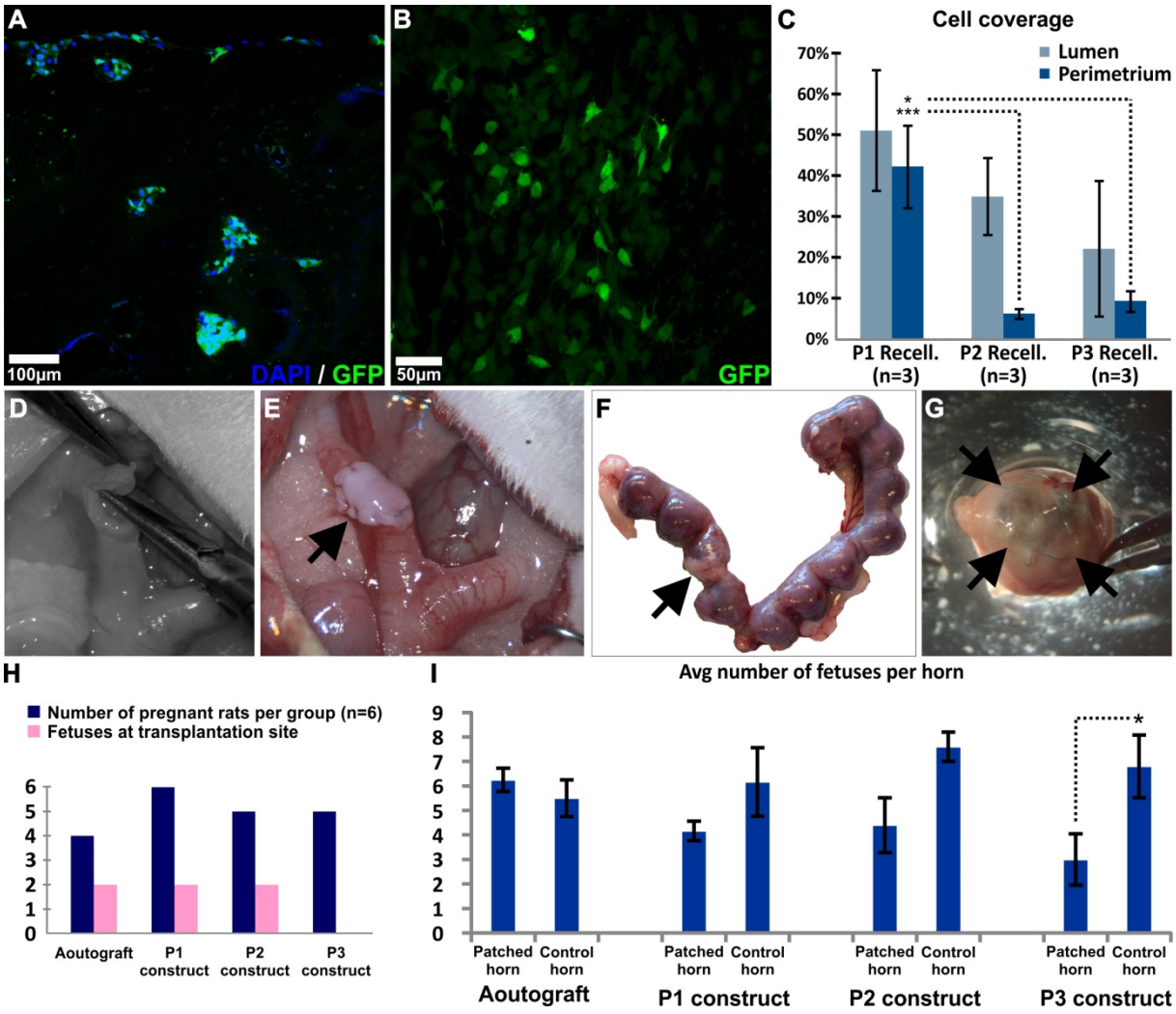
A
100µm
DAPI / GFP
B
50µm
GFP
C
Cell coverage
Lumen
Perimetrium
70%
60%
50%
40%
30%
20%
10%
0%
*
***
P1 Recell. (n=3)
P2 Recell. (n=3)
P3 Recell. (n=3)
D
E
F
G
H
Number of pregnant rats per group (n=6)
Fetuses at transplantation site
6
5
4
3
2
1
0
Aoutograft
P1 construct
P2 construct
P3 construct
I
Avg number of fetuses per horn
9
8
7
6
5
4
3
2
1
0
*
Patched horn
Control horn
Aoutograft
P1 construct
P2 construct
P3 construct

Fig 1

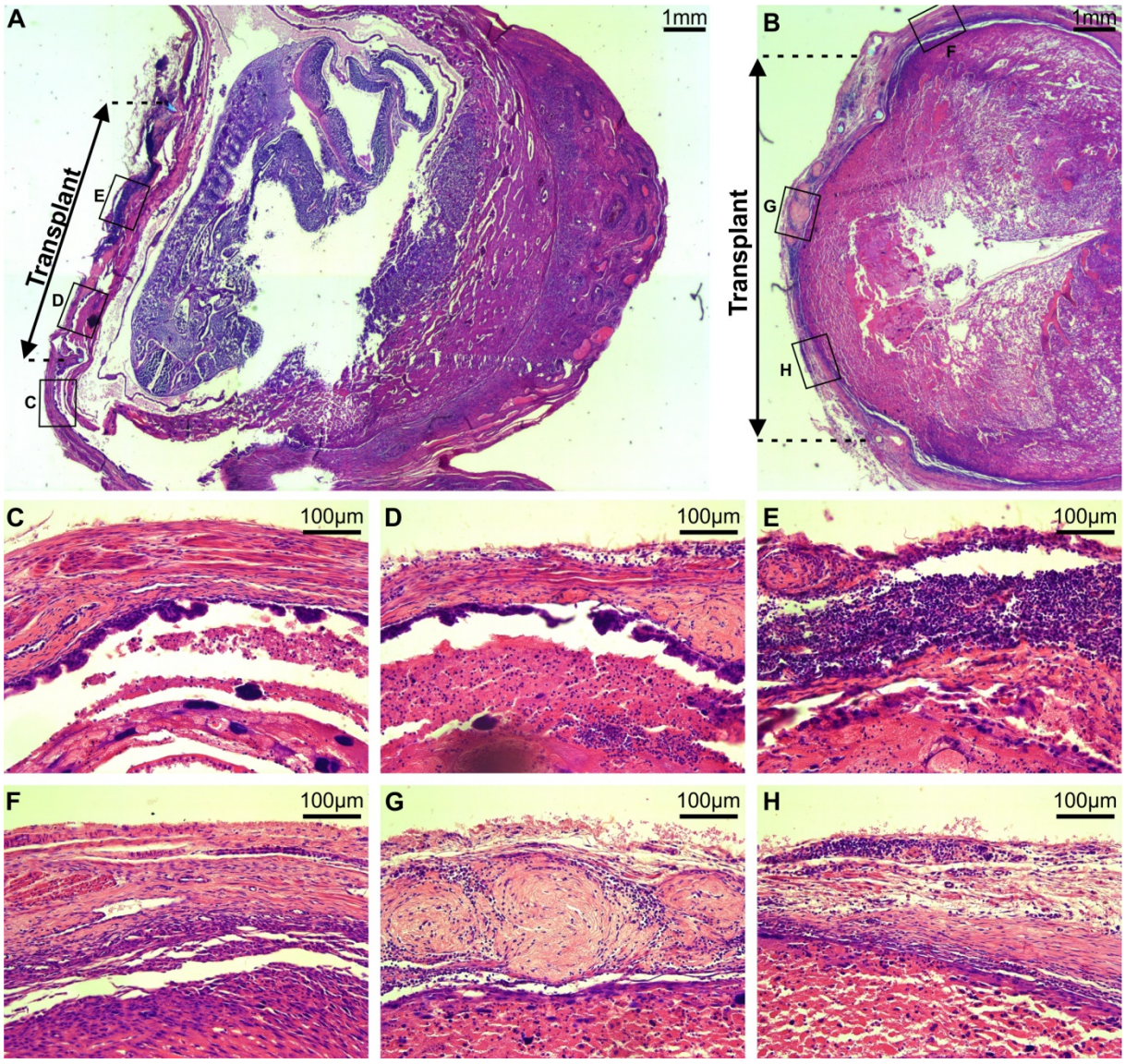
A
1mm
Transplant
E
D
C
B
1mm
F
G
Transplant
H
C
100μm
D
100μm
E
100μm
F
100μm
G
100μm
H
100μm

Fig 2

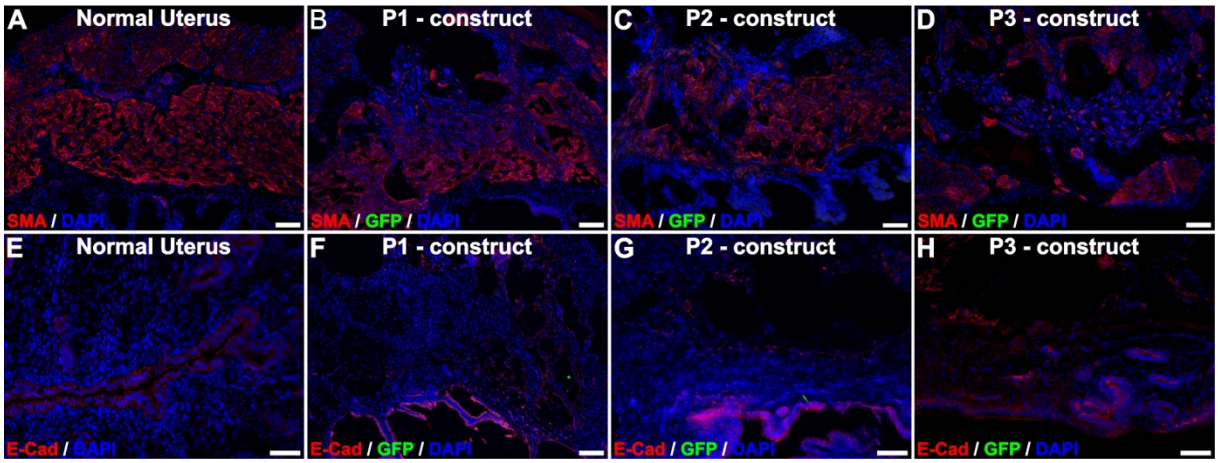
A
Normal Uterus
SMA / DAPI
B
P1 - construct
SMA / GFP / DAPI
C
P2 - construct
SMA / GFP / DAPI
D
P3 - construct
SMA / GFP / DAPI
E
Normal Uterus
E-Cad / DAPI
F
P1 - construct
E-Cad / GFP / DAPI
G
P2 - construct
E-Cad / GFP / DAPI
H
P3 - construct
E-Cad / GFP / DAPI

Fig 3

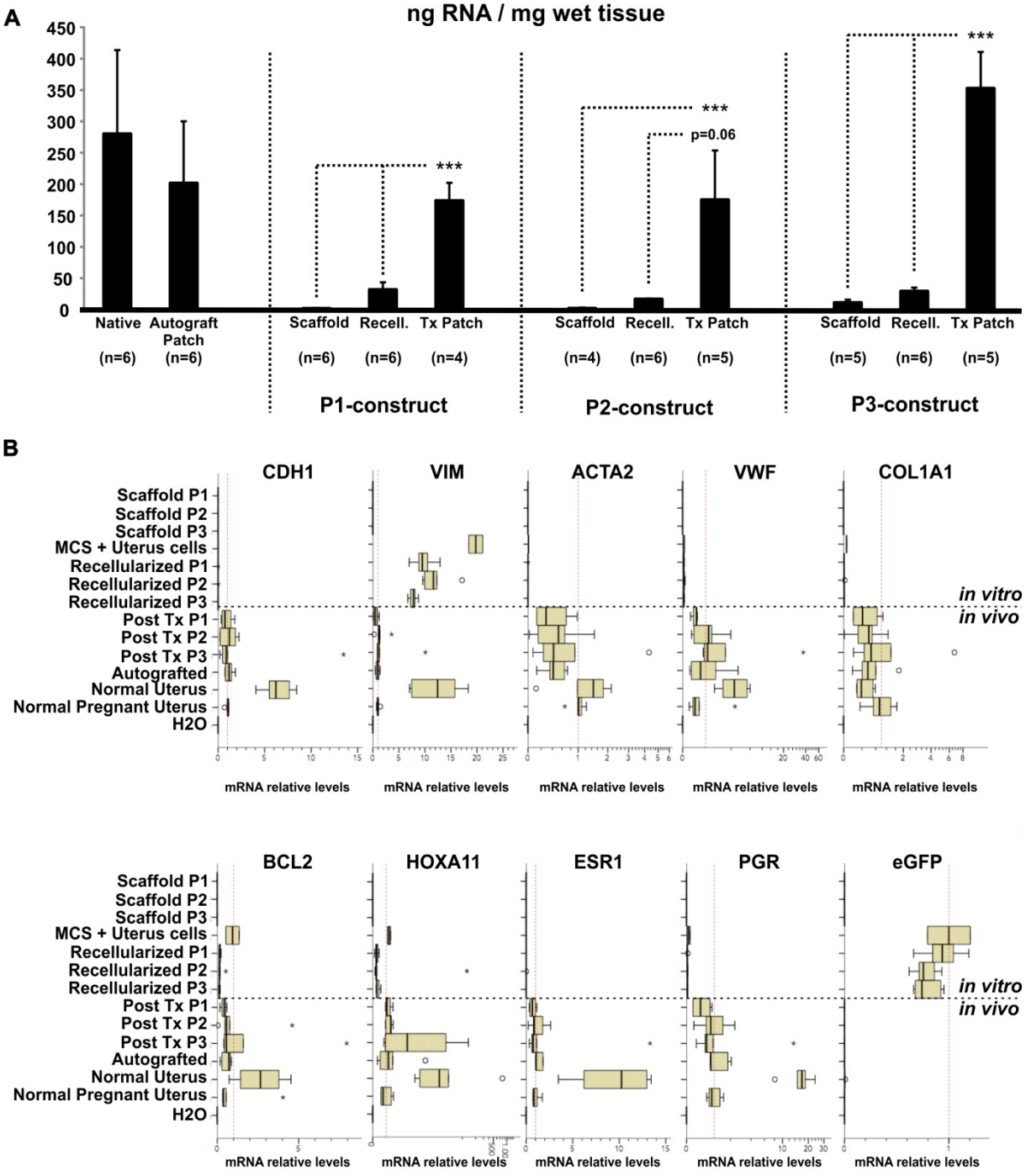
A
ng RNA / mg wet tissue
450
400
350
300
250
200
150
100
50
0
***
***
p=0.06
***
Native
Autograft Patch
Scaffold
Recell.
Tx Patch
Scaffold
Recell.
Tx Patch
Scaffold
Recell.
Tx Patch
(n=6)
(n=6)
(n=6)
(n=6)
(n=4)
(n=4)
(n=6)
(n=5)
(n=5)
(n=6)
(n=5)
P1-construct
P2-construct
P3-construct
B
CDH1
VIM
ACTA2
VWF
COL1A1
Scaffold P1
Scaffold P2
Scaffold P3
MCS + Uterus cells
Recellularized P1
Recellularized P2
Recellularized P3
Post Tx P1
Post Tx P2
Post Tx P3
Autografted
Normal Uterus
Normal Pregnant Uterus
H2O
in vitro
in vivo
mRNA relative levels
BCL2
HOXA11
ESR1
PGR
eGFP
Scaffold P1
Scaffold P2
Scaffold P3
MCS + Uterus cells
Recellularized P1
Recellularized P2
Recellularized P3
Post Tx P1
Post Tx P2
Post Tx P3
Autografted
Normal Uterus
Normal Pregnant Uterus
H2O
in vitro
in vivo
mRNA relative levels

Fig 4