Tissue- engineering as an adjunct to pelvic reconstructive surgery

New potential concepts evaluated in animal studies

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STUDIES INCLUDED IN THE PHD THESIS

The thesis is based on the following studies, which will be referred to by their number.

1. Jangö H, Gräs S, Christensen L, Lose G. Muscle fragments on a scaffold in rats: a potential regenerative strategy in urogynecology. Int Urogynecol J. 2015 Dec 24;26(12):1843–51.[1]
2. Jangö H, Gräs S, Christensen L, Lose G. Tissue-engineering with muscle fiber fragments improves the strength of a weak abdominal wall in rats. Int Urogynecol J. 2017 Feb 16;28(2):223–9.[2]
3. Jangö H, Gräs S, Christensen L, Lose G. Examinations of a new long-term degradable electrospun polycaprolactone scaffold in three rat abdominal wall models. J Biomater Appl. 2017 Feb;31(7):1077–86.[3]
4. Jangö H, Gräs S, Christensen L, Lose G. Modification and evaluation of a transabdominal vaginal model – a pilot study in rabbits. (unpublished data)

INTRODUCTION AND BACKGROUND

DEFINITION OF PELVIC ORGAN PROLAPSE

Pelvic organ prolapse (POP) is defined as descent of the anterior vaginal wall, the posterior vaginal wall, the uterus (cervix), or the apex of the vagina (vaginal vault or cuff scar after hysterectomy)[4]. The descent can involve one or more compartments and should be correlated with relevant POP symptoms[4]. Typical POP symptoms include seeing or feeling a bulge in the vaginal introitus, increased heaviness, and compartment-related symptoms such as urinary incontinence or voiding dysfunction (anterior compartment; cystocele), anal incontinence and defecation problems (posterior compartment, rectocele)[4]. Symptoms often occur when the descent reaches the level of the hymen or beyond, but are often not specific for the compartment involved[4].
Anatomic findings of POP can be found in more than 50% of women over 40 years of age[5], but most of these women are asymptomatic. POP related symptoms are reported by 8-12% of women[6,7] and the diagnosis is based on both subjective symptoms and objective findings[8]. In women with POP referred from general practitioners, 75% have affected quality of life[9] and women with advanced stage of POP have affected quality of life[10].
Conservative approaches for POP treatment consist primarily of pelvic floor muscle training[11] and the use of a pessary, a passive mechanical device that supports the vagina[12,13]. Surgical treatment of POP aims at restoring the normal vaginal anatomy and restoring or maintaining normal bladder, bowel and sexual function[14]. In Denmark, 80-year old women have a 18.7% lifetime risk of undergoing POP repair[15]. The corresponding risks in the UK and the US are 9.5%[16] and 12.6%[17], respectively.

RECONSTRUCTIVE SURGERY FOR PELVIC ORGAN PROLAPSE

The most common surgical procedures for POP are anterior and posterior repair (colporrhaphy) for anterior and posterior POP, respectively[14]. In colporrhaphy, the native tissue is repaired by plication, i.e. folding and suturing of the fascia underneath the vaginal epithelium. Together, anterior and posterior compartment surgery account for more than 90% of reconstructive surgery of POP[18]. However, several other vaginal approaches exist along with different abdominal approaches[14].
Anatomical failure rate after POP surgery has previously been reported to vary between 29% and 70%[18,19]. The recognition of this initiated a time period with the introduction of biological and synthetic materials to reinforce the tissue at POP surgery[20], but according to more recent results the risk of repeated POP surgery is still 15.8%[16]. In contrast, a Danish study found that 2% had repeat surgery 1-5 years after conventional vaginal repair[21].
The use of degradable biological materials combined with native tissue repair has generally failed to improve clinical outcome compared to native tissue repair alone[22]. Synthetic permanent meshes, with polypropylene being the most common, have shown improved anatomical outcome and reduced recurrence rate compared to native tissue repair[14,23]. However, the studies

comparing anterior colporrhaphy with and without mesh reinforcement failed to find any differences in symptoms and quality of life between groups[14]. Furthermore, POP in the other compartments was found to be more common after mesh surgery than after native tissue repair[14]. This supports the theory that POP is caused by failure of both local fascia tissue support and of ligament suspension[24]. Failure of local fascia tissue support, i.e. of the pubocervical and the rectovaginal fascia (midlevel vaginal support) causes prolapse of the anterior and posterior compartment (cystocele and rectocele, respectively), while failure of the suspensory fibers of the paracolpium and parametrium (upper vaginal support) causes vaginal and uterine prolapse[25,26].

In 2008 the FDA (the US Food and Drug Administration) issued a public warning because of recognition of serious and potentially life-threatening adverse events related to the use of permanent synthetic meshes placed transvaginally during reconstructive surgery[27]. In 2011, the FDA launched an update, stating that these adverse events are not rare and that the use of mesh does not improve outcome[28]. In 2016, the FDA announced two orders sharpening the requirements for using transvaginal surgical mesh in POP repair[29]. The first order was a reclassification of mesh from class II to class III, which generally includes high-risk medical devices. The second order was a requirement for the manufacturers to submit rigorous premarket approval applications to prove safety and effectiveness of the mesh.

Correspondingly, in 2015 the SCENIHR (Scientific Committee on Emerging and Newly Identified Health Risks) published an opinion for the European Commission on the use of transvaginal meshes for POP surgery[30]. They concluded that these mesh types should only be considered in complex cases, in particular after failed primary repair surgery or when primary surgery was expected to fail. They also emphasized the need for further improvement in the composition and design of synthetic meshes for future progress in pelvic reconstructive surgery.

REGENERATIVE MEDICINE AND TISSUE-ENGINEERING

Stem cells are generally defined by their ability to confer self-renewal, their ability to form clonal cell populations, and their ability to differentiate into a number of different cell-types[31]. During the last two decades, intensified stem cell research has been employed with the purpose of developing new tissues or even new organs in different medical fields.

In regenerative medicine, the aim is to create a functional tissue for use in the repair or replacement of a tissue function that has been lost due to damage, disease or age. Tissue-engineering is often used as a synonym to regenerative medicine, but traditionally it refers to the use of a scaffold in combination with stem cells[32]. Most regenerative medicine strategies use ex vivo culturing of cells[33]. The use of trophic factors and other biologically active molecules can be used as alternatives or as adjuncts to cell-based therapies. Facilitated endogenous repair represents a branch of tissue-engineering, which uses biological stimuli and manufactured scaffolds while avoiding the culturing of cells[33].

Regenerative medicine in urogynecology

In urogynecology, the use of stem or progenitor cells has been most intensely studied in connection with injection treatments for stress urinary incontinence, where primarily muscle derived cells have been injected into the urethral sphincter with the objective to restore its function[34]. Numerous animal studies[35–37] and a growing number of clinical studies[36–40] have been carried out to evaluate the effect of these treatments, but although results from animal studies have provided promising proof of concept, results obtained from clinical studies have only shown moderate effect[34,37].

Similarly, several animal studies[37] and one clinical study[41] have been published and five ongoing clinical studies (www.clinicaltrials.gov) investigate the potential of treating anal incontinence with muscle derived stem cell injections to improve the function of the external anal sphincter.

In 2009, Ho et al. were the first to evaluate a tissue-engineering strategy for the treatment of POP in a rat vaginal model by seeding skeletal muscle-derived stem cells on scaffolds of porcine small intestine submucosa[42]. They demonstrated that muscle-derived stem cells differentiated into smooth muscle cells when implanted in the rat vagina, which could be beneficial in the treatment of POP[42]. However, transdifferentiation of muscle derived stem cells has not been demonstrated by others. A yet limited number of experimental studies have since examined different candidate cells and scaffolds for potential POP repair[43–50], but to date no clinical studies have been identified (www.clinicaltrials.gov and www.clinicaltrialsregister.eu).

CANDIDATE SCAFFOLDS

Generally, three classes of scaffolds for tissue-engineering exist[51]:

- Naturally derived materials, e.g. silk or collagen
- Acellular tissue matrices, e.g. small intestine submucosa or dermis
- Synthetic polymers, e.g. polypropylene or MPEG-PLGA

Naturally derived materials and acellular tissue matrices might be advantageous due to biological recognition, but synthetic polymers can be produced in a large scale and allow for controlling and alteration of specific properties[51]. Mangera et al. evaluated seven different natural or synthetic candidate scaffolds with regard to cell attachment, the formation of extracellular matrix (ECM) and biomechanical properties resembling those of native tissue[52]. They seeded oral fibroblasts on the scaffolds and found that the synthetic electrospun poly(L)-lactic acid and the natural small intestine submucosa both showed superior cell attachment and increased collagen and elastin formation[52]. Poly(L)-lactic acid showed biomechanical properties that were closest to those of native tissue[52] and it was concluded that this scaffold might be the preferable for tissue-engineering application in POP surgery[52,53]. However, several unanswered questions regarding the choice of scaffold still remain. Degradation time, biocompatibility and the modification of biomechanical properties, which is caused by in vivo implantation as well as the specific combination effect of added cells, must all be considered.

For the purpose of POP repair, a tissue-engineering concept should contribute to restoration of normal function by restoring native, healthy tissue properties and anatomy. Ideally, the scaffold should function as an anchor for the added stem cells, providing a three-dimensional structure for the cells to grow on, and at the same time provide tissue reinforcement. The added cells would grow on and into the scaffold and be integrated with the host tissue while the tissue is held in place by the scaffold. When the scaffold is fully degraded, the cells should have formed new functional tissue, capable of providing durable anatomical correction and thus, functional restoration.

CANDIDATE CELL SOURCES

Embryonic stem cells are derived from totipotent cells of the early embryo and are capable of unlimited, undifferentiated division[54,55]. The culturing of these cells was first described from

mouse embryos in 1981[54,55] whereas culturing of human embryonic stem cells was first described in 1998[56]. The embryonic stem cells can differentiate into all adult cell types and have a great therapeutic potential. However, the clinical use of these cells is limited by 1) the risk of tumorigenicity, 2) immunogenicity since the cells are allogenic, 3) ethical concerns, and 4) extensive regulatory demands[57,58].

Adult (somatic) stem cells are multipotent or unipotent cells that are capable of proliferating and differentiating into one or more phenotypic cells and tissues[59]. Bone-marrow derived mesenchymal stem cells (MSCs) were the first adult stem cells to be described[60] and can be obtained from almost all tissues of the body[61]. The use of adult stem cells is also subjected to strict regulatory demands, but is considered safe and without ethical concerns.

There are three groups of candidate cell sources for tissue-engineering application in POP reconstructive surgery[62]:

- Muscle-derived stem cells, harvested from skeletal muscle
- MSCs, e.g. adipose or bone marrow MSCs
- Fibroblasts

The specific combination of cell source, scaffold and biological stimuli is complex, and although several cells have shown promising results for POP repair[42–45,50,63] a superior cell source remains to be identified[64].

Muscle fiber fragments

The inclusion of in vitro cultured cells for clinical use is considered an 'advanced therapy' medicinal product by EMEA (European Medicines Agency) (European Union Regulation 1394/2007) and is thus limited by strict regulatory demands[36]. In addition, an increasing number of animal studies have demonstrated that the proliferative potential of muscle cells may be compromised by the culturing process[65,66].

In contrast, minced skeletal muscle has a remarkable regenerative ability, which was described already decades ago by Studitsky[67] and Carlson[68]. This technique has recently been reintroduced by Corona et al. for potential use in tissue-engineering of volumetric loss of muscle tissue[69]. Skeletal muscle tissue contains muscle stem cells called satellite cells[70] that can undergo asymmetric division into new, quiescent satellite cells and proliferating myoblasts[71]. The preparation of autologous fresh muscle fiber fragments (MFFs) is easy, quick, inexpensive, and does not depend on advanced facilities or technologies[64]. The MFFs can be harvested, prepared and applied during the same surgical procedure, which makes the method clinically attractive. Moreover, the MFFs are exempted from the strict regulatory demands associated with the clinical use of cultured muscle cells[64].

Our research team has previously shown that MFFs formed striated muscle tissue when they were seeded on methoxypolyethyleneglycol-poly(lactic-co-glycolic acid) (MPEG-PLGA) scaffolds and implanted in the rat abdominal wall[44]. Our team has further performed a clinical study, injecting MFFs into the urethral sphincter of women with stress urinary incontinence[39]. This study showed a beneficial effect, which was comparable to that found by others using cultured stem cells[72–76].

TROPHIC FACTORS

Tissue-engineering strategies for POP repair may benefit from the addition of bioactive substances to the cell scaffold complex[64], since the tissue metabolism of the vaginal wall is altered in women with POP[77].

Estrogen has an important role in the regulation of lower urinary tract function[78]; however, its importance for the development of POP is controversial[64]. Clinical data have indicated that systemic administration of estrogens in postmenopausal women with urinary incontinence would actually worsen the symptoms[79]. This may be caused by the fact that estrogen decreases the production of vaginal smooth muscle cells and tropoelastin[80]. Yet, locally administered estrogen might still improve urinary incontinence[79] and symptoms of POP[81,82], although robust evidence is lacking. The mechanisms of estrogens in the treatment of POP needs to be further evaluated[78].

In patients with stress urinary incontinence, platelets have been added to the autologous cells injected to the urethral sphincter to improve the regenerative potential[83]. Platelet-rich plasma is known to release bioactive factors that affect the response in muscle regeneration[84], since it contains growth factors and bioactive proteins with fundamental effects on tissue repair[85].

Several studies have shown that only a small percentage of added MSCs for regenerative purposes are actually integrated into the host damaged tissue despite substantial tissue repair[86]. This indicates that the tissue repair is caused indirectly by a paracrine effect of the MSCs and not by the MSC engraftment[86]. The stem cell secretome is defined as a complex set of secreted molecules from stem cells that are essential for several biological functions, for example cell growth, differentiation, signaling, adhesion and angiogenesis[31]. Studies have shown that MSCs probably exert their regenerative function by the secretion of cytokines, growth factors and extracellular vesicles[31]. The secretome strategy circumvents the implantation of cells[86], which underlines the crucial role of the trophic factors in the regenerative process.

CANDIDATE ANIMAL MODELS

No consensus exists with regard to the optimal animal model for POP repair[87], and different animal models and species have been explored in basic urogynecology research. Currently, rodents are the most frequently used animals. They are inexpensive and easy to work with in large numbers[87], but vaginal implantation is difficult. Larger animals like rabbits and sheep allow the use of vaginal implantation[87], but transvaginal implantation of POP meshes in these species generally results in high exposure rates[88]. This high risk of exposure may be caused by the contaminated environment and anatomical differences amongst species. In addition, the rabbit vagina is regionally different in terms of histology and function[89] and this may affect the outcome if the meshes are implanted at different sites. Non-human primates like squirrels or rhesus macaque monkeys are bipedal and can spontaneously develop POP after delivery. They allow testing in a model that largely mimics that of women with POP[90], but the use of these primates is subjected to strict ethical regulations and considerable costs[87,90].

Despite several feasible models, no consensus regarding the optimal animal model for POP repair exists[87,90,91]. The models are thus chosen according to the specific research question, since different models provide different aspects in terms of structural and biomechanical responses[87,91].

AIMS

The overall aim of this thesis was to evaluate new tissue-engineering strategies that could serve as adjuncts to reconstructive pelvic surgery.

The specific aims of the thesis were:

- To confirm our previous results and to further evaluate the potential regenerative effect of MFFs seeded on an MPEG-PLGA scaffold in terms of histological and biomechanical properties in two rat abdominal wall models.
- To evaluate if a newly developed electrospun PCL scaffold would be able to 1) provide biomechanical tissue reinforcement and 2) act as a carrier for muscle stem cells in the form of MFFs in different rat abdominal wall models.
- To assess a new rabbit vaginal model for the evaluation of tissue-engineering concepts in POP repair.

MATERIALS AND METHODS

COMMON METHODOLOGY STUDY 1-3 – RAT STUDIES

ANIMALS

Animal housing and caretaking was provided by the Animal Facility at Panum Institute, University of Copenhagen, Denmark. The Danish Animal Experiments Inspectorate approved the study (permission no. 2012-15-2934-00242), and their guidelines for care and use of laboratory animals were followed. We used a total of 72 Sprague Dawley retired female breeders, weighing 245-315 grams (Taconic, Lille Skensved, Denmark).

SCAFFOLDS

MPEG-PLGA

MPEG-PLGA is a quickly degradable, freeze-dried scaffold - spongy and porous - with multiple interconnected pores[92]. Compared to PLGA (Vicryl, polyglactin mesh), the MPEG-PLGA is more hydrophilic[92]. The MPEG-PLGA scaffold has been tested in animal models for cartilage repair where it was found to enable regeneration of the cartilage defects[93], and the MPEG-PLGA scaffold has been CE-marked for cartilage repair.

Our research team has previously demonstrated that the MPEG-PLGA was fully degraded eight weeks after subcutaneous implantation in rats[44,92], and that MFFs seeded on the scaffold had generated fragmented striated muscle tissue[44].

PCL

Since the MPEG-PLGA scaffold is fragile, with no inherent strength, we wanted to evaluate a stronger, degradable scaffold. PCL is an FDA approved polymer, which was used to form an electrospun scaffold comprising a thin layer of randomly spun fibers with a mean thickness of 1.58 µm (standard deviation (SD) ±0.96 µm) (Coloplast A/S, Humlebaek, Denmark). The electrospinning process allows engineering of scaffolds to mimic native ECM with high porosity and a nanoscale topography[94,95]. The estimated degradation time of PCL has been found to be approximately two to four years[96]. The electrospun PCL scaffold used in our studies has previously been tested in vitro and it was demonstrated that cultured human fibroblasts can successfully be seeded on the scaffold[97]. The PCL scaffold has not previously been tested in vivo.

ABDOMINAL WALL MODELS

In total, we used four different rat abdominal wall models (Table 1). The more fragile MPEG-PLGA scaffold was tested in two models, and the PCL scaffold was tested in three models.

Table 1. Overview over scaffolds and models used in the different rat studies.

Study	Scaffold	Models used; with and without MFFs			
		Native tissue repair model	Partial defect model	Full thickness defect model	Subcutaneous model
Study 1	MPEG-PLGA	X			
Study 2	MPEG-PLGA		X		
Study 3	PCL		X	X	X

MPEG-PLGA models: Study 1 and 2

The two different models for the MPEG-PLGA scaffold were:

- Native tissue repair model (Study 1): a small full-thickness sample of the abdominal wall was removed, the defect was sutured and the scaffold was placed on the repaired defect (Figure 1). The model was modified from Ozog et al.[98].
- Partial defect model (Study 2): The most superficial muscle layer was removed and replaced by the scaffold (Figure 2). The partial defect model was modified from Valentin et al.[99].

Figure 1. Native tissue repair model: overview of the three groups in Study 1. Adapted from[1].

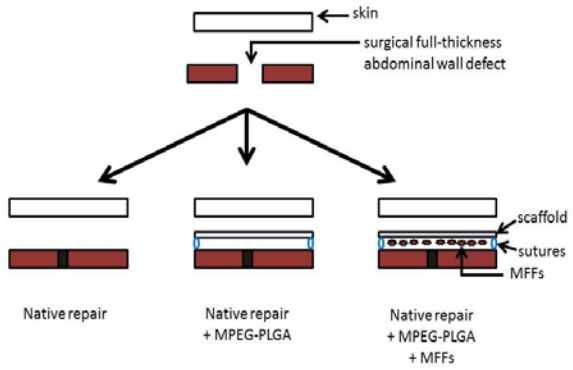
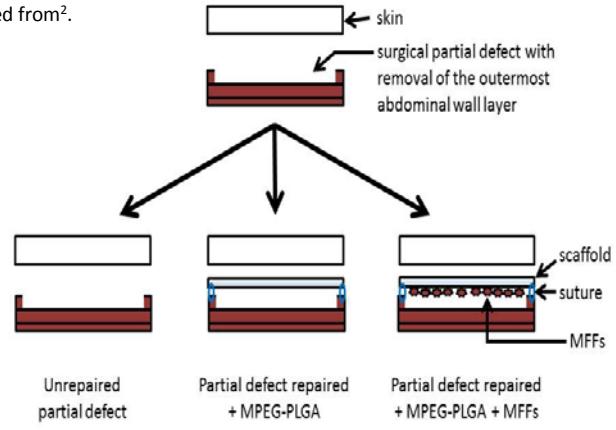


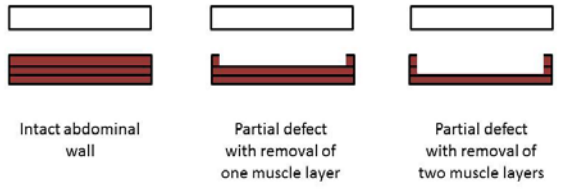
Figure 2. Partial defect model: overview of the three groups in Study 2. Adapted from[2].



Thus, both Study 1 and 2 consisted of 3 groups each, with six animals in each group.

Prior to Study 2, we performed an initial evaluation of the surgical feasibility and the biomechanical strength of the partial defect in euthanized rats. We compared the normal abdominal wall (n=9) with a partial defect, where 1) only the outermost layer was removed (n=3) and where 2) the two outermost layers were removed (n=3) (Figure 3). As removal of a single layer was easier to perform and the two partial defect models appeared to be comparable upon biomechanical testing the partial defect with removal of one muscle layer only was chosen for Study 2.

Figure 3. Two different partial defect models: overview of the three groups compared in the initial study for Study 2.



PCL models: Study 3

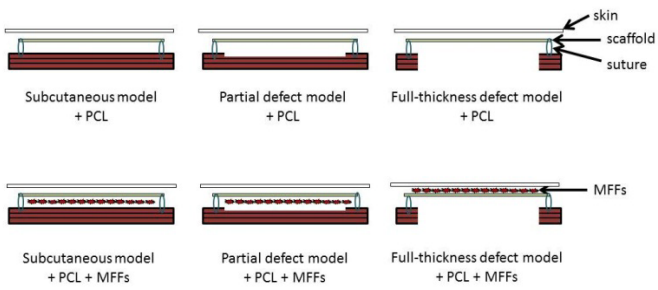
In study 3, we evaluated the tissue and the biomechanical responses to the PCL scaffold in three abdominal wall models with different loads (Figure 4):

1. Subcutaneous placement, corresponding to the model tested by Boennelycke et al.[44,92], in which the scaffold was placed on the intact abdominal muscle layers. There was no load on the scaffold.
2. Partial defect model, corresponding to the aforementioned model in Study 2 and modified from Valentin et al.[99], where the outermost muscle layer was removed. The scaffold was subjected to some load.
3. Full-thickness defect model, where all three muscle layers were removed[100] and replaced by the PCL scaffold. The scaffold was subjected to maximal load.

The full-thickness model was chosen to evaluate whether the PCL scaffold was capable of providing biomechanical tissue reinforcement even in case of considerable load.

Each of the three models had implanted PCL scaffolds with and without MMFs, thus leading to a total of six groups with six animals in each group:

Figure 4. Overview of the six groups in Study 3. Adapted from[3].



IMPLANTATION

The rats were anesthetized with Hypnorm/Midazolam 0.3 ml/100 g (Hypnorm, VetaPharma Ltd., Leeds, UK and Midazolam, Hameln Pharmaceuticals GmbH, Hameln, Germany). We performed a midline skin incision on the abdomen followed by subcutaneous blunt dissection.

Surgeries for the different models were performed as follows:

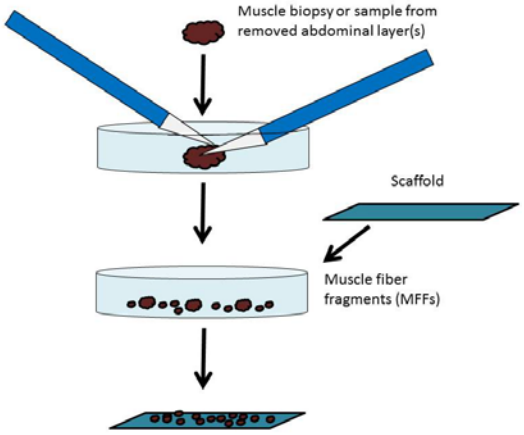
- In the native tissue repair model (Study 1), a longitudinal full-thickness portion of the abdominal wall measuring approximately 3.0×0.1 cm was resected. The defect was sutured continuously with Vicryl 4-0. For later identification and location, one non-absorbable Prolene 5-0 suture was placed in each end of the repaired defect.
- In the partial defect model (Study 2 and 3), a defect was created by removing the outermost muscle layer lateral to the rectus muscle, over an area measuring 3.0×1.5 cm.
- In the subcutaneous model (Study 3), the scaffold was placed on the intact abdominal wall layers.
- In the full-thickness model (Study 3), all three muscle layers were removed over an area measuring 3.0×1.5 cm, lateral to the rectus muscle.

For partial and full-thickness defect models, the defected areas were marked using a grid. In all cases of scaffold implantation, these were placed longitudinally to the midline covering the defect. All scaffolds measured 2.5×4.0 cm, thus, oversizing the defect with 0.5 cm on each border for the partial defect and the full-thickness defect. The implants were held in place by four non-degradable Prolene sutures, one stitch in each corner for later identification, followed by a continuous degradable Vicryl 4-0 suture along the borders. The skin was closed using staples (Reflex One, REF 3036, ConMed, Utica, NY, USA). Antibiotic prophylaxis and analgesia were administered according to veterinarian recommendations.

PREPARATION AND LABELING OF THE MFFS

In the native tissue repair model, the partial defect model and in the full-thickness defect model, the removed abdominal wall muscle was used for preparation of the MFFs. In the subcutaneous model, a muscle biopsy from the thigh was obtained, using a biopsy punch of 4 mm. The muscle tissue was placed in a sterile petri dish and cut into fine pieces using two scalpels (Figure 5). The resulting MFFs were labeled with the fluorescent dye PKH26 (Sigma-Aldrich, St. Louis, MO, USA). The PKH26 have long aliphatic tails that bind irreversibly to lipid regions of the cell membrane[101], and was chosen since it is traceable several weeks after in vivo implantation[42,102].

Figure 5. Preparation of MFFs. Adapted from[64].

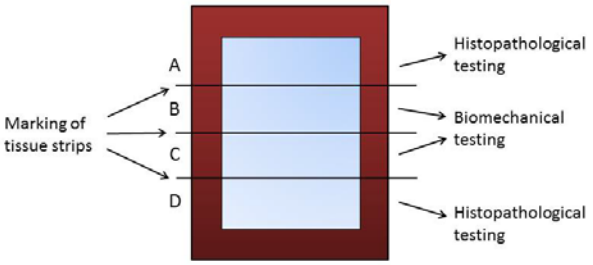


The labeled MFFs were then applied to the scaffold as a thin layer on the surface. The preparation and labeling of the MFFs were performed while the animal was anesthetized. At implantation, the scaffold was placed with the MFFs facing down, i.e. between the scaffold and the abdominal muscle layers, in all models except the full-thickness model, where the MFFs were located on the superior surface of the scaffold instead, i.e. between the scaffold and the skin (Figures 1-3).

EXPLANTATION

After eight weeks, the rats were euthanized by cervical dislocation. A midline skin incision was performed followed by subcutaneous blunt dissection to present the abdominal wall musculature with attached subcutaneous and fascia tissue. Sutures in the corners were located and 1.0 cm wide tissue strips were marked prior to removal (Figure 6). A full-thickness sample of all abdominal layers was removed en bloc, including the area of implantation and surrounding tissue. After removal, the tissue was cut into four strips (Figure 6), where strips A and D were used for histological testing and strips B and C for biomechanical testing.

Figure 6. Marking of tissue-strips before removal. Adapted from[1–3].



Histopathology and immunohistochemistry

Tissue samples were fixed in 10% buffered formalin, embedded in paraffin and cut in 5 µm sections. All samples were stained with hematoxylin and eosin (H&E). Samples from Study 1 and 2 were stained with van Gieson/alcian blue (van Gieson acid fuchsin solution, Sigma Aldrich, HT 254; alcian blue, Dako, pH 2.3, code no. AR 160) and samples from Study 3 were stained with Masson trichrome (Sigma-Aldrich, St. Louis, MO; USA). The neighboring sections were immuohistochemically analyzed for desmin (Dako, Glostrup, Denmark), a cytoplasmic marker of smooth and skeletal muscle. The paraffin embedded sections were stained using the EnVision FLEX+ (Dako, Glostrup, Denmark) polymer peroxidase diaminobenzidine system, and a Trisethylenediaminetetraacetic acid (EDTA) solution pH9.0 (Dako) was used to perform heat-induced epitope retrieval. Anti-human desmin mouse monoclonal antibody (Dako IR 606 ready-to-use), which cross-reacts with both mouse and rat proteins, was applied for 20 min at ambient temperature in the Dako Autostainer Link 48.

In Study 3, the quantity of giant cells was calculated as a percentage of nuclei using H&E stained specimens. Also, in Study 3, we performed orcein staining (Orcein Stain Kit, Artisan, Dako, Denmark) to stain the elastin fibers.

The slides were viewed under an Olympus BX60 Microscope (Olympus, Center Valley, PA, USA). Images were analyzed using the Image-Pro Plus 7.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Bonar score

In Study 1 and 2, we evaluated histopathological characteristics of the connective tissue after full degradation of the MPEG-PLGA scaffold using the semi quantitative Bonar score. The Bonar score (range 0-20), which was recently published by Fearon et al.[103], provides a standardized evaluation of five distinct parameters of the regenerative process of connective tissue: cell morphology, collagen arrangement, cellularity, vascularity, and ground substance tissue response. The score uses a predefined number of fields that require evaluation at a predefined magnification[103].The score was originally established for the evaluation of tendon injuries[104,105] and a higher score represents a more advanced stage of the regenerative repair process. We assessed collagen arrangement with the additional use of polarization filter imaging of the van Gieson-stained fibers. The ground substance was evaluated using the alcian blue staining for mucopolysaccharides; the other outcomes were assessed using the H&E stained specimens. Assessment of Bonar score was performed by senior pathologist L.C. who was blinded to group allocation.

The Bonar score was not used in evaluation of the PCL implants (Study 3) because the PCL was not degraded, and apart from a few collagen fibers and scattered blood vessels no measurable signs of de novo connective tissue production were found.

FLUORESCENCE

To detect PKH26 fluorescence, frozen samples were cut into 16-µm sections and evaluated in a fluorescence microscope Olympus BX51 (Olympus, Center Valley, PA, USA). If no fluorescence was detected, the sample was re-cut and examined further to ensure the absence of fluorescence-positive cells in nearby foci.

UNIAXIAL BIOMECHANICAL TESTING

Directly after removal, the tissue for biomechanical testing was placed in sterile petri dishes with sterile phosphate-buffered saline (PBS). Approximately 2-4 hours after removal, the samples were tested using a TA.XT plus Texture Analyser (Stable Micro Systems, Godalming, Surrey, UK) with a 5 kg load cell and TA 94 Pneumatic Grips (Thwing-Albert Instrument Company, West Berlin, NJ, USA), using a pressure of 3 bar. Testing was performed in a controlled environment with a constant temperature of 23°C and a relative humidity of 50%. To secure a tight grip without squeezing the tissue, the clamps were modified with a grip paper (3M). A preload of 0.1 N was applied to the inserted tissue strips to remove slack and the grip-to-grip distance was measured and defined as elongation of zero. Two tissue samples, strips B and C, from each rat were tested (Figure 6). Strip B was inserted with a grip-to-grip separation of 1.0 cm, while strip C was inserted with a grip-to-grip separation of 3.0 cm. Only the part of the tissue placed between the grips was subjected to testing. Thus, strip B-testing only involved the area with scaffold and underlying tissue or defect, whereas strip C-testing also involved the surrounding normal tissue (Figure 7). For strip B, the clamps moved with a speed of 0.333 mm/second. For strip C, they moved with a speed of 1 mm/second. The difference in speed between the two grip-to-grip distances was chosen to ensure constant strain-rate.

Load (N) and elongation (mm) were recorded until failure. The biomechanical results were analyzed with Exponent Version 6,1,3,0 software (Stable Micro Systems, Surrey, UK). Load was plotted against elongation, forming bilinear curves with a low- and a high stiffness zone (Figure 8). Data were reported as stiffness in the low- and high-stiffness zones (N/mm), load at failure (N) and elongation at failure (mm).

Figure 7. Biomechanical testing of tissue strip B, only including the central part of the scaffold, and strip C, also including the surrounding tissue. Adapted from[3].

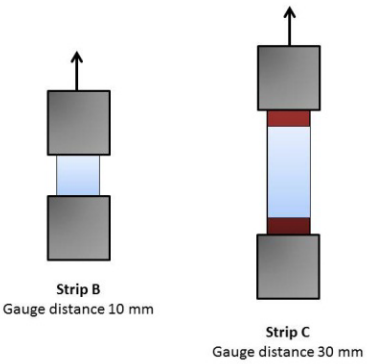
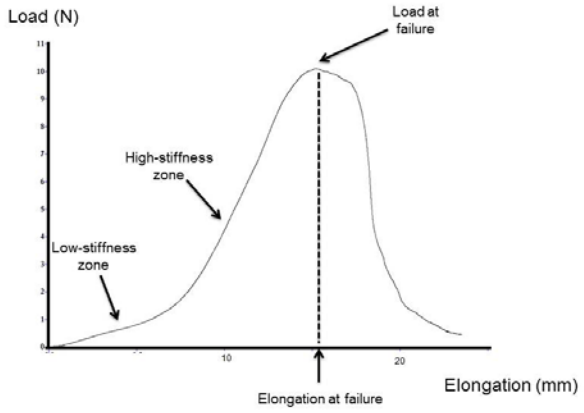


Figure 8. Load-elongation curve visualizing the low- and the high-stiffness zones, load at failure and elongation at failure. Adapted from[1].



Methodology Study 4 – Pilot study in rabbits

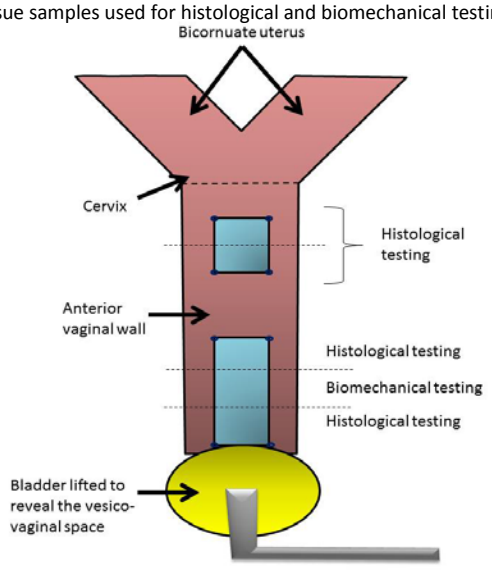
We performed a feasibility pilot study to evaluate a new vaginal model in rabbits. The study was approved by the Danish Animal Experiments Inspectorate (permission no. 2013-15-2934-00842/ACHOV) and guidelines for care and use of laboratory animals were followed. Animal husbandry was provided by the Department of Experimental Medicine, Frederiksberg Campus, University of Copenhagen, Denmark.

The vaginal model was based on a model previously presented by Zhang et al. who used an abdominal approach and placed polypropylene meshes in the vesico-vaginal space of the rabbit[106]. We used a total of four female New Zealand white rabbits, weighing 3.3-4.0 kg. The animals were anesthetized with an intramuscular injection of Ketamin 35 mg/kg and Xylazin 10 mg/kg, supplemented with inhalation of Isoflurane.

The experiments were performed in a conventional operating theatre; the abdomen was shaved, disinfected and draped in a sterile fashion. An intramuscular injection of antibiotics (Streptocillin 0.1 ml/kg) and a subcutaneous injection of analgesics (Rimadyl 4 mg/kg and Temgesic 0.1 ml/kg) were administered. A vertical midline incision through the skin, measuring approximately 5 cm, was performed, the bicornuate duplex uterus was identified and lifted up through the skin incision to present the vaginal wall, and the peritoneum between the bladder and the vagina was cut open to access the anterior vaginal wall. We performed a partial vaginal wall defect by creating two superficial longitudinal incisions: one incision of 1.5-2.0 cm in length was performed on the anterior vaginal wall in the vesico-vaginal space and another incision measuring 1.0-1.5 cm in length was performed on the anterior vaginal wall close to the cervix. The distal ends of both incisions were marked by single Prolene 5-0 sutures for later identification of the area. In two rabbits, the incisions were left unsutured, and no scaffolds were inserted. In one of the other two rabbits, a scaffold measuring 1.0×2.0 cm was placed longitudinally covering the incision in the vesico-vaginal space, and another scaffold measuring 1.0×1.0 cm was placed covering the incision close to the cervix (Figure 9). In one of the two rabbits the scaffold material was MPEG-PLGA and in the other the scaffold was the electrospun PCL, both scaffolds are described earlier. In both animals with scaffolds, a muscle biopsy from the abdominal wall incision was harvested and used to produce MFFs labeled with PKH26 as described above. After having placed the MFFs between the scaffolds and the vaginal wall, the vagina and uterus were again placed in the abdomen, the fascia was closed using continuous Vicryl 4-0 suture, and the skin was closed using staples (Reflex One, REF 3036, ConMed, Utica, NY, USA). A total of 30 ml of physiological saline was injected subcutaneously at the different injection sites to ensure rehydration. Intramuscular injection of Antisedan (Zoetis, New Jersey, US) was given immediately postoperatively to reduce sedation time. The animals were given postoperative antibiotics and analgesia as recommended by veterinarians and were checked daily for the entire observation period. After eight weeks, the rabbits were euthanized by injection of pentobarbital with lidocaine into the ear veins, followed by cervical dislocation.

At autopsy, the vagina and bladder were identified and evaluated macroscopically, and defects and scaffolds were measured prior to their removal en bloc. The posterior vaginal wall and the bladder were cut open to evaluate erosions and/or signs of infection, and the tissue samples were harvested for histological, fluorescence and biomechanical testing (Figure 9). Biomechanical testing was performed as described for tissue strip B in Study 1-3. The parts of the anterior vaginal wall that were not used for histological or biomechanical testing served as controls for the biomechanical testing.

Figure 9. Overview of placement of scaffolds in the rabbit vaginal model and tissue samples used for histological and biomechanical testing.



STATISTICAL ANALYSES

In Study 1 and 2, the Bonar score was evaluated and data were reported as median and range. Groups were compared using the non-parametric Kruskal-Wallis test of variance. Significant findings were further analyzed with post hoc Mann-Whitney test with inbuilt Bonferroni correction.

Biomechanical data were reported as mean ± SD since data were assumed to be normally distributed after evaluation of quantile-quantile plots. Differences between groups were evaluated with one-way Analysis of Variances (ANOVA). Levene's test was used to test variance of homogeneity between groups. For outcomes with significant difference in variance, one-way ANOVA with Welch correction was used. If significant differences between groups were found, using ANOVA, post hoc multiple comparisons analyses were performed with inbuilt Tukey's correction.

For Study 3, three different models were used with and without MFFs. If no significant differences between the six groups were found, the groups with or without MFFs in the same model were pooled to evaluate the possible differences between models. Discrete data regarding localized infection were presented as numbers (%) and groups were compared using Fisher's exact test.

For all statistical analyses, *P* values <0.05 were considered statistically significant. All statistical analyses were performed using the statistical software R[107].

RESULTS

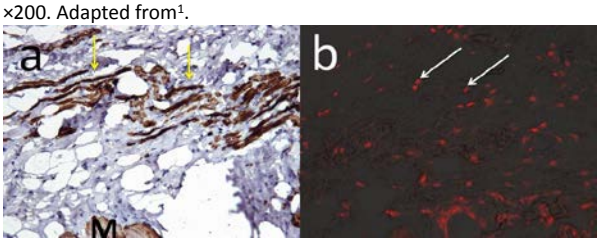
In all studies, surgery and the postoperative period were well tolerated, and no animals developed erosions or hernia. Both the MPEG-PLGA scaffold and the PCL scaffold were easy to handle, although the MPEG-PLGA scaffold was more fragile.

RESULTS: STUDY 1

Histological findings

In the native tissue repair model, we found that the MPEG-PLGA scaffold was completely degraded after eight weeks in all animals. The Bonar score ranged from 5 to 7, and although there was a borderline significant difference between groups (p=0.044), a post hoc test comparing groups revealed no significant differences (p=0.16). In the animals with MPEG-PLGA seeded with MFFs, we found desmin-positive cells forming extra muscle fibers with striation (Figure 10a). PKH26 fluorescence-positive cells were visible in all six animals that had MFFs seeded on the MPEG-PLGA scaffold (Figure 10b), but not in those without MFFs that served as negative controls. The staining pattern of desmin- and PKH26-positive cells differed, but samples were prepared from different locations and not from neighboring sections.

Figure 10. Extra muscle fibers in the group with native tissue repair with MFFs. (a) Desmin immunostaining, with arrows pointing out the extra muscle tissue stained dark brown. Striated muscle tissue of the abdominal wall is marked M. (b) PKH26 fluorescence labeling with arrows pointing out examples of red fluorescence-positive cells. Original magnification ×200. Adapted from[1].



Biomechanical findings

Uniaxial biomechanical testing (see Table 2 in[1]) revealed that the group with MPEG-PLGA seeded with MFFs was significantly stiffer in the high-stiffness zone than the group with MPEG-PLGA alone when the smaller tissue strip B was tested (p=0.032). The group with MPEG-PLGA seeded with MFFs was also borderline significantly stiffer than the native tissue repair group (p=0.054) (Figure 11). Furthermore, we found a decreased elongation at failure for the group with MFFs seeded onto the scaffold compared to the native tissue repair group (p=0.046) (Figure 12). There were no significant differences in load at failure or stiffness in the low-stiffness zone for the smaller tissue strip B. No differences were found between groups when comparing the biomechanical properties of the larger tissue strip C that also included the surrounding normal tissue.

Figure 11. Boxplot of stiffness in the high-stiffness zone in the repair near tissue strip B.

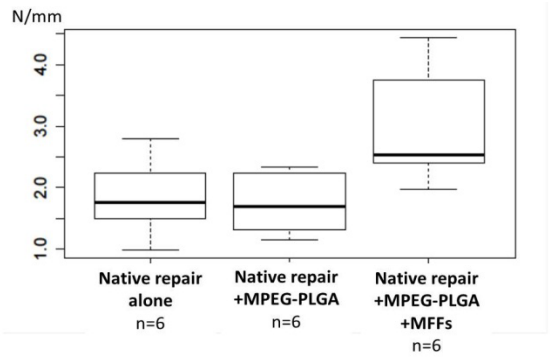
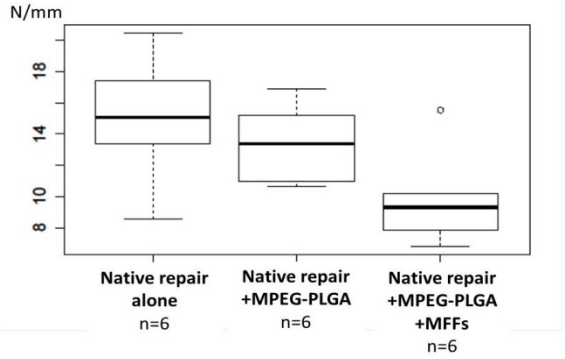


Figure 12. Boxplot of elongation at failure in the repair near tissue strip B.



RESULTS: STUDY 2

Histological findings

When evaluating the MPEG-PLGA scaffold with and without MFFs in the partial defect model, we found no remnants of the MPEG-PLGA scaffold after eight weeks. However, in some animals we found remnants of Vicryl sutures used to secure the scaffold (Figure 13). No significant differences in Bonar score (ranging between 5 and 6) were found (p=0.35).

Irregular desmin-positive muscle cells were found adjacent to the more well-defined remaining muscle layers in all groups (Figure 14). In the partial defect model, the outermost muscle layer had been removed, leaving an uneven surface, and it was not possible to differentiate between the irregular superficial muscle layer and

cells originating from the MFFs (Figures 13 and 14). PKH26 fluorescence-positive cells were only found in those animals that had had their scaffold seeded with MFFs (Figure 15).

Figure 13. Van Gieson/alcian blue staining. Black arrow points out remnants of Vicryl suture, yellow arrows point out fragments from the removed muscle layer or from the MFFs. M marks the normal underlying muscle layers. Original magnificantion ×40.

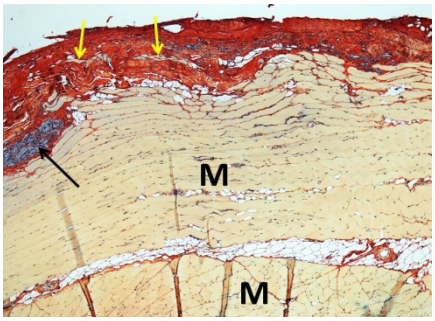


Figure 14. Desmin immunostaining of specimens in Study 2. Arrows point out examples of desmin-positive cells stained dark brown separated from the normal underlying muscle layers. Original magnification ×200. Adapted from[2].

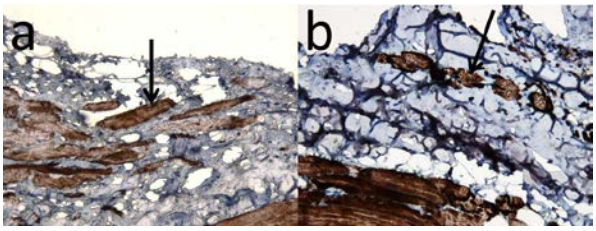
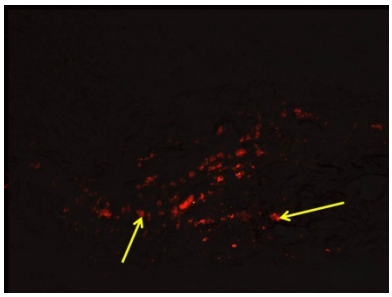


Figure 15. PKH26 fluorescence positive cells found in the group with MFFs in Study 2 are marked with yellow arrows. Original magnification ×200. Adapted from[2].



Biomechanical findings – initial study

In the initial study, we evaluated how removal of one and two muscle layers affected the biomechanical load at failure compared to the normal abdominal wall in rats (Table 2). There was a significant weakening of the abdominal wall when one layer was removed (p=0.006) and also when two layers were removed (p=0.001), but as no differences were found in the weakening between the two defect models (p=0.75), the partial defect model with removal of a single muscle layer was chosen for the final part of Study 2.

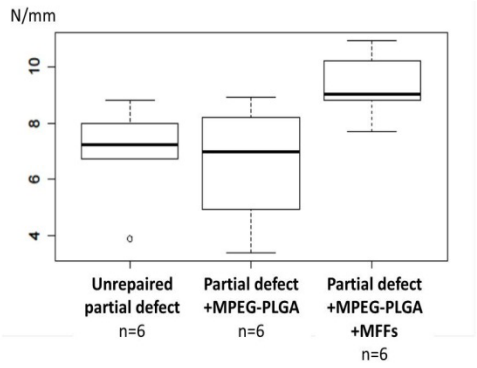
Table 2. Initial study prior to Study 2, evaluation of different partial defect models.

	Normal abdominal wall	**Partial defect, removal of one muscle layer**	**Partial defect, removal of two muscle layers**	**p-value** (ANOVA)
Number of rats	9	3	3	
Number of samples tested	51	10	11	
Load at failure (N) mean (SD)	10.1 (2.5)	4.4 (1.4)	3.2 (0.6)	<0.001

Biomechanical findings – final study

In the final study (see Table 3 in[2]), we found that the group with MPEG-PLGA seeded with MFFs had an increased load at failure compared with the group with MPEG-PLGA alone (p=0.034), for the smaller tissue strip B (Figure 16). This could not be found when comparing load at failure of the larger tissue strip C that also included the surrounding tissue (p=0.25). No significant differences between groups were found for the remaining biomechanical properties in strip B and strip C.

Figure 16. Boxplot of load at failure in tissue strip B that included the partial defect area only.



RESULTS: STUDY 3

Macroscopic findings

In Study 3, we compared three models with different loads. Macroscopically, the PCL scaffold was intact and clearly not degraded after eight weeks (Figure 17). Six animals from different groups (Table 3) had signs of localized infection on the superficial surface of the scaffold (facing the skin). The thickness of the PCL scaffold was increased from approximately 50 µm to 1.6 mm with no differences between groups (p=0.70) (Table 3) or models (p=0.31). The mean shrinkage in the area (measured as length × width before removal of the scaffold) was 11.1% with borderline non-significant differences between groups (p=0.05) (Table 3). When comparing the models, this difference became significant (p=0.014), revealing that scaffolds in the full-thickness model shrunk less than scaffolds in the subcutaneous model (p=0.011).

Figure 17. Macroscopic picture of intact scaffold. Arrow pointing out blue non-degraded corner suture. Adapted from[3].

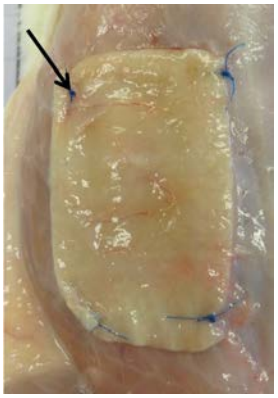


Figure 18. Histological response eight weeks after implantation of the electrospun PCL scaffold (a) H&E staining showing numerous giant cells (orange arrows) located around and between the non-degraded PCL fibers (white arrows). (b) Some collagen formation (stained blue by Masson trichrome staining and marked by yellow arrow) was found between fibers and inflammatory cells. Original magnification ×200. Adapted from[3].

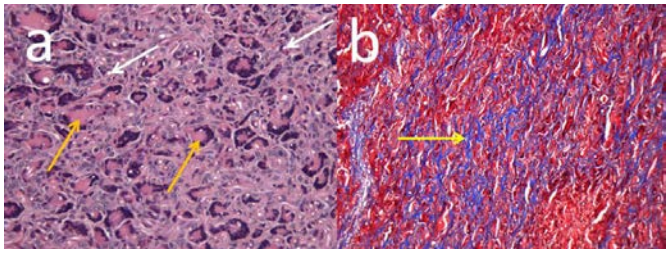


Table 3. Study 3, comparing properties of neo-tissue PCL construct between the groups (adapted from[3]).

Variable of interest	Subcutaneous model		Partial defect model		Full thickness defect model		*p-value* (ANOVA)
	without MFFs	with MFFs	without MFFs	with MFFs	without MFFs	with MFFs	
Thickness (mm)	1.42 (0.30)	1.50 (0.32)	1.63 (0.58)	1.76 (0.40)	1.47 (0.36)	1.58 (0.32)	*0.70*
Shrinkage (%)	15.7 (6.6)	16.2 (5.7)	10.6 (6.8)	12.7 (7.3)	9.6 (14.3)	1.7 (4.3)	*0.05*
Abscess present	2 (33%)	1 (17%)	0	1 (17%)	1 (17%)	1 (17%)	*0.98**
Nuclei (%)	2.4 (0.3)	2.5 (0.4)	2.5 (0.2)	2.6 (0.4)	2.7 (0.3)	2.7 (0.5)	*0.11*

Data are presented as means (SD) or numbers (%). *Fisher's exact test.

Histological findings

Microscopically, we found a massive in-growth of inflammatory cells with large and numerous giant cells located around and between the PCL fibers (Figure 18a). When calculating the percentage of nuclei as a representative of the quantity of giant cells (Figure 19), we found no significant differences between groups (p=0.11) (Table 3) or models (p=0.33). There was some collagen formation (Figure 18b) and scattered vessels, which along with the many inflammatory cells formed a cellular "neo-tissue PCL construct" responsible for the increased thickness of the scaffold. The central middle "layer" of the PCL-tissue construct was acellular or partially acellular (Figure 20). The width of the acellular layer varied slightly, but there were no systematical differences between groups or models. No desmin or PKH26 fluorescence-positive cells were observed inside or around the construct.
In animals with macroscopic signs of infection, we found tissue with necrosis and hemosiderin-laden macrophages, plasma cells, lymphocytes and granulocytes corresponding to different stages of abscess formation (Figure 21).

Figure 19. Calculation of percentage of nuclei as a measurement of number of giant cells inside the PCL scaffold. (a) H&E staining and the corresponding mask used for calculation of nuclear percentage (b). The percentage was calculated as the white area in (b) divided by the total area in (b). Original magnification ×400. Adapted from[3].

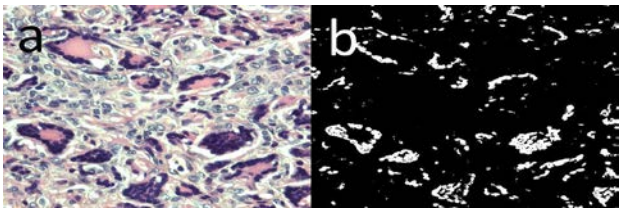


Figure 20. (a) Central acellular or partially acellular sample in Masson trichrome staining and (b)H&E staining, magnification ×40 and ×200, respectively. Adapted from[3].

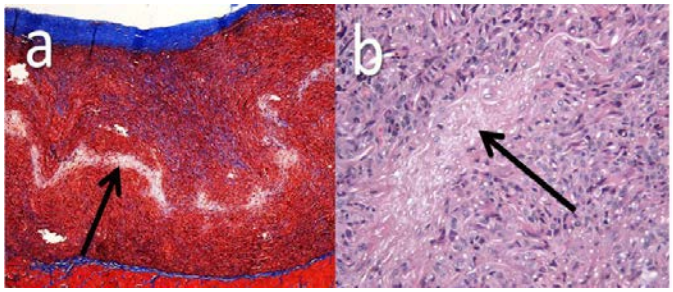
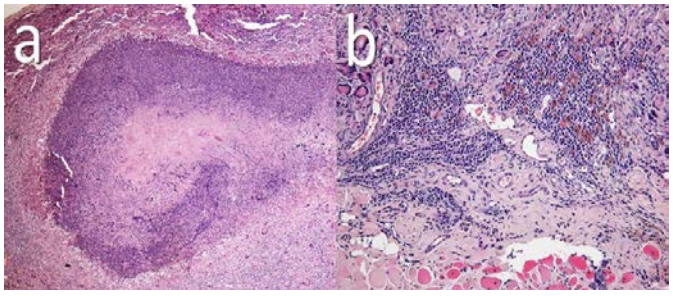


Figure 21. Abscess with necrosis and hemosiderin-laden macrophages, lymphocytes, plasma cells and granulocytes (H&E staining, magnification ×100 (a) and ×200 (b)). Adapted from[3].



Biomechanical findings

We found no significant differences between groups for any of the biomechanical properties (see Table 2 in[3]), but there was a significant difference in elongation at failure between models (see Table 3 in[3]). For the smaller tissue strip B, the full-thickness model had reduced elongation at failure compared to the subcutaneous model (p=0.008) (Figure 22). For the larger tissue strip C, the full-thickness model had reduced elongation at failure compared with the partial defect model (p=0.002) (Figure 23).

Figure 22. Boxplot of elongation at failure, testing strip B (the defect/scaffold area).

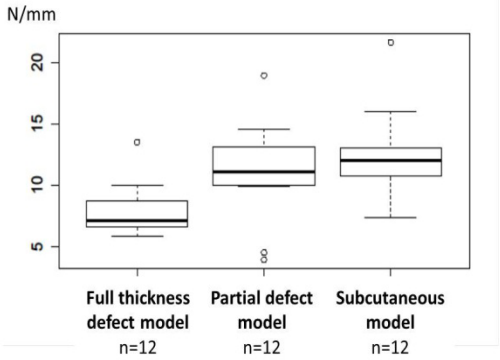
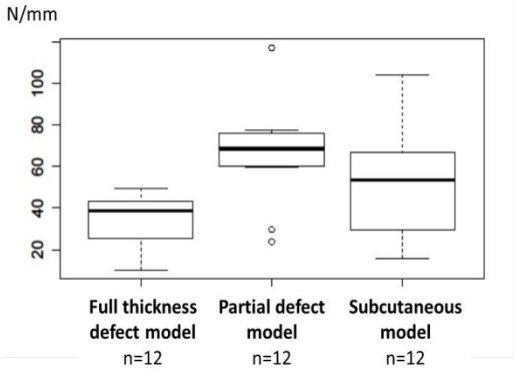


Figure 23. Boxplot of elongation at failure, testing strip C (the defect/scaffold area and the surrounding tissue).



RESULTS: STUDY 4

This feasibility study was carried out in four rabbits, where we performed a partial defect model and implanted scaffolds in the vesico-vaginal space and on the anterior vaginal wall close to the cervix (Figure 24). The surgical procedure was easy to perform and was well tolerated by all animals without causing erosions or infections. However, using rabbits rather than rats as laboratory animals required a more advanced setup, especially in terms of surveillance during and after anesthesia.

One rabbit had implantation of MFFs seeded on the MPEG-PLGA scaffold. These cells could be traced by fluorescence after eight weeks (Figure 25).

Figure 24. Placement of the two MPEG-PLGA scaffolds. Arrow marks the bladder.

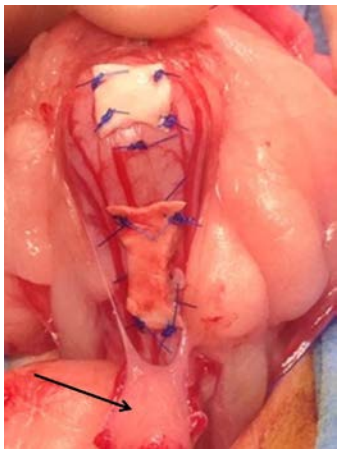
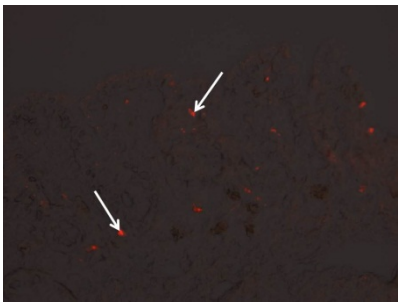


Figure 25. Fluorescence positive cells in the rabbit implanted with MFFs seeded on the MPEG-PLGA scaffold. Examples of these cells are marked with white arrows. Original magnification ×200.



The other rabbit had implantation of MFFs seeded on the PCL scaffold, which caused a marked inflammatory response with numerous and large giant cells located around and between the PCL fibers, lying both individually and in clusters (Figure 26). Fluorescence positive cells could not be identified. The biomechanical results are presented in Table 4.

Figure 26. Van Gieson Alcian blue staining of tissue sample of rabbit vaginal wall implanted with MFFs seeded on the electrospun PCL scaffold. Red arrows mark giant cells, the black arrow marks a single PCL fiber and blue arrows mark bundles of PCL fibers. Original magnification ×200.

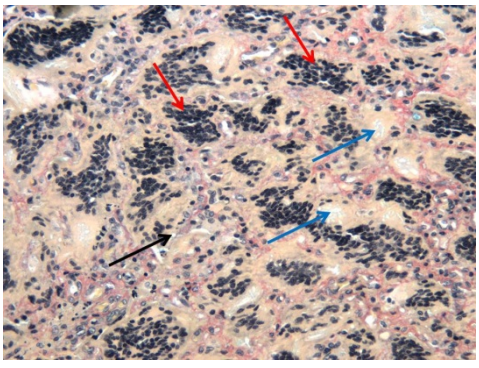


Table 4. Biomechanical properties of the vaginal tissue eight weeks after a partial defect was created with or without MFFs added to the scaffold in the vesico-vaginal space.

Model tested	Number of rabbits	Number of samples tested	Stiffness in the low-stiffness zone (N/mm)	Stiffness in the high-stiffness zone (N/mm)	Load at failure (N)	Elongation at failure (mm)
Normal vaginal wall*	4	7	1.49	11.24	2.36	47.06
Unrepaired partial defect*	2	2	1.24	6.64	1.60	45.23
MPEG-PLGA + MFFs	1	1	1.06	13.93	3.90	65.39
PCL + MFFs	1	1	3.29	13.42	2.56	32.72

* Data are presented as medians.

In one rabbit that had an unrepaired partial defect, the Prolene suture marking the caudal end of the defect in the vesico-vaginal space, had penetrated the bladder and caused formation of a bladder stone measuring 2×3×2 mm. The stone was adherent to the end of the Prolene suture.

DISCUSSION

This thesis was carried out to evaluate new tissue-engineering strategies that could serve as adjuncts to pelvic reconstructive surgery. We evaluated scaffolds of MPEG-PLGA and PCL, with or without seeded MFFs and to simulate different POP repair scenarios different animal models were used.

In two rat abdominal wall models (Study 1 and 2), we confirmed previous findings that MFFs seeded on the short-term degradable MPEG-PLGA scaffold resulted in the formation of new tissue fibers or cells adjacent to the existing muscle layers. Also, we confirmed that this scaffold was completely degraded after eight weeks and did not appear to cause any connective tissue response when implanted alone[44,92]. Cells from the MFFs could be traced and the biomechanical properties were affected. These results suggest that MFFs seeded on a scaffold of MPEG-PLGA participate in the regenerative process and could provide a beneficial cell-delivering strategy for a potential new tissue-engineering approach to improve pelvic reconstructive surgery. However, the scaffold did not provide any initial tissue reinforcement. Moreover, a long-term animal study is needed to evaluate whether this combination of scaffold and cells has a durable regenerative effect before a clinical study can be initiated.

In contrast, cells from MFFs seeded on the long-term degradable PCL scaffold (Study 3) could not be traced by fluorescence or detected by immunohistochemistry, and did not affect the biomechanical properties. The PCL scaffold was clearly not degraded after eight weeks and a massive inflammatory foreign-body reaction inside the PCL scaffold resulted in the formation of a strong cellular neo-tissue PCL construct. This construct showed a remarkable ability to adapt and provided biomechanical tissue reinforcement, even in the model where the scaffold was subjected to maximal load. Cultured human fibroblasts have proven capable of being seeded and cultured in vitro on an electrospun PCL scaffold similar to that used in our study[97]. Therefore, it is plausible, that the milieu within the construct was too unfavorable for the added MFFs to survive in vivo. Thus, a balanced inflammatory process seems essential for survival of added cells. Other studies using PCL have also found that cells can be seeded and cultured in vitro[108–110], and several studies have shown that cells can grow onto PCL in vivo[109,111]. In our study, the extent of the inflammatory foreign-body response was unexpectedly high, presumably causing a substantial release of tissue-degrading factors. Therefore, as a scaffold material, with the function of delivering cells to a specific anatomical site, the electrospun PCL scaffold used in Study 3 seems to be poor. Nevertheless, the neo-tissue PCL construct could replace the normal abdominal wall indicating a considerable potential for biomechanical tissue reinforcement. Although previous studies using degradable meshes at reconstructive POP surgery have failed to prove superior results compared with native tissue repair alone[14,22], the concept of using electrospun PCL might be an alternative to polypropylene meshes. Ideally, tissue-engineering strategies added to pelvic reconstructive surgery should restore tissue function by restoring anatomy. Thus, the scaffold should provide initial tissue reinforcement to enable both local fascia tissue support and ligament suspension. Our results indicate that the neo-tissue PCL construct could provide initial strength comparable to local tissue support, but it is plausible that the construct even could provide strength comparable to that required for ligament suspension, although testing at even higher load is required to verify this. Furthermore, a long-term animal study until full PCL degradation would be necessary to ensure formation of a functional tissue construct.

To evaluate a vaginal tissue-engineering model for POP repair, we modified a new trans-abdominal rabbit model, creating a partial defect in the vaginal wall (Study 4). This was a pilot study with a low number of animals included and comparisons between histological and biomechanical properties of the implanted scaffolds were not meaningful. However, no erosions or exposure of the implanted scaffolds could be found. Cells from the MFFs seeded onto the MPEG-PLGA scaffold could be traced after eight weeks, and again, no trace of these cells was seen in the implanted PCL scaffolds. Similar to Study 3, the PCL scaffold caused a massive foreign-body response with numerous and large giant cells located around and between the PCL fibers. Obviously, larger studies are needed before the tissue response can be evaluated and compared using this model. However, it could be a promising alternative model for future tissue-engineering studies, especially those requiring long-term follow-up.

EIGHT WEEKS FOLLOW-UP

Our results were limited to the follow-up time of eight weeks in all studies and represented just a snapshot of the ongoing regenerative process. In Study 1, we found increased stiffness in the group with MFFs, but it is uncertain whether this increase would have persisted at later time points. The degradation time of PCL is long and in Study 3, our results only reflected the initial tissue response. Long-term follow up to evaluate tissue response after full degradation of the PCL scaffold would require different animal

models and species. With the gradual disappearance of the scaffold fibers, collagen formation may take over the load and eventually form scar tissue, but it is also possible that the foreign-body response to the PCL affects the collagen formation or the regenerative response. These questions can only be answered in studies with longer follow-up time.

ANIMAL MODELS

When designing studies to evaluate the potential of new meshes or scaffolds, preclinical studies in laboratory animals are ethically necessary to understand the host response and mechanisms of action[91]. A variety of different animal models have been used[91], but there is no consensus on the optimal animal model in the studying of reconstructive POP surgery per se[87,91]. Therefore, the choice must depend on the research question under consideration[87]. The vaginal tissue response of laboratory animals like rats, rabbits and sheep have shown to differ from that of humans and erosion rates ranging from 50 to 100% have been reported after transvaginal implantation of synthetic meshes in these animals[91]. It seems therefore reasonable that the hernia abdominal wall model should be considered a first line approach when new materials for POP repair are to be evaluated in vivo[91].

The MPEG-PLGA scaffold has previously been implanted subcutaneously in a rat abdominal wall model[44,92]. By using the native tissue repair model (Study 1) we wanted to evaluate whether performing and repairing a defect of the native tissue at the site of scaffold implantation would affect the regenerative response. In the partial defect model (Study 2), we induced a weakening of the abdominal wall to test the influence of MFFs added to the MPEG-PLGA scaffold on the biomechanical strength. In Study 1, the native tissue repair model itself was found to be strong, which was in accordance with a study using a small intestine submucosa-scaffold in a similar model[112]. Therefore, the use of the weakened abdominal wall model in Study 2 supplemented the results of Study 1; the MFFs were found to increase the load at failure when seeded on the MPEG-PLGA scaffold. Thus, the combination of MFFs and MPEG-PLGA could provide a cell-delivering approach, creating a functional tissue construct although the scaffold per se did not provide any initial tissue reinforcement. The electrospun PCL scaffold used in Study 3 had not previously been tested in vivo. Therefore, we chose to evaluate the tissue response in three abdominal wall models, where increasing loads were applied to the scaffolds. The inherent strength of the PCL scaffold enabled testing of a full-thickness model, which would not have been possible with the MPEG-PLGA scaffold. Indeed, the PCL scaffold did provide biomechanical tissue reinforcement, even when exposed to the maximum load in the full-thickness model.

Using models with different loads allowed assessment for diverse possible clinical applications. We found that the properties of the resulting neo-tissue PCL construct were similar in all groups. If we had gradually increased the load to the mesh further the scaffold might have proven even stronger until eventual failure, resulting in herniation. Using a model with further load would have revealed whether the PCL scaffold could provide strength necessary for ligament suspension in POP surgery.

Although rodent abdominal wall models should be first-line studies when evaluating new reinforcement strategies for POP repair, the anatomy of the abdominal wall is clearly different from the elastic/collagenous, fibrous, smooth muscle and epithelial tissue of the vaginal wall. Therefore, we set out to develop a rabbit vaginal model. In order to avoid the high exposure rate previously found when meshes have been placed transvaginally[113–116], we chose a transabdominal approach. Placing the scaffold in the vesico-vaginal space has not been associated with vaginal erosion previously[106], and neither did our partial defect of the anterior vaginal wall with implanted scaffolds reveal any erosions, although this result must obviously be verified in a larger study.

BIOMECHANICS AND STATISTICAL CONSIDERATIONS

To fully understand the pathogenesis of POP, in which a weakening of the supporting tissues are causing the vaginal tissue to prolapse, testing of the vaginal wall in women with and without POP is crucial. Knowing the area of the female pelvic floor and the pressure acting on it, one can estimate the load that applies to the vaginal tissues. The area of the pelvic floor is approximately 94 cm^2 in women without POP[117] . The peak load that applies to the pelvic floor is thus 129 N[118], whereas the loads that apply in quiet standing and in supine posture are considerably lower (37 N and 19 N, respectively)[118].

The substantial side effects shown in relation to vaginal reconstructive surgery using the non-degradable polypropylene mesh are probably caused by the mismatch between the strength of the native tissue and the mesh itself. Since the polypropylene mesh is strong and stiffer than the surrounding native tissue, the mesh bears the load that applies to the pelvic floor. Meanwhile, the native tissue becomes increasingly atrophic, eventually causing erosion and mesh protrusion through the vaginal wall. This phenomenon, known as “stress-shielding”[119], is also known from other medical fields, especially in orthopedics with casting of fractured extremities. Thus, the perfect material for prosthetic purpose in POP repair, with biomechanical properties similar to those required for both local fascia tissue support and of ligament suspension of the patient’s own tissue, is necessary in order to avoid potential severe adverse events.

We used uniaxial biomechanical testing in the studies, as we had access to this method. Multiaxial ball-burst test[120,121] or testing of active biomechanical properties[122] , to which we had no access, could probably have added further information that more realistically would imitate the biomechanical loads that apply to the vaginal wall in patients with POP. However, comparison to other materials used for POP repair is difficult since models, sample geometry and testing techniques differ[123]. To evaluate differences between groups, we performed quantile-quantile plots to assess whether data on biomechanical properties were normally distributed. Normal distribution should be evaluated within groups and given the small numbers in each group, normality was difficult to evaluate. However, we assumed that data were normally distributed to allow parametric testing.

The low number of animals in each group increased the risk of type II error, i.e. failure to reject a false null hypothesis (page 169)[124]. This means that although there might have been a significant difference between groups, we could not find this difference due to the low number within each group. Parametric testing is superior to non-parametric testing in detecting even small differences (p. 189)[124]. Further, Tukey correction for multiple testing is more likely to find significant differences than the more robust Bonferroni correction[125].

A central challenge when aiming for a tissue-engineering strategy in POP repair is to decide which biomechanical forces we are up against. The local fascia tissue support provided by the midlevel vaginal support (pubocervical and rectovaginal fascia) is clearly weaker than the upper vaginal support by ligament suspension (suspensory fibers of paracolpium and parametrium). An ideal scaffold material should provide initial tissue reinforcement for

both applications. Until such an ideal scaffold has been found, the biomechanical properties of a specific combination of scaffold and cells in vivo must be matched with a surgical procedure that allows regeneration of the native tissue.

CONCLUSIONS

We sought to develop a tissue-engineering adjunct to improve the outcome of pelvic reconstructive surgery and we evaluated potential concepts that need to be further developed before clinical studies can be initiated.

- We confirmed previous findings that MFFs seeded on the quickly degradable MPEG-PLGA scaffold survive and participate in the regenerative process to form extra striated muscle fibers in different rat abdominal wall models.
- Cells from MFFs seeded on MPEG-PLGA could be traced by fluorescence labeling and also affected some biomechanical properties.
- The long-term degradable PCL scaffold caused a marked foreign-body response, forming a neo-tissue construct that provided biomechanical tissue reinforcement even when subjected to substantial load.
- MFFs seeded on the PCL scaffold did not survive in the inflammatory milieu.

Taken together, MFFs seeded on MPEG-PLGA may provide a new cell-delivering strategy for pelvic reconstructive surgery in combination with native tissue repair, if a long-term study finds a durable regenerative effect. Furthermore, the electrospun PCL scaffold might be beneficial in POP or hernia repair to provide initial biomechanical reinforcement, but long-term animal studies showing the tissue response that follows the full degradation of PCL after 2-4 years are required. An optimal tissue-engineering strategy, that provides both initial biomechanical reinforcement and at the same time delivering cells to restore durable functional tissue, remains to be found.

PERSPECTIVES

THE SECRETOME

The potential clinical use of secretome introduces a new dimension of the therapeutic use of regenerative strategies[86]. Cell-free regenerative or tissue-engineering approaches have obvious benefits compared to cell-based strategies, and the use of the secretome may bypass issues related to tumorigenicity, immune compatibility and the risk of infection transmission associated with cell-based therapies[31]. Further, the biological variability can be minimized allowing more accurate dosing and thus, safer and more effective therapies[86]. The challenges with the culturing of cells could be avoided, and secretome therapies could be prepared in advance and be immediately available for treatment when desired, even in the treatment of acute conditions[31].

In the field of urogynecology, this secretome effect has been studied in a stress urinary incontinence rat model. It showed that proteins released by stem cells in conditioned media in vitro[126] gave results equivalent to those following systemic administration of MSCs when delivered locally as periurethral injections[127] and systemically by intraperitoneal injections[128]. Neither of these studies, however, identified the active cytokines of the secretome[127,128]. In POP, the metabolism of the vaginal wall is altered[77] and the regenerative potential of the pelvic structures may be compromised. A secretome strategy may therefore be superior, provided the altered tissue is still capable of undergoing regeneration upon stimulation.

Although the use of secretome mainly focuses on MSCs, similar mechanisms are evident for muscle stem cells that contain numerous signaling proteins that participate in the regenerative processes[129]. In relation to our studies using MFFs, the functions of the secretome were, at least partially, responsible for the effects seen in the groups with added MFFs, but it should be noted that the MFFs also contained other cells and signaling molecules that may have affected the regenerative response.

Before secretome treatment can be considered for clinical studies, all the components of the secretome should be identified to increase our understanding of how it functions and how it can be used to obtain clinically optimal outcomes[86].

GENETICS

Increasing evidence points to a genetic component in the etiology of POP. Pelvic tissue in women with a genetic predisposition for POP shows chronic abnormal ECM remodeling with increased proteolytic activity[130]. Also, aging, vaginal delivery and other factors known to modulate the tissue and alter the normal architecture with effect on biomechanical properties, may cause POP[130]. Studies on connective tissue properties in women with POP have indicated alterations in the remodeling and composition of collagen and elastin, but also in the up- and downregulation of matrix metalloproteinases[131–133]. Furthermore, differences in the gene expression between women with and without POP have been identified[131], and a recent systematic review also found that polymorphisms of the *COL1A1*-gene was associated with POP[134]. However, they concluded that testing for gene polymorphism could not be recommended based on the current evidence[134]. Extensive research would be needed before the pathogenesis of POP has been fully revealed[132].

The idea of being able to modify specific genes could be an interesting treatment option for POP in the future. Especially, in the field of tissue-engineering, the regenerative potential may benefit from local gene therapy in order to optimize the clinical outcome. Gene delivery by electroporation is an increasing field of research, mainly focusing on cancer treatment options, but it has also been used for vaccination purposes and for the delivery of genes coding for other therapeutic molecules[135]. Electroporation was first described in 1982 by Neumann et al. and is a technique in which an electrical field is applied to cells or tissues to increase the permeability of the cell membrane, allowing DNA, chemicals or drugs to enter the cell[136].

POSSIBLE APPLICATIONS

There is an obvious need for new strategies to improve the outcome of POP surgery. Tissue-engineering could provide such an adjunct, ideally contributing to both initial biomechanical reinforcement while allowing the added cells or secretome to create a new, strong, elastic and functional regenerated tissue construct. Based on the results of this thesis, we have not found this new ideal concept. However, our results provide knowledge that enhances our understanding of how tissue-engineering can function. MPEG-PLGA does not per se provide biomechanical reinforcement, but the added cells were found to participate in a regenerative response. The PCL on the other hand provided initial biomechanical reinforcement, but here the seeded cells did not survive. Both strategies need to be evaluated with long-term follow up studies to assess whether the effects are durable. For MPEG-PLGA, a long-term study would probably require a six-month follow up, whereas a long-term PCL study would require 2-4 years of follow up due to the long degradation time. A recent

study evaluated an electrospun composite consisting of poly(lactide-co-glycolide) blended with PCL[110]. This composite material could possibly combine the positive effects related to the two scaffolds used in our studies. Furthermore, another co-electrospun material of poly(L-lactide-co-caprolactone) and fibrinogen has been implanted in a canine abdominal wall model and in a clinical setting, where it showed comparable results to implantation of polypropylene meshes, although long-term results were absent[111].

The combination of scaffold/mesh, cells and/or secretome, as well as the local microenvironment possibly modified by gene transfer generates a broad spectrum of strategies for potential future treatment options, which may be tailored to different surgical procedures for POP. Other fields of urogynecology may also benefit from these strategies. Clearly, stress urinary incontinence treatment share many resemblances with POP, both in terms of pathophysiology[130] and treatment options with the use of synthetic woven materials[137]. In contrast, defects of the levator ani muscle (avulsions) have just recently gained interest. No standard treatment options exist[138], but it is possible that MFFs may help in treating this condition, since they are capable of forming new striated muscle fibers.

The different tissue-engineering combinations for possible POP repair have an obvious overlap with hernia repair, and common strategies for both POP and hernia should be considered.

Currently, neither MPEG-PLGA nor PCL are available as commercial products for POP repair. To our knowledge, no other tissue-engineering products or ongoing clinical trials using stem cells or a secretome strategy for reconstructive POP surgery exist. Despite increasing interest in alternative reinforcement options for pelvic reconstructive surgery, the large gap between basic science and clinically expected outcome remains and awaits further exploration before tissue-engineering can be a realistic adjunct to restore functional tissue at POP surgery.

FUTURE RESEARCH

Tissue-engineering as an adjunct to improve pelvic reconstructive surgery while avoid complications related to permanent synthetic meshes seems promising. Future research is clearly warranted before tissue-engineering strategies can be implemented for treatment of POP in women:

- Long-term studies to evaluate whether MFFs seeded on MPEG-PLGA have a durable regenerative effect.
- Evaluation of survival and possible differentiation into smooth muscle tissue of the MFFs when implanted on MPEG-PLGA in a vaginal model.
- Short- and long-term studies to evaluate whether MFFs can be used alone, without scaffold, in order to assess whether they impose a regenerative effect on the tissue.
- Evaluation of the electrospun PCL scaffold in a long-term study with the purpose of uncovering properties of the regenerated tissue after full degradation of the PCL fibers have taken place.
- Evaluation of the electrospun PCL scaffold in a model with further increased load to evaluate the full biomechanical potential of the neo-tissue PCL construct.
- Development of new scaffolds that can provide both initial biomechanical reinforcement and at the same time function as stem cell carriers to provide an optimal tissue-engineering strategy to restore functional tissue.
- Studies of basic science to fully reveal the pathophysiological mechanisms of POP and the regenerative potential of the local supportive and suspensory tissues in women with POP.

SUMMARY

This PhD-thesis is based on animal studies and comprises three original papers and unpublished data. The studies were conducted during my employment as a research fellow at the Department of Obstetrics and Gynecology, Herlev University Hospital, Denmark.

New strategies for surgical reconstruction of pelvic organ prolapse (POP) are warranted. Traditional native tissue repair may be associated with poor long-term outcome and augmentation with permanent polypropylene meshes is associated with frequent and severe adverse effects. Tissue-engineering is a regenerative strategy that aims at creating functional tissue using stem cells, scaffolds and trophic factors. The aim of this thesis was to investigate the potential adjunctive use of a tissue-engineering technique for pelvic reconstructive surgery using two synthetic biodegradable materials; methoxypolyethyleneglycol-poly(lactic-co-glycolic acid) (MPEG-PLGA) and electrospun polycaprolactone (PCL) - with or without seeded muscle stem cells in the form of autologous fresh muscle fiber fragments (MFFs). To simulate different POP repair scenarios different animal models were used.

In Study 1 and 2, MPEG-PLGA was evaluated in a native tissue repair model and a partial defect model of the rat abdominal wall. We found that the scaffold was fully degraded after eight weeks. Cells from added MFFs could be traced and had resulted in the formation of new striated muscle fibers. Also, biomechanical changes were found in the groups with added MFFs.

In Study 3, the long-term degradable electrospun PCL scaffold was evaluated in three rat abdominal wall models representing different loads on the scaffold. Surprisingly, cells from the MFFs did not survive. After eight weeks, a marked inflammatory foreign-body response was observed with numerous giant cells located between and around the PCL fibers which appeared not to be degraded. This response caused a considerable increase in the thickness of the mesh, resulting in a neo-tissue PCL construct with strength comparable to that of normal rat abdominal wall. The foreign-body inflammatory response did not differ between the groups in terms of cellularity, cell types or thickness, and no differences were found between groups when comparing biomechanical properties.

In study 4, we modified a new transabdominal rabbit vaginal model to avoid the erosions known to occur following vaginal mesh implantation. A partial defect was created on the anterior vaginal wall in the vesico-vaginal space and on the anterior vaginal wall close to the cervix. This was a feasibility study aimed at obtaining results comparable to those seen in the rat model. The model was easy to perform and no vaginal erosions were observed.

In conclusion:

- In two rat abdominal wall models, cells from autologous MFFs, seeded on the quickly degradable MPEG-PLGA scaffold, survived implantation and contributed to the regenera-

tive process by forming extra striated muscle fibers and influencing the biomechanical properties of the regenerated tissue.

- Consequently, MFFs seeded on an MPEG-PLGA scaffold is a potentially advantageous cell-delivering strategy to regenerate tissue at pelvic reconstructive surgery.
- In three rat abdominal wall models, a long-term degradable PCL scaffold caused a marked foreign-body response and formed a neo-tissue PCL construct that provided biomechanical tissue reinforcement to the abdominal wall, even at maximal load.
- Consequently, the PCL scaffold might be beneficial in pelvic reconstructive surgery, providing initial biomechanical reinforcement, although long-term studies showing the tissue response at full degradation are required.
- Cells from the MFFs did not survive in or around the neo-tissue PCL construct, possibly because of the massive inflammatory response.
- Consequently, as a scaffold material, with the purpose of delivering cells to a specific anatomical site, the PCL scaffold seems poor.
- A transabdominal rabbit vaginal model was feasible and might be advantageous in the evaluation of meshes used for pelvic reconstructive surgery, especially when long-term studies are needed.

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