Principles of scaffold generation for bioengineering of the ovary and uterus

A study focusing on decellularisation

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Cover illustration: The principle of ovulation, fertilization and implantation

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Dedicated to my family (My parents, Faizah Alkholi and Bakor Alshaikh, my brothers Hashem and Ali, my sisters Ala'a and Reema). I lack words to define your contribution to this research. You made all my challenges easy. When I almost gave up, you reminded me that I could do better. My daughter Zaina and my son Bakor (my miracles) for loving me even when I was stressed. Thanks for cheering me up when I almost gave up. Being away for so long is not easy for a parent.

ABSTRACT

Introduction: Cancer therapy often results in fertility problems due to inflicted injury to the reproductive organs. Since most women survive cancer, fertility preservation has become an important consideration during cancer therapy. However, options for young women with blood-related cancers are missing. Papers I-II describe the development and characterization of mouse ovarian scaffolds derived from ovarian extracellular matrix (ECM). Such scaffolds may be used for future ovarian bioengineering applications as a supporting matrix for the expansion of immature follicles isolated from young cancer patients to preserve their fertility. Paper III-IV use the rat model to analyse similar scaffolds for uterus bioengineering applications and evaluate if these scaffolds are immunologically inert after engraftment.

Methods: Three decellularisation protocols based on sodium dodecyl sulphate (SDS) and sodium deoxycholate (SDC) were developed for mouse ovary scaffold production (Paper I). Scaffolds were then characterised using histology and quantification methods for ECM components. Recellularisation was tested using mesenchymal stem cells (Paper II). Previously established uterus scaffolds were grafted to syngeneic (Paper III) or allogeneic rats (Paper IV) to investigate if the decellularisation process generated any detrimental damaged associated molecular products (DAMPs; Paper III) and if the allogeneic recipient's immune system remained stable after scaffold engraftment (Paper IV). Immunohistochemistry and gene expression analysis with digital droplet PCR were used to quantify infiltrating immune cells and the expression of proinflammatory signals.

Results and conclusions: Papers I-II developed three novel mouse ovarian scaffolds. The SDS and the SDC protocols were found promising, whereas a protocol based on both detergents was found to be too aggressive on the ECM. Paper III showed that a mild, yet effective decellularisation protocol generated less amounts of DAMPs, and that this scaffold type also remained the most inert to the recipient's immune system in an allogeneic setting (Paper IV).

SAMMANFATTNING PÅ SVENSKA

Introduktion: Cancerbehandling orsakar vanligen fertilitetsproblem som biverkning. Eftersom de flesta kvinnor överlever sin cancer är fertilitetsbevarande åtgärder före cancerbehandling numera en vanlig företeelse. Dock finns ingen effektiv fertilitetsbevarande åtgärd för unga kvinnor som drabbas av blodcancer. Delarbete I - II i denna avhandling undersöker framställningen av ett äggstocksbiomaterial genom en metod som kallas avcellularisering. Ett biomaterial skulle möjligen kunna användas som stödstruktur under odling av omogna äggblåsor som isolerats från patienten före behandlingen och fungera som en fertilitetsbevarande åtgärd. Delarbeten III - IV använde en råttmodell för att analysera immunförsvarsreaktionen mot liknande biomaterial framtagna för att reparera livmodersrelaterad infertilitet.

Metoder: Tre avcellulariseringsmetoder som baserades på natriumdodekylsulfat (SDS) och natriumdeoxykolat (SDC) utvärderades för musovarier (Delarbete I - II). Dessa äggstocksbiomaterial analyserades med immunohistokemi och förekomsten av viktiga bindvävskomponenter kvantifierades. Biomaterialens förmåga att stimulera stamcellstillväxt undersöktes också eftersom de har potentiellt en förmåga att stimulera mognaden av äggblåsor. I Delabete III - IV transplanterades tre olika typer av livmodersbiomaterial för att undersöka om dessa biomaterial accepterades av värddjurets immunförsvar. Genom att använda immunohistokemi, gen expressions analyser (ddPCR) och en inavlad råttstam undersöktes det om själva avcellulariseringsprocessen påverkade immunogeniciteten (Delarbete III). Den immunologiska reaktionen undersöktes även på liknande sätt i en allogen (genetiskt olik) situation (Delarbete IV).

Resultat och kommentarer: Delarbete I - II visade att SDS och SDC var effektiva för framställningen av biomaterial för musäggstockar, men att ett kombinationsprotokoll av dessa detergenter till stor del förstörde biomaterialet. Delarbete III - IV visade att en mild men effektiv avcellulariseringsdetergent var mer fördelaktig jämfört med en starkare detergent för framställningen av ett immunologiskt inert biomaterial.

LIST OF ARTICLES

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. **AB Alshaikh**, AM Padma, M Dehlin, R Akouri, MJ Song, M Brännström, M Hellström. Decellularisation methods for the mouse ovary: Scaffold generation for future ovarian bioengineering studies. J. Ovarian Res. 2019; 12:58.

II. **AB Alshaikh**, AM Padma, M Dehlin, R Akouri, MJ Song, M Brännström, M Hellström. Decellularisation and recellularisation of the ovary for bioengineering applications: Studies in the mouse. Reprod Biol Endocrinol. 2020; 18:75.

III. Padma AM, **Alshaikh AB**, Song MJ, Akouri R, Oltean M, Brännström M, Hellström M. Decellularisation protocol-dependent DAMPs in rat uterus scaffolds differentially activate the immune response after transplantation. Tissue Eng Regen Med. In Press

IV. Padma AM, **Alshaikh AB**, Song MJ, Akouri R, Oltean M, Brännström M, Hellström M. An assessment of the immune response after allogenic decellularised uterus tissue engraftment in the rat. Biomed Mater. Under 2nd revision

CHAPTERS

ABBREVIATIONS

3D	three-dimensional
AB	alcian blue
ALL	acute lymphocytic leukaemia
AML	acute myeloid leukaemia
ART	assisted reproductive technology
BM-MSCs	bone marrow derived mesenchymal stem cells
CD	cluster of differentiation
cDNA	coding DNA
DAMPs	damage associated molecular patterns
DAPI	4´,6-diamidino-2-phenylindole
ddPCR	digital droplet polymerase chain reaction
dH_2O	deionized water
FSH	follicle stimulating hormone
h	hour(s)
hCG	human chorionic gonadotropin
H&E	haematoxylin and eosin
IFN-γ	interferon γ

IL	interleukin
IVF	in vitro fertilization
LH	luteinising hormone
MCP1	monocyte chemoattractant protein 1
MIP-1α	macrophage inflammatory protein 1 alpha
MIP-3α	macrophage inflammatory protein 3 alpha
MT	Masson's trichrome
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NSAID	nonsteroidal anti-inflammatory drug
P1	protocol 1
P2	protocol 2
P3	protocol 3
PBS	phosphate buffered saline
PPIH	peptidyl-prolyl cis-trans isomerase H
SD	Sprague Dawley
SDC	sodium deoxycholate
SDS	sodium dodecyl sulphate

SEM	scanning electron microscopy
sGAGs	sulphated glycosaminoglycans
STAT-3	signal transducer and activator of transcription 3
TNF	tumour necrosis factor
Tx	transplantation
UTx	uterus transplantation
VVG	Verhoeff Van Geison

INTRODUCTION

Assisted reproductive technology (ART) is a collective name for typically used fertility treatments for men and women, when any intervention will assist with reproduction. Examples of these are insemination in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI). As knowledge progressed, protocols for these techniques were modified and made more sophisticated. For example, novel ART methods recently explored in the clinic have included the “three-parent baby” (that is, mitochondrial oocyte replacement) (1, 2), rejuvenation (3) and stem cell ovarian transplantation for poor responders (4, 5). Additionally, ART may also include fertility preservation methods for women who undergo gonadotoxic cancer treatment through ovarian cortex cryopreservation and re-transplantation (6) or include surgery methods such as uterus transposition (7) or uterus transplantation (8). Many of these techniques were difficult to contemplate 25 years ago. Thus, regimens that may seem unbelievable today may very well be in clinical practice in the near future.

Human ovarian anatomy and physiology

The ovary is an endocrine organ that produces mainly oestrogens and progesterone, and smaller amounts of androgens, during the fertile life of a woman. It houses the ovarian reserve of all primordial follicles from birth and is where follicular development occurs. This is at first independent of gonadotropins but is at later stages controlled by follicle-stimulating hormone (FSH), which is released from the pituitary gland. During follicular development, the primordial follicle (about 40 µm in size) has a single cell layer of squamous granulosa cells surrounding the oocyte. A primary follicle then develops further when the granulosa cells turn into a stratified columnar epithelium and when the follicle growth reach approximately 100 µm in size. The granulosa cells then start to proliferate which results in a multi-layered granulosa inner cell mass which surrounds the pellucid zone and the oocyte. These follicles

are classified as secondary follicles and are around 200 µm in size. They also attract stroma cell-like theca cells which attach to the basal lamina that separates the follicle from the surrounding tissue. Secondary follicles may grow further into a tertiary (or antral) follicle (>400 µm in size) which is identified by a fluid-filled cavity (antrum) in the granulosa cell layer (Figure 1). Theca cells stimulate vascularization around the follicles at this stage, and the continued follicular growth becomes hormone dependent (9).

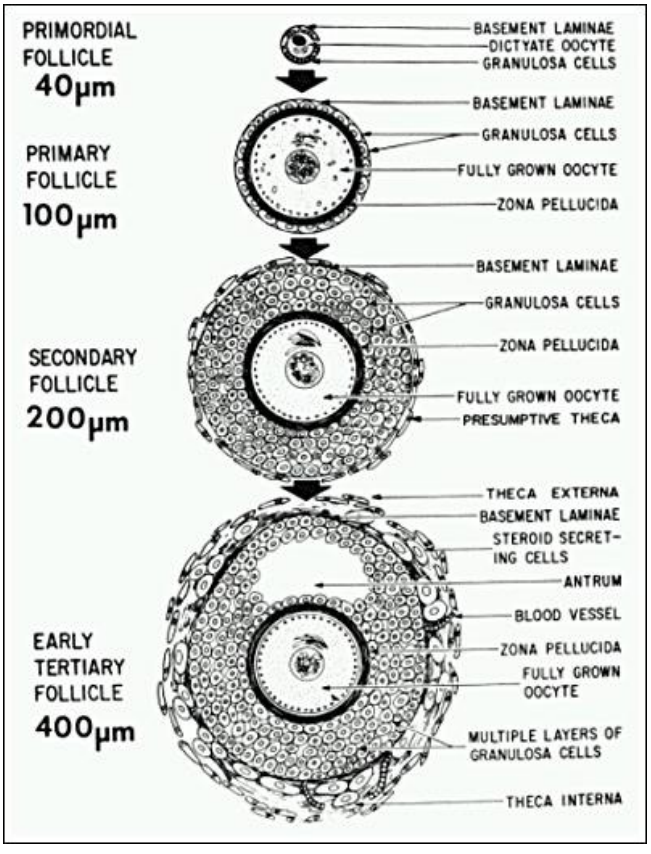

Figure 1; Illustration of the early development of human follicle. Figure adopted with permission from (9).

Granulosa cells mainly produce oestradiol by aromatase-conducted conversion of theca-cell derived testosterone. This cooperation between theca and granulosa cells is referred to as the two-cell hypothesis. As oestradiol levels increase with follicular growth, the dominant follicle can reach a size of up to 2.5 cm (now called a Graafian follicle). The oestradiol level eventually reaches a threshold at which oestradiol converts from enforcing negative feedback to instead initiating a positive feedback on the hypothalamic gonadotropin-releasing hormone, which regulates the FSH and luteinising hormone (LH) release from the pituitary gland. This specifically creates a surge in LH and induces ovulation from the largest dominant follicle in the ovary. After ovulation, the ruptured follicle will store cholesterol as a component for further synthesis of mainly progesterone, and thereby acquire the yellow colour (corpus luteum) (Figure 2).

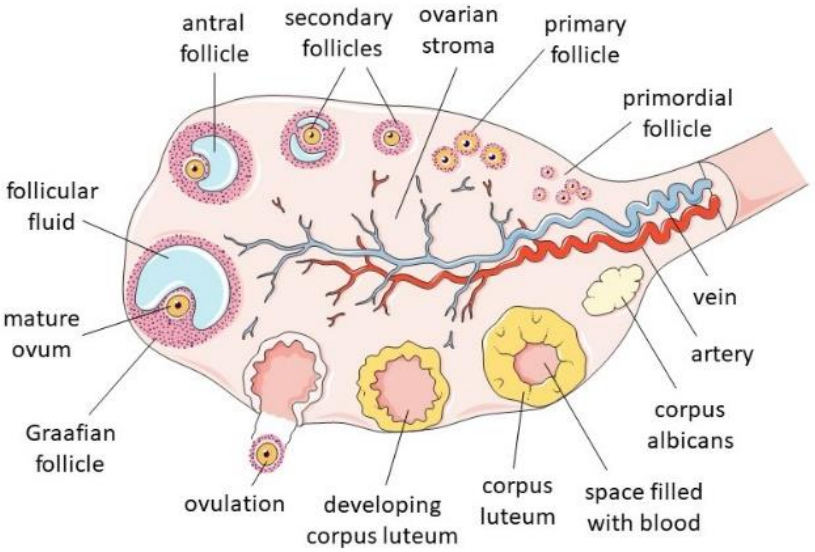

Figure 2. An illustration of the different follicular stages in a fertile woman. This figure was obtained from Smart Servier Medical Art.

The large amount of progesterone production from cells in the corpus luteum starts a negative feedback loop on the hypothalamus and the pituitary gland, inhibiting LH and FSH release. If pregnancy does not occur, the corpus luteum slowly regresses because of lack of luteotropic stimulation by human chorionic gonadotropin (hCG), which leads to a decline in progesterone and oestradiol production. The loss of progesterone makes the endometrial layer in the uterus shed, which defines the start of menstruation. Then, the entire process repeats, with a slow accumulation of oestradiol from a new pool of growing follicles (Figure 3).

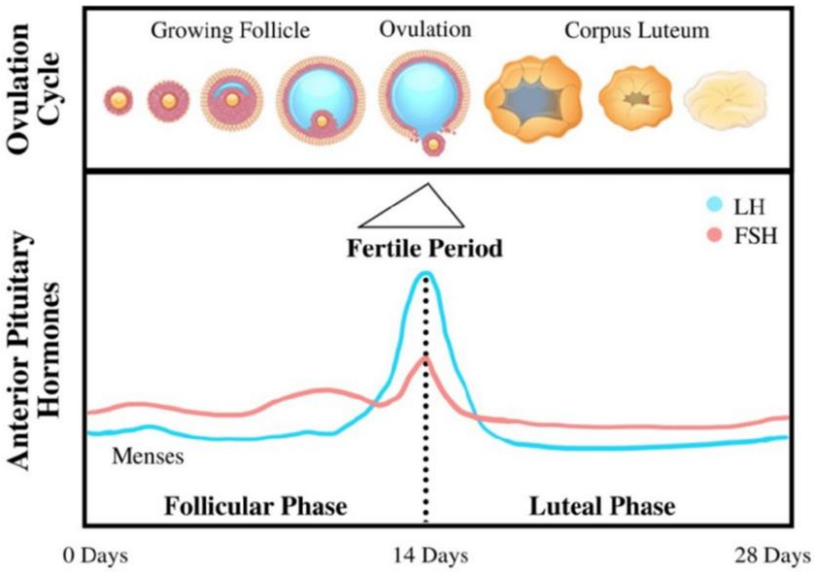

Figure 3; Levels of luteinising hormone (LH) and follicle-stimulating hormone (FSH) during the human menstrual cycle. Adopted with permission from (10).

Unless the oocyte is fertilised, a woman repeats this cycle about 13 times a year. In a lifetime, about 300 – 400 oocytes will reach maturity, while the rest of the follicles will degenerate. A woman is born with an absolute number of primordial follicles and enters menopause when the ovarian reserve becomes depleted; consequently, her hormonal cycle stops.

Human anatomy and physiology of the uterus

The uterus is linked to the ovary via the Fallopian tube (Figure 4) and is a muscular organ with an inner mucus lining — the endometrium. The endometrial thickness depends on the oestradiol production from the ovaries and becomes the thickest just after ovulation to facilitate successful implantation and placentation. Progesterone addition to an oestradiol-primed endometrium renders the endometrium to convert from a proliferative to secretory profile. During this phase, the spiral arteries and glandular structures are plentiful within the endometrium closest to the lumen (stratum functionalis). However, this inner endometrial layer sheds completely if there is no embryo implantation. Only the innermost lining of the endometrium (stratum basalis) that sits next to the myometrium remains after menstruation. This layer is rich in uterine specific stroma/stem cells (11-13) and can regenerate a new stratum functionalis during the follicular and luteal phases of each menstrual cycle. The thick muscle layer (myometrium) is structured as two perpendicular layers so that the uterus can contract during childbirth.

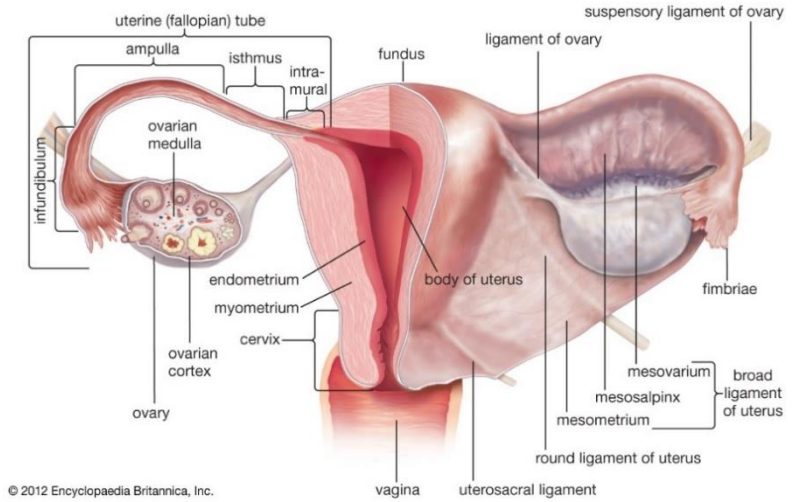

Figure 4. General anatomy of the human uterus. Figure reused with permission from Britannica, The Editors of Encyclopaedia. "Uterus". Encyclopaedia Britannica, https://www.britannica.com/science/uterus. Accessed 13 April 2021.

Ovarian and uterine anatomy and physiology in mice and rats

Female mice reach sexual maturity at around six to eight weeks of age, and the equivalent for rats is at eight to twelve weeks of age. The onset of puberty in rodents is established by an increasing frequency of pulsatile release of LH. Rodents do not have a menstrual cycle with menses (bleeding). Instead, the cyclic events of the rodent female genital tract are referred to as the oestrus cycle with the phases dioestrus, prooestrus, oestrous and metoestrus. The oestrous cycle in rodents is considerably shorter compared to humans and is repeated every four to five days. A five-day oestrous cycle would have metoestrus I and metoestrus II, but in all cycles, ovulation occurs in the night with transition from pro-oestrus to oestrous. As described above, rodents do not menstruate; instead, the endometrium reabsorbs itself. The rodent cycle becomes irregular in animals older than around eight months and eventually becomes acyclic between 10 and 16 months of age (14, 15). The anatomy of the rodents' uterus is a bicornuate uterus (Figure 5) and can normally harbour between four and six foetuses per uterine horn during the 19–21 days (mice) or 21–23 days' (rats) gestation.

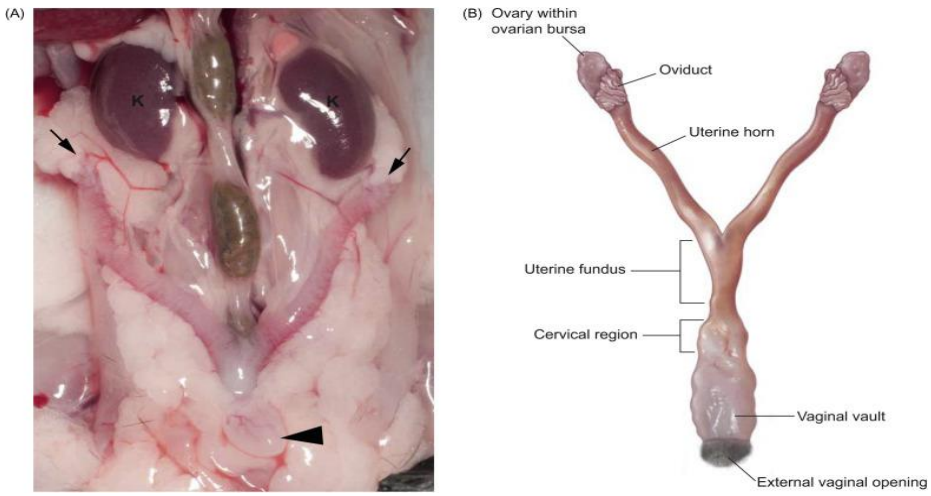

Figure 5. The reproductive organs of the mouse and rat. Figure adopted with permission from the publisher and authors (16).

Female rodents have remarkable reproductive capability and may become pregnant again only a few days after giving birth to a litter. They are also easy to keep for experimental purposes. For these reasons, rodents are treasured animals for reproductive research, even if there are several significant differences compared to the human reproductive system. Moreover, since rodents are used extensively in biomedical research the knowledge about biological processes of these animals are extensive and there exist ample research-tools, such as monoclonal antibodies, designed for experimental use in rats and mouse.

Female infertility following cancer therapy

Female infertility following cancer therapy is common due to gonadotoxic treatment side-effects, Aggressive cancer treatments (some specific chemotherapeutic agents and total body radiation) are known to cause secondary menopause and therefore infertility in a large proportion of patients. The different chemotherapeutics exhibit a range of gonadotoxic effects, both concerning mechanisms of action and in their gonadotoxic potency. For example, actinomycin, bleomycin methotrexate, vincristine, and fluorouracil are less prone to cause amenorrhea, which is commonly used as an indication of infertility, while alkylating agents have potent gonadotoxic effects and more often lead to premature ovarian failure by damaging the pool of primordial follicles (that results in amenorrhea) (17-19). Anthracyclines treatment used for induction remission and post remission chemotherapy in girls with acute myeloid leukaemia (AML) and acute lymphocytic leukaemia (ALL) are also associated to cause permanent infertility (20).

Additional cancer treatment-induced damage may affect the ovarian vasculature and induce ovarian cortical fibrosis which can prevent normal follicular development and ovulation (21, 22). Thus, many female cancer survivors may experience premature ovarian insufficiency, early menopause, and infertility. Over the years, improvements in therapy protocols have significantly increased survival

rates among cancer patients, and more focus has been placed on factors related to the quality of life after cancer, including fertility.

Fertility preservation for female cancer survivors

Ovarian cryopreservation

There are various strategies through which fertility can be preserved in female cancer victims of fertile age (e.g., embryo or oocyte cryopreservation or ovarian transposition during radiation treatment). However, regarding prepubertal girls, fertility is significantly more challenging to preserve since they lack antral follicles that can be hormone stimulated to produce a mature oocyte. Cryopreservation of ovarian tissue is currently the only available option for these girls. The first successful case of ovarian cortex re-transplantation after cryopreservation was that of a 32-year-old woman cured of Hodgkin's disease. She received a freeze-thawed ovarian autograft collected before her cancer therapy six years earlier and gave birth to a healthy child (6). Following this successful first case of autologous ovarian cortex transplantation, about two hundred babies have now been born worldwide using this technique, which proves that this method can be used to successfully preserve fertility for many women who overcame cancer (23, 24). However, transplantation of cryopreserved ovarian tissue can be associated with the risk of re-implanting malignant cells and thereby recurrent cancer, particularly for patients who suffer from hematopoietic cancers, including leukaemia, neuroblastoma and Burkitt's lymphoma (25). These women have a high risk (> 11%) of malignant cells being present within the ovarian tissues, while there is a moderate risk (0.2%–11%) for women who suffered from progressed breast cancer, cervical adenocarcinoma, or colon cancer (24, 26). Therefore, women with these types of cancers are restrained from undergoing cryopreserved ovarian tissue re-transplantation. Yet, their ovarian tissue is usually harvested and cryopreserved before cancer

treatment, with the hope that new and safer fertility treatment options will be developed in the future (25, 26).

In cases where there is a prominent risk of malignant cells in the ovarian tissue, the risk of transmission of malignant cells may be overcome by (1) assessing the graft to ensure that there is no contamination of malignant cells before autotransplantation or (2) by the isolation and in vivo or in vitro culturing of the small follicles (primordial—primary—secondary follicles) that should not carry the risk of including malignant cells. Isolated immature follicles may then be stimulated to mature and grow into large follicles with oocytes that are competent enough to undergo the last step of meiosis and thereby fertilisation. A drawback of the first procedure with in vivo maturation of isolated follicles is that some malignant cells might be overlooked. However, the second procedure is considered relatively safe since the ovarian follicles are typically surrounded by a basal membrane, which separates them from the stromal environment, capillaries, and nerves (27). Based on these morphological observations, one can assume that these follicles are free from cancer cells. However, a major challenge with this second procedure is to develop an in vitro culture system that can appropriately support the survival and expansion of growing follicles in vitro (28). This is particularly challenging for human follicles since they instigate a size expansion from an early-stage follicle diameter size of about <100 µm to a mature human follicle size of >20 mm. In rodents, the follicular growth difference is considerably smaller, with a young (primordial/secondary) follicle size of about 20 µm–120 µm to a mature follicle size of about 400 µm (29).

Follicular in vitro maturation

Several human babies have been born from in vitro-matured oocytes derived from relatively large follicles (starting over 5 mm in size) by classical transvaginal oocyte-pick up from women (30). The oocytes would grow in vitro to assume competence to undergo resumption of the second meiotic phase and thereby fertilization by IVF. This ART

may serve as an optional fertility treatment alternative for women suffering from polycystic ovary syndrome (PCOS) who could not undergo conventional IVF stimulation because of factors such as high risk for ovarian hyperstimulation syndrome (OHSS). However, it is not helpful to prepubertal women who undergo cancer therapy since their ovaries harbour only follicles that have not reached the size a clear antral follicle, and these follicles will not respond well to gonadotropin stimulation.

However, pioneering work has shown that it is feasible to grow rodent primary follicles using a two-step in vitro culture system, and the aspirated mature oocytes can be fertilised to generate offspring (31, 32). A similar two-step in vitro system was then evaluated for human follicles (33). This was improved further using a multi-step protocol, which resulted in oocytes possessing a metaphase II spindle conformation (34, 35). Although these studies showed low efficiency rates (about 10% of the isolated secondary follicles) and they did not investigate the fertilisation probabilities of the isolated ova, they still serve as proof-of-concept that human follicles can be matured in vitro from immature follicles under the right circumstances.

An alternative technique for the in vitro maturation of immature follicles is the use of three-dimensional (3D) matrices that can appropriately encapsulate the isolated follicles. Such biomaterial should ideally be able to give 3D structural support for the ovarian follicles and be able to undergo rapid remodelling to allow follicle expansion. At a suitable moment, either the whole biomaterial construct is transplanted back to the patient who can then complete the follicular growth in vivo, or the construct will continue the follicular maturation in an in vitro environment. The objective for both principles is to be able to aspirate a mature oocyte that can be used for standard IVF procedures. These principles come under the terms ‘ovarian bioengineering’ or ‘artificial ovary’ (Figure 6).

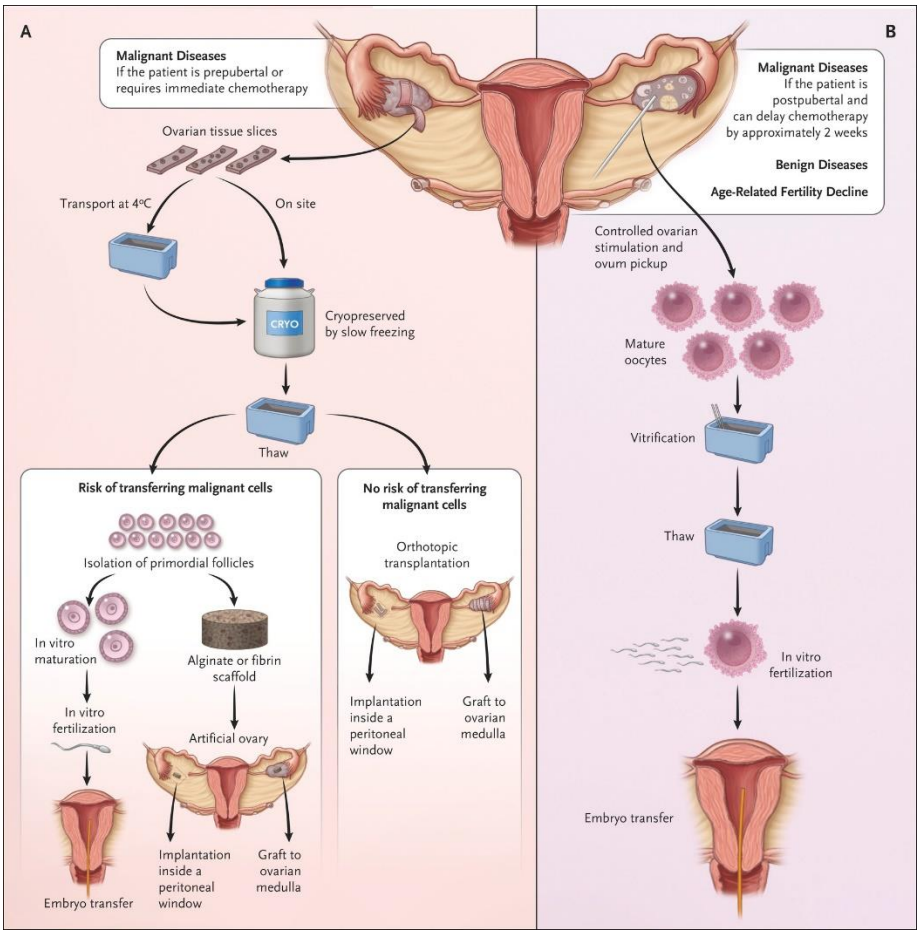

Fig. 6. If the cancer patient needs urgent chemotherapy, or is prepubertal (A), cryopreservation of isolated ovarian cortex tissue is conducted for fertility preservation strategies. However, if the patient is fertile and can delay cancer treatment for about two weeks, fertility is preserved by ovarian stimulation and aspiration of mature oocyte for future IVF (B). Figure reproduced with permission from (23).

Ovarian bioengineering

Much research has been conducted on trying to create a 3D culturing system that can support the in vitro growth of immature follicles, as described above One of the first biomaterials evaluated for this application was based on an alginate-based hydrogel that provided

growth support to mouse secondary follicles (150 – 180 µm in size) during in vitro folliculogenesis (36). Follicles in this substrate could grow to a size of about 350 µm in 8 days, and the culture medium showed increased concentrations of oestradiol and progesterone during the process. Metaphase II oocytes were harvested, which could be fertilised in vitro. Embryos were then transferred to pseudo-pregnant mice which then delivered live fertile offspring after normal gestation time (36). Later, the alginate-based biomaterial for mouse follicles was improved by adding fibrin which increased the plasticity of the biomaterial and gave better structural stability during follicular growth (37). Simultaneously, human follicles were cultured under similar conditions which showed that small secondary follicles of about 43 µm could survive in alginate culture (38). When starting from larger human follicles (175 µm), these could be cultured to an average size of 715 µm using a 30-day in vitro encapsulation protocol with a culture media that included bovine fetuin (a serum substitute) and 0.1 IU/ml FSH (39).

Rather than allowing complete folliculogenesis to occur in vitro, multiple labs evaluated the possibility of grafting encapsulated follicles and letting the recipient support follicle growth in vivo. Consequently, the aim of this bioengineered ovary is not only to facilitate reproduction but also to restore ovarian endocrine function. This idea was already explored in the 1990s using collagen or plasma clots to encapsulate mouse follicles, which were then transplanted to the kidney capsule. Mature oocytes were harvested, and the animals produced offspring after IVF (40, 41). Since then, several groups have explored follicle encapsulation and subsequent transplantation (Tx) to restore fertility using alginate-fibrin scaffolds (42-44), fibrin-collagen scaffolds (45), and more recently, a 3D-printed gelatine-based scaffold (46). These rodent studies also proved functionality by the birth of normal offspring. However, all these methods are not easily translated to human follicles due to the significant differences in the size expansion and growth time of the human follicles. Hence, further studies are required to optimise the methods and find a suitable biomaterial that will provide unique requirements for human follicle growth.

A significant body of literature has suggested that ovarian biomaterials based on extracellular matrix (ECM) components are advantageous (47). The ECM forms a structural network between cells and tissues and plays a significant role in the crosstalk between ovarian stromal cells and the cells of the primordial follicles. It further provides structural support during follicle expansion and acts as a growth factor reservoir (27).

It has been proposed that an ovarian-specific ECM-derived scaffold suitable for ovarian bioengineering applications can be made by a process called ‘decellularisation’. This topic is discussed in detail later in the Introduction and is the research focus of Papers I–II in this thesis.

Uterus transplantation

Before IVF development, there was much research interest in uterus transplantation (UTx) as a means to cure tubal factor infertility by transplantation of the uterus together with the oviducts. However, when Steptoe and Edwards (1978) reported the first live birth after IVF and presented an efficient method to cure tubal infertility, the idea of UTx became redundant (48). Tubal infertility was rather prevalent and due to previous damage of the delicate mucosa and tubal structure of the oviducts, typically caused by salpingitis. At that time little scientific attention was paid to the smaller group of women with uterine factor infertility, caused by absence of the uterus from birth, after hysterectomy, or the presence of a non-functional uterus. Reasons for uterine non-functionality could be uterine malformation or severe intrauterine adhesions. These women had the motherhood options of adoption or use of gestational surrogacy.

However, transplantation protocols have become significantly more successful with the introduction of calcineurin inhibitors (cyclosporine and tacrolimus) as immunosuppressive drugs, and organ transplantation as a mean to increase function and/or gain quality-of-life parameters has obtained much attention lately. For example,

transplantation of a new face, forearm or hand transplants were successfully accomplished (49, 50). Therefore, professor Brännström started a research programme to investigate whether UTx could become a treatment option to cure uterus factor infertility. This is a condition that affects about 3–5% of women of fertile age due to an acquired disease or trauma caused to the uterus (e.g., intrauterine adhesions, partial uterine malformation, or hysterectomy) or from a developmental malformation that induces an underdeveloped, or a completely absent uterus from birth (Mayer–Rokitansky–Küster–Hauser–syndrome) (51). A breakthrough in UTx research occurred in a mouse model in 2003 when Akouri and Brännström et al. published the first successful results on UTx with subsequent live births (52, 53). The surgical methods were improved and translated into the rat animal model, with subsequent live births (54-56). The surgical protocols were also rapidly developed and evaluated on larger animal models, such as pigs (57), sheep (58), and non-human primates (59, 60). During this time, two human UTx attempts were conducted by two other groups (61, 62). However, none of these two procedures was successful.

The significant preparation and collection of important parameters for UTx protocols established by professor Brännström and his team in the animal models enabled the first organised clinical trial on UTx to start in 2013. Two years later, the first baby was delivered through a UTx (8). To date, several other groups have succeeded with this treatment regimen, and there are now around 25–30 babies born worldwide (63-67). Hence, uterus factor infertility can now be considered a curable condition. Most successful cases involved live donors. Using this donor type is somewhat easier to logistically organise, and the donor's uterus can be meticulously assessed for an optimal functional outcome after Tx. However, two groups have reported successful birth after using a brain-dead multi-organ donor (65, 68). Naturally, the advantage of this method is that risky live donor surgery can be avoided. However, organised comparative studies with both modalities used (69), and well-planned selection criteria and cohorts, will be required to conclude which donor source is the most favourable for UTx. Currently, the organ donor criteria are strict, and multiple UTx trials now report similar

problems for other organs used for Tx; That the availability of good quality donor organs is limited (70).

Apart from risky donor surgery procedures that include isolating long uterus vascular pedicles located deep in the pelvic region, negative side effects of immunosuppressive treatment can induce an increased sensitivity to infections, nephrotoxicity, and lymphoproliferative disorders (71-73). Hence, finding an alternative donor source would be an attractive option.

Uterus bioengineering

Recent principles in the field of bioengineering have stipulated that tissues and organs may be created using an appropriate scaffold together with the patient's own cells (74). This is an attractive concept regarding UTx. A bioengineered donor uterus bypasses problem related to the limited number of donor organs and donor surgery risks. Additionally, if such constructs were based on the patient's own cells, immunosuppressive drugs would not be needed (Figure 7).

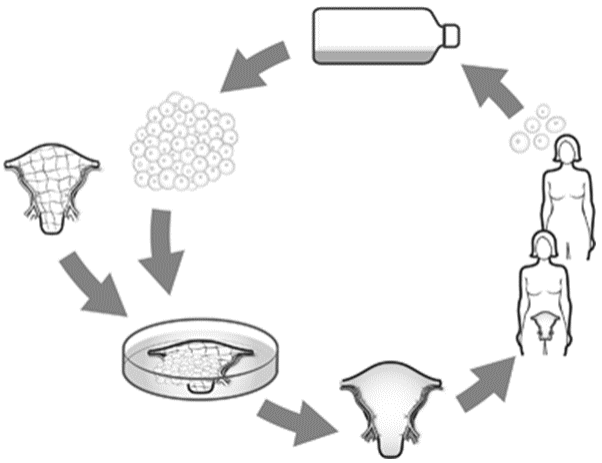

Fig. 7 The principles of uterus bioengineering. Autologous cells are isolated from the patient, expanded in vitro and then introduced to a biomaterial which then is further cultured in vitro prior to the engraftment for its clinical application. Figure reused with permission from publisher and author (75).

Uterine bioengineering may also be useful for treating acquired uterine wall injuries that impact implantation or create a risk for uterine rupture during pregnancy. Such injuries may be caused by repeated uterine incisions following caesarean, placental tumour resections, extensive myomectomy or adenomyomectomy. A bioengineered uterus tissue may be used to reduce scarring after such procedures or even replace damaged uterine tissue with a grafted bioengineered uterus patch.

However, the first bioengineering studies conducted using uterine cells were performed in vitro on different types of substrates to evaluate implantation mechanisms and uterine cancer cell migration (76, 77). Most of these studies used scaffolds based on collagen combined with Matrigel (an ECM gelatinous mixture isolated from mouse sarcoma cells), or silk structures (78-81). One of the initial studies that evaluated a bioengineered uterus construct in vivo was performed on rats by Campbell et al. in 2008 (82). The constructs were made from autologous tubular-shaped myofibroblast tissue that was transformed to uterus-like tissue during the 12-weeks' observation time after engraftment. This new tissue was strong enough to support pregnancy to full term. Ding et al. used a bone marrow-derived mesenchymal stem cell (BM-MSCs) filled collagen scaffold in a similar rat animal model with comparable results (78). Hydrogels derived from collagen or gelatine (the denatured product of it) are therefore interesting for uterus bioengineering applications, particularly since hydrogels can be combined with supporting reagents that enable 3D printing of uterine scaffolds (83).

Perhaps the most impressive uterus bioengineering study to date is a recent study performed on rabbits by professor Atala's team (84). In that study, they used a similar biomaterial that was previously evaluated in patients requiring a new bladder (85) or a neovagina (86). The uterus scaffold was based on poly-lactide-coglycolide and poly-glycolic acid. These polymers turn into biological by-products during degradation. When seeded with autologous uterine cells taken from the removed contralateral uterine horn, this cell-containing biomaterial was used to replace a U-shaped segment of the remaining native uterus horn (6–8

cm long and 2.5 cm wide). Four out of ten rabbits gave birth to healthy pups after this procedure (84). However, the histological evaluation showed that embryo implantation and placentation had occurred on the native tissue that was retained during the engraftment procedure of the bioengineered construct. If the implantation of the embryos and placentation had occurred in the bioengineered graft, it would have provided solid evidence that the bioengineered construct was fully functional. However, the regenerative ability following the Tx of this particular bioengineered construct was impressive, and their results provide hope that large bioengineered uterine grafts can be created in a size relevant for human applications. Yet, whole bioengineered organs and large constructs will eventually need to be connected to the recipient's vasculature system via vascular anastomoses in order to prevent necrosis after engraftment.

Scaffolds derived from decellularised tissue

Collectively, scaffolds made up of collagen, hyaluronic acid, fibrin, and other ECM components have shown promising results in many bioengineering experiments, including ovarian and uterus bioengineering applications (29, 87). Therefore, a novel scaffold production technique called decellularisation has attracted much interest lately.

Decellularisation aims to remove all immunologically active cellular and nuclear materials from a tissue of interest while preserving its native ECM ultrastructure and composition (88-91). Thus, a decellularised tissue/organ induces a 3D ECM structure that mimics the acellular components of the tissue from which it has originated. Therefore, this may be a suitable scaffold for bioengineering applications. Hence, these principles have been evaluated on some rat models, including the construction of whole-organ scaffolds of the heart (89), kidney (92), lung (90), liver (91), ovary (93, 94) and uterus (95, 96).

Scaffolds derived from decellularised tissue have been shown to influence cell mitogenesis and chemotaxis (97), direct differentiation of stem cells (98-102), and induce constructive host tissue remodelling responses (103-105). It is likely that the 3D ultrastructure, surface topology, and composition of the ECM contribute collectively to these positive effects. However, there is also evidence that residual cellular material from an incomplete decellularisation process can decrease or fully negate the constructive tissue remodelling advantages of these scaffolds after engraftment in vivo (106-108). Therefore, the type and extent of tissue-processing methods, such as decellularisation protocols, are critical determinants for the success of using this type of scaffold for bioengineering applications.

Methods for decellularisation

A selective cell removal from a tissue or organ without compromising the functionality, structure and mechanical properties of the ECM is difficult to achieve. There is a significant body of literature that is based on various optimisation protocols for all kinds of tissues. However, in this complex area every protocol must be adjusted to each specific tissue/organ and its composition (density, cellular abundancy, thickness, and vascularity). General decellularisation principles may be divided into physical and chemical measures, and most established decellularisation protocols include various combinations of these methods.

Physical elements in decellularisation

One common physical decellularisation method includes repeated freeze-thawing cycles, where the formed intracellular ice crystals will disrupt the lipid layers of the cell-membrane and cause cell lysis. However, this process may also irreversibly disrupt the morphology of the ECM and limit scaffold functionality. Agitation may be considered another physical method. The tissue is submerged into a

decellularisation reagent, which is then stirred or shaken to enhance the passive diffusion of the decellularisation reagent into the tissue. The reagent itself usually acts as a chemical decellularisation factor that disrupts the cell membranes and facilitates cell component removal (see below). This technique is usually deployed for tissues and organs that cannot be perfused through the vasculature. Vascular perfusion is a very effective way to deliver decellularisation reagents to all tissue compartments in vascularised tissue. Additionally, multiple studies have shown that such vascular perfusion methods induce scaffolds with remaining vascular conduits that may be connected to the recipient's vascular system during engraftment via vascular anastomosis (89, 109) including decellularised uterus tissue Another interesting physical decellularisation strategy evaluated on rat uterus tissue is to force decellularisation reagent through the tissue with high hydrostatic pressure (110). For tough and robust tissues like bone and cartilage, sonication may be another interesting physical decellularisation method to consider. In this method, cell destruction is caused by the physical vibrations generated by ultrasound. However, sonication may also destroy important ECM structures.

Chemical elements in decellularisation

As mentioned above, chemical strategies to decellularise tissue are usually applied in combination with physical strategies. The commonly used chemical reagents are detergents of different types, which disrupt the cell membrane and break molecular bonds so that cellular components can be removed from the ECM with subsequent water or buffer washes (111). Two popular decellularisation detergents are sodium dodecyl sulphate (SDS) and sodium deoxycholate (SDC). These detergents are ionic and disrupt cells by osmotic forces, and by disrupting the lipid layer in the cell membranes. An example of a much milder non-ionic detergent used for decellularisation is Triton X-100. This detergent is less effective than SDS or SDC in removing cellular and nuclear material from the tissue. However, it is gentler on the remaining ECM. SDS is by far the most popular detergent used for

decellularisation protocols, including for the ovary (94, 112, 113) and the uterus (96, 110, 114, 115).

Other chemical factors include solutions of different osmolarities or solutions with enzymes or different pH. For example, a popular decellularisation strategy is to alternately expose the tissue to hypertonic and hypotonic solutions. Deionised water (dH_2O) is commonly used to remove cellular debris after using the hypertonic detergent SDS or after applying the hypertonic solution dimethyl sulfoxide (DMSO; which is not a detergent but an organic compound). These alterations cause substantial osmotic cellular stress that effectively lyses the cells and simultaneously washes out the cellular remnants from the tissue. Enzymes such as trypsin or DNase are also common decellularisation reagents. Trypsin is a strong and effective enzyme, but due to its non-specificity, it also degrades ECM components. DNase, however, is precise and effectively degrades nucleic acid bonds, thereby reducing remnants of large genomic strands of DNA in decellularised tissue. Acids are also commonly used in decellularisation protocols. However, acid is more commonly used as a scaffold sterilisation method at the end of the protocol than as a key decellularisation reagent.

Components of decellularised tissue

The ECM comprises large glycosylated protein molecules secreted from nearby cells. This important structure provides tissue organisation and handles the organ/tissue-specific physical attributes. It also acts as a reservoir for growth factors and other beneficial molecules for nearby cells that affect cell proliferation, migration, and differentiation. The ECM of the ovary is highly plastic and is continuously being modified by surrounding cells, which is essential for successful folliculogenesis (116).

The most prevalent ECM components are different types of collagens. Naturally, the collagen amount is tissue dependent. Collagen type I is

typically found in the skin, tendons, bones, and other tough tissues (117). However, together with collagen type III, type I is also abundant around ovarian follicles in many species, including humans (118). The same collagen types are major constituents of uterine ECM, which also contain large amounts of type IV and V collagens (119). Interestingly, there is a sevenfold increase in collagen during human pregnancy to give extra tissue strength to the uterus during this condition (120).

Elastin is another major ECM component that also significantly increases during pregnancy (121). As the name suggests, this molecule is important for mechanical stability during tissue expansion and contraction and is particularly abundant in elastic aortic vessels, the bladder, and the pregnant uterus. The elastin content of the ovary is widely distributed in both the ovarian medulla and cortex regions, suggesting multiple roles, including the maintenance of follicular integrity during growth.

Laminin is a major component of lamina propria (the basement membrane) and is another ECM molecule that is important for cell-cell interactions. It has a high affinity to collagens and other ECM molecules and has been shown to be an important component in stimulating primordial follicle growth during ovarian in vitro cultures in mice (122). Laminin is particularly ample in the endometrial layer compartment that includes the basal epithelial membranes around the lumen, glandular structures, and capillaries (123), and laminin is also vital for the embryo implantation process (124).

Fibronectin is another major configuration of the ECM. Fibronectin interacts with cells via integrin receptors and affects multiple cellular functions, such as cell adhesion, migration, and organisation. It has also been found to be important for embryonic stem cell self-renewal. Hence, fibronectin is vital for embryo implantation and growth (125).

Sulphated glycosaminoglycans (sGAGs) are large protein-carbohydrate multi-bifurcated molecules that are hydrophilic and make the ECM retain water-soluble growth factors, including signalling molecules. Its inclination to homeostasis and to provide important

growth cues for cells and angiogenesis is indicated by the abnormally high sGAGs-content in ECM from ovarian cancer tissue (126, 127).

Decellularised tissue for ovarian bioengineering

Six years ago, Laronda et al. reported the first ovarian bioengineering application using a decellularised matrix as a scaffold to support grafted murine follicles in vivo (94). The functionality of the bioengineered ovaries was neatly confirmed by the induction of puberty in ovariectomised mice with restored hormone production. The same study also showed that it was possible to use SDS to decellularise bovine and human ovaries. Liu et al. developed SDS-based decellularisation protocols for pig ovaries and used a xenograft transplantation model (in rats) to briefly assess its immune-activating properties (128). Though their results were only based on the cell infiltration of three different immune cell types, they indicated that xenogeneic decellularised ovarian tissue only caused a modest immune reaction after Tx.

Since then, only a limited number of publications on decellularised ovarian tissue have been published, and the majority of these have used decellularisation protocols based on the detergent SDS (112, 113, 129-131). However, as described earlier in this thesis, the protocols affect the quality of the scaffolds, and variations in the decellularisation protocols induce unique scaffold types that may benefit or hamper recellularisation abilities, along with its ability to support follicular growth and its immunological properties after engraftment. Hence, further studies in these research areas are needed and have been the research focus of this thesis (Papers I–IV).

Decellularised tissue for uterus bioengineering

Similarly, the attention to decellularised tissue in uterus bioengineering research has increased during the last decade (87, 132). Interestingly, numerous decellularisation protocols have been evaluated for uterine tissue compared with the SDS-dominant decellularisation protocols developed for ovarian bioengineering. For example, ethanol, water and trypsin were used to decellularise segments of rat and human myometrium (133). In that study, it was shown that human and rat myocytes could recellularise the scaffolds and form a multilayered in vitro structure with some elementary contractility functions. However, in 2014, three studies that together described seven different decellularisation protocols for the rat uterus were published (95, 96, 110). Most of these protocols were based on detergents, including Triton X-100 (which can be considered a mild detergent), SDC (an intermediary detergent) and SDS (which can be considered a strong aggressive detergent). However, an alternative protocol developed by Santoso et al., showed that a high hydrostatic pressure treatment was more advantageous than the evaluated SDS-based protocol (110). One major advantage of these seven decellularisation protocols is that the vascular conduit network in the produced whole-uterus scaffold was remained intact. Hence, these scaffold types may be used in future experiments for whole organ bioengineering studies (Figure 8). The scaffolds' vascular conduit network may then be used as a route to distribute cells during recellularisation, and these cells may also be fed by perfusing culture medium through the vasculature in a bioreactor. Additionally, such scaffolds could be transplanted via vascular anastomosis, like standard UTx protocols. However, to accomplish this method, initial effective endothelial recellularisation of the vasculature will also need to be developed.

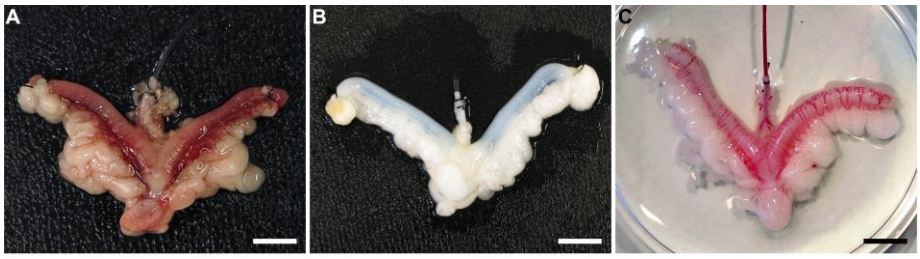

Figure 8. A native rat uterus was isolated with intact vasculature (A) enabling the organ to be cannulated and perfused with decellularisation reagents for five days to remove cellular components, leaving only the uterine extracellular matrix (B). The decellularised organ have patent vascular conduits, evident in (C) by the perfused coloured oil. Figure used with permission from the publisher and authors (95).

Some of these types of scaffolds were evaluated in vivo in a rat model. Scaffold segments were recellularised with uterus-specific cells together with rat MSCs and then grafted into the uterine wall to repair uterus injury. These novel treatment procedures have been shown to restore fertility (96, 134) even without the recellularisation step, although with a reduced therapeutic effect (110). It has also been shown that it is important to maintain the correct scaffold topography during engraftment for correct morphological regeneration (135). Furthermore, the regenerative response following transplantation of decellularised uterus scaffold of the mouse was dependent on signal transducer and activator of transcription 3 (STAT-3) and interleukin (IL) 6, rather than being oestrogen and progesterone dependent as would be expected (136).

These successful rodent studies have encouraged translational evaluation and the development of uterus decellularisation protocols for larger animals. For example, Campo et al. developed an SDS-based protocol for decellularising the uterus in rabbits (114) and pigs (115). They also showed that the scaffold composition varied significantly depending on which cycle stage the uterus was harvested from before decellularisation. Their results indicated that it was advantageous to

collect donor material from the luteal phase when the endometrium was at its maximal thickness and in a secretory phase (114). The uterine scaffolds derived from the stimulated animals were composed of more ECM products, and the hydrogel that was produced from these uterine scaffolds supported embryo growth better than the non-stimulated counterpart. Additional uterus decellularisation protocols have now been established for goats (137) and sheep (138, 139). The latter is a significantly better large animal model than most other species for reproductive research for its close resemblance to the human uterus in size and vascular anatomy.

Most of the studies mentioned above have focused on establishing effective decellularisation protocols and to find efficient ways to recellularise the produced scaffolds. Naturally, these steps are crucial for translating the successful bioengineering applications developed in rats to more clinically relevant larger animal models.

Immune response towards decellularised tissue

The recipient's immune response after the engraftment of decellularised tissue involves both the innate and the adapted immune response. Initially, there is a rapid inflammatory response that includes recruitment of mast cells, dendritic cells, fibroblasts, and many types of leukocytes. Depending on how well the biomaterial integrates and remains inert to the host immune system, proinflammatory or anti-inflammatory signals will be expressed by these infiltrating cells which will decide graft survival or destruction. Hence, these events are of critical importance for the success or failure of the transplanted tissue. However, only a few studies have investigated the immune response following the engraftment of decellularised tissue. It is critical for the treatment outcome that the bioengineered scaffold remains inert or only induces transient activation of the immune response following engraftment. Decellularised tissue is of allogeneic origin, but the decellularisation process is assumed to remove the major immunogenic tissue components. Most scientists seem to rely on the genetically close

homology of ECM molecules between different species and between individuals of the same species (140) which, in theory, should allow ECM based scaffolds to be inert if the allogeneic donor cells are removed. In particular, when following the criteria specified in a highly influential review paper which states that decellularised tissue should contain less than 50 ng donor DNA per mg dry scaffold weight, and that the remaining DNA should be of less than 200 base pairs in size to avoid a detrimental immune response after engraftment (111). This article is cited more than 2000 times, yet no references were used to support these decellularisation norms.

Decellularisation protocols include denaturing detergents and tissue-degrading enzymes, which will expose new antigens and epitopes that may act as damage-associated molecular patterns (DAMPs). These include fragmented DNA, mRNA, fibronectin, sGAGs and collagen (141-143). Thus, DAMPs are allo-independent immunogenic compounds that may be further potentiated in an allogeneic or xenogeneic transplantation setting. Yao et al. were able to show that decellularised rabbit uterus tissue did not cause a significant immune response in a xenotransplantation model in rats (144). However, the study only investigated the infiltration of $CD68^+$ macrophages and the proinflammatory response of tumour necrosis factor-alpha (TNF-α). A great number of different cell types, cytokines and chemokines are involved in the inflammatory response that dictates the graft's success or failure. These are critical mechanisms requiring elucidation to develop optimal scaffold types for reproductive bioengineering applications, alongside effective translation to larger animal models and novel clinical fertility treatments for women.

Hence, the immunogenicity of decellularised uterus scaffolds was also studied in this thesis. Using the rat model, Paper III evaluated whether any decellularisation protocol-dependent allo-independent DAMPs were present in the uterus scaffolds, and Paper IV investigated the general immunological response following allogeneic uterus scaffold Tx.

AIM

This PhD thesis primarily aimed to establish sound methodologies for constructing suitable scaffolds for future ovarian bioengineering applications in the mouse model (Paper I–II).

Furthermore, it is important to ensure that scaffolds based on decellularised tissue do not provoke a negative immune response following engraftment. However, the physiological effects after engrafting ECM-derived scaffolds are not well established. Therefore, this thesis also assessed the immunological events following the engraftment of uterus scaffolds derived from three decellularisation protocols in the rat animal model (Paper III–IV).

Research questions

Paper I	Can ECM-derived scaffolds be developed by decellularisation for future ovarian bioengineering studies in the mouse model?
Paper II	What is the composition of mouse ovary ECM derived scaffolds and can the scaffolds be repopulated with stem cells?
Paper III	Do ECM-derived rat uterus scaffolds contain any allo-independent immunogenic molecules as a consequence of the decellularisation process that may be detrimental for graft survival after transplantation?
Paper IV	Do ECM-derived rat uterus scaffolds contain any allogeneic antigens that may be detrimental for graft survival after transplantation?

MATERIAL AND METHODS

Animal work

All the animal studies conducted in this thesis followed the guidelines outlined in document 114–2014, which was approved by the Animal Welfare Committee at the University of Gothenburg.

Mouse ovary isolation (Papers I-II)

Eighty-three female C57BL/6N mice were used in Paper I to optimise new decellularisation protocols and to characterise the ECM after the scaffold generation. An additional 108 female mice of the same strain were needed for the studies presented in Paper II. All mice used for these studies were aged between 10 and 20 weeks (Charles River, Sulzfeld, Germany) and were housed under controlled 12 h light and dark cycles with free access to food and water.

Each ovary was harvested from isoflurane-anaesthetised mice by a long midline incision through the abdominal wall. The ovaries were exposed, cut out, and placed in the organ preservation solution Perfadex (Exvivo, Gothenburg, Sweden). Each organ was then individually frozen in the same solution at –20°C until used for the experiments specified below. The mice were euthanized during the surgical procedure to harvest the ovaries.

Rat uterus isolation (Papers III-IV)

For Papers III and IV, 12 uteri per study were isolated from eight to ten weeks old female Lewis rats (Janvier labs, Le Genest-Saint-Isle, France). Each uterus was explanted as described earlier in detail (95, 145). Briefly, a midline incision through the abdominal tract was made on the isoflurane anaesthetised rat. The uterus was dissected out by first ligating all branching vessels from the descending aorta and

ascending vena cava, only leaving the common iliac, internal iliac, and uterine artery and veins open so that the uterus could be extracted with an intact vasculature that facilitated organ perfusion via aortic cannulation (Figures 7 and 8). These uteri were used for scaffold production using the three decellularisation protocols outlined below.

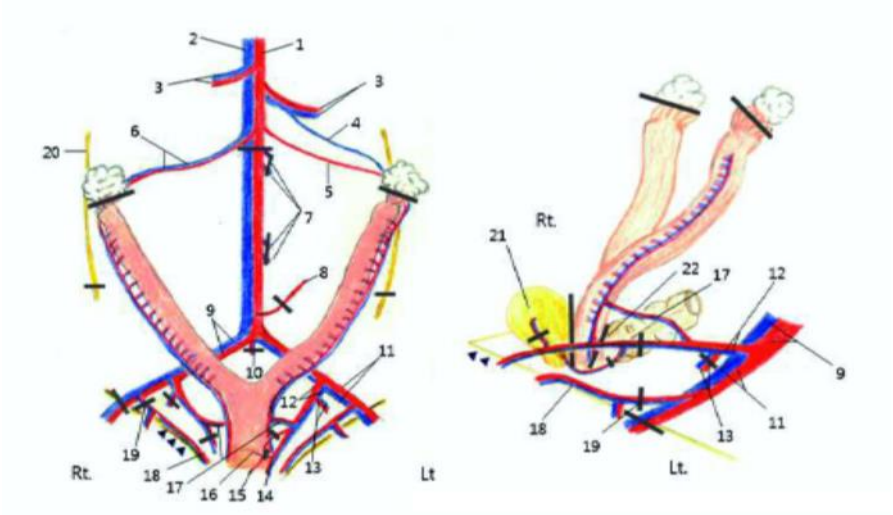

Figure 8. Schematic drawing of vascular anatomy of the abdomen and the pelvis of the rat. Ligations are indicated by bold black/gray bars. 1=abdominal aorta; 2=vena cava; 3=renal vessels; 4= left renal vein; 5=left renal artery; 6=right renal vessels; 7=lumbar vessels; 8=inferior (caudal) mesenteric artery; 9=common iliac vessels; 10=caudal vessels; 11=external iliac vessels; 12=internal iliac vessels; 13=superior gluteal vessels; 14=umbilical vessels; 15=inferior vesical vessels; 16=superior vesical vessels; 17=uterine vessels; 18=external pudendal vessels; 19=inferior epigastric vessels; 20=ureter; 21=urinary bladder; 22=recto-sigmoid colon (Rt., right side; Lt. left side). Figure and legend reused with permission from the publisher and authors (145).

Transplantation of decellularised rat uterus

For Papers III and IV, 12 decellularised uteri isolated from Lewis rats were used for each study. Four uteri were decellularised by each of the three developed decellularisation protocols for rat uterus described below. Once decellularised, the uterus scaffolds were cut into full-thickness uterus segments 1 cm x 0.5 cm in size. Three pieces of the same scaffold type were grafted subcutaneously to the nape of a recipient rat, creating three experimental groups receiving one scaffold type, respectively (n = 6 for each group). The difference between Paper III and Paper IV was that for Paper III, the allo-independent immunological response was investigated using a Tx model with inbreed animals (syngeneic animals). For Paper IV, an outbreed Tx model was used to investigate potential allogeneic immunological factors. Hence all recipient animals in Paper III were of the inbreed Lewis strain and recipients were of the outbreed SD strain in Paper IV (Table 1). Donor animals in both papers were of the inbred Lewis strain, except for groups described below.

Two additional animal groups were created for each study; one group of rats received grafts in the same location based on three similar sized pieces of its own isolated uterus (autologous control group). The other group received uterine tissue grafts isolated from three allogeneic donor rats (outbreed Sprague Dawley, SD, rats in Paper III; inbreed Lewis rats in Paper IV). Table 1 summarises the animal groups established for Papers III and IV (Table 1). All animals were given a nonsteroidal anti-inflammatory drug (NSAID) by carprofen during the first 72 h after surgery (5 mg/kg once daily; Rimadyl®, Orion Pharma AB, Danderyd, Sweden) and a dose of buprenorphine during surgery for pain relief (0.05 mg/kg; Temgesic®, RB Pharmaceuticals, Berkshire, UK).

Table 1. A summary of the animal experimental groups used in Papers III and IV. SD, Sprague Dawley. P, protocol.

Group 1 (Paper III)	Autologous uterus tissue, Lewis recipient, n=6
Group 1 (Paper IV)	Autologous uterus tissue, SD recipient, n=6
Group 2 (Paper III)	Allogeneic SD donor uterus, Lewis recipient, n=6
Group 2 (Paper IV)	Allogeneic Lewis donor uterus, SD recipient, n=6
Group 3 (Paper III)	Lewis decellularised uterus (P1), Lewis recipient, n=6
Group 3 (Paper IV)	Lewis decellularised uterus (P1), SD recipient, n=6
Group 4 (Paper III)	Lewis decellularised uterus (P2), Lewis recipient, n=6
Group 4 (Paper IV)	Lewis decellularised uterus (P2), SD recipient, n=6
Group 5 (Paper III)	Lewis decellularised uterus (P3), Lewis recipient, n=6
Group 5 (Paper IV)	Lewis decellularised uterus (P3), SD recipient, n=6

Graft retrieval (Papers III and IV)

Five days after the initial engraftment of the uterus tissues or the decellularised uterus tissues, one grafted piece was isolated from each rat under isoflurane anaesthesia by reopening the surgical site. The same procedure was conducted on day 15 post Tx, and on day 30. Each graft was divided into two-halves; one was placed in 4% buffered formaldehyde (Histolab, Gothenburg, Sweden) and used for histological analysis, and the other half was trimmed further to ensure that only grafted tissue was kept. The biopsy was placed in RNALater® (Qiagen, Sollentuna, Sweden) for future gene expression analysis.

Mouse ovary decellularisation (Papers I and II)

Each isolated ovary was weighed and then immersed in a decellularisation solution of 0.5% SDS (Protocol 1; P1) for 10 h or of 2% SDS (Protocol 2; P2) for 16 h. The solutions were then replaced with deionised water (dH_2O) to wash the ovaries from cellular debris and residual detergent. A third decellularisation protocol was also evaluated based on reducing the duration of detergent exposure to half of the respective chemicals used in P1 and P2. Hence, the ovaries in Protocol 3 (P3) were exposed to 0.5% SDS for 5 h, then dH_2O for 15 h, followed by a second detergent treatment with 2% SDC for 8 h. These ovaries were then washed for 24 h in dH_2O to remove detergents and cellular debris. Each ovary was then buffered in phosphate buffered saline (PBS) for 1 h and submerged in a DNase I solution (40 units/ml; Sigma-Aldrich, Stockholm, Sweden) for 30 min at 37°C. Each ovary was then washed in PBS for 24 h and sterilised by exposing them to 0.1% peracetic acid (Sigma-Aldrich) in normal saline for 30 min. Each ovary was then rinsed repeatedly with sterile PBS for a total of 24.5 h and was then extensively analysed in Papers I and II by the various methods described below.

Rat uterus decellularisation (Papers III and IV)

The rat uterus decellularisation protocols used for scaffold production in Papers III and IV have been developed and meticulously assessed previously (95, 134, 145). Briefly, the aorta attached to each explanted uterus was cannulated and the uterus was perfused through the vasculature with various decellularisation solutions according to the protocols stated in Table 2 below. Once the decellularisation procedure was completed for each organ by respective protocol, the rat uterus scaffolds were stored at –20°C until further used. These protocols have routinely generated scaffolds with varying degrees of remaining donor DNA with P1.

Before the uterus scaffold transplantation procedure was conducted in Papers III and IV, each scaffold segment was sterilised a second time using gamma radiation at 25 kGy/h for 3 min and 25 s to ensure that the immunological response assessed was not caused by a contaminated graft.

Table 2. Decellularisation protocols for rat uterus scaffold production for the experiments used in Papers III and IV.

Protocol 1	**Protocol 2**	**Protocol 3**
4% DMSO (4h)	4% DMSO (4h)	2% SDC (6h)
1% Triton X-100 (4h)	1% Triton X-100 (4h)	dH_2O (18h)
PBS (16h)	dH_2O (16h)	
Repeated five times (for a total of five days) Scaffold sterilisation with 0.1% peracetic acid perfusion (0.5h) Scaffold washing with sterile PBS perfusion (72h)		

DNA quantification (Paper I)

To evaluate the decellularisation efficiency of the developed protocols for generating mouse ovary scaffolds, the remaining donor DNA content was assessed in the decellularised tissue. Whole ovaries were homogenised using a tissue lyser (Qiagen, Stockholm, Sweden). The homogenate was then applied directly on a DNA binding column from the DNeasy Blood & Tissue Kit (Qiagen). The DNA was then washed in several centrifugation steps and then eluted in 30 µl of dH_2O. The DNA concentration of the elute was measured using a NanoDrop1000 (Thermo Fisher Scientific, Gothenburg, Sweden). Values were then calculated and plotted as ng DNA per ovary for the respective scaffold type.

Protein, collagen, glycosaminoglycans and elastin quantification (Papers I and II)

The total protein content was established on normal and decelluralised mouse ovaries using the Coomassie (Bradford) Protein Assay Kit (Thermo Fisher Scientific), which is a colorimetric-based method. Simply described, the samples were homogenised (n = 6 per group) in T-per buffer (Thermo Fisher Scientific) which is a lysing buffer optimised for protein extraction. Due to the low protein content of the decellularised mice ovaries, the ovaries were pooled two and two, giving a statistical value of n = 3 per group. The extracted protein was mixed with Coomassie dye from the kit, which colours all protein blue. A standard curve established from several samples of known protein concentrations was also included so that the absolute protein concentration from the experimental samples could be determined using a spectrophotometer at 595 nm (plate reader; Varioscan Lux, Thermo Scientific, Finland). The results were presented as protein content per two ovaries.

The collagen, sGAGs and the elastin content of normal and decellularised mice ovaries (n = 12 per group) were also measured colorimetrically using the same spectrophotometer. Sample values were compared to a standard curve based on reagents included in the commercially available kits from BioColor (Carrickfergus, Northern Ireland; Fastin for elastin; Blyscan for sGAGs; Sircol for soluble and insoluble collagen, respectively). As in the protein assay, the samples were conducted in pairs so that the amount of ECM products would be quantified and were within the detection limits for the kits. Hence, the results were presented as total content per two ovaries and gave a statistical power of n = 6 per group.

Evaluation of scaffold toxicity (Paper II)

A common way to evaluate whether the scaffolds contain any toxic residual decellularisation reagents is to conduct a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

This method indirectly measures cellular metabolic activity by measuring the conversion of MTT to a dye called formazan, which depends on the NAD(P)H activity in any type of cells. Hence, if the cell metabolism is in any way compromised when exposed to toxic products, this assay will detect a colour change between the wavelengths of 500 – 600 nm, which will indicate potential toxicity. Paper II describes such measurements based on the metabolism of human embryonic kidney cells exposed to scaffold washing solution. Roche's cell proliferation kit 1 was used following the manufacturer's instructions (MTT; Merck KGaA, Darmstadt, Germany).

Histology (Papers I – IV)

Histological assessments were conducted on paraformaldehyde fixed normal and decellularised tissue in Papers I and II, and on grafted tissue in Papers III and IV. Tissue was then dehydrated by processing the tissue with increasing concentrations of ethanol and finally in xylene before being saturated with melted paraffin. The paraffin embedded tissue was then cut into 5 µm thickness sections that were transferred to glass slides. Sections were then processed for various standard staining protocols to visualise the general morphology (haematoxylin and eosin; H&E) or collagen (Masson's trichrome; MT), elastin (Verhoeff van Geison; VVG) or sGAGs (alcian blue; AB).

Stem cell recellularisation (Paper II)

Ovarian scaffolds were recellularised to evaluate whether they may support cells and be used for future follicular cocultures. For this, a red fluorescent protein (RFP) labelled mouse bone marrow mesenchymal stem cell line was used (MSCs; RFP-MSCs; MUBMX-01201C57BL/6, Cyagen, Santa Clara, CA, USA). The RFP labelling had been genetically engineered into the genome of each cell so that each cell progeny also remained labelled. The major benefit of using labelled

cells for recellularisation studies is that the cells used for recellularisation can be distinguished from any potential reaming donor cells (unlabelled) within the scaffold.

The RFP-MMCs were cultured under standard cell culturing conditions in a humidified chamber with 5% CO_2 enriched air. Cells were fed three times a week with DMEM medium containing 10% foetal calf serum and 1% antibiotics-antimycotics (penicillin, streptomycin, amphotericin B; Thermo Fisher Scientific). The cells were split once per week or when they reached confluence.

For the recellularisation, $1x10^6$ RFP-MMCs per ovarian scaffolds were used. Cells were injected into each scaffold (n = 12 per ovarian scaffold type) using a 30G needle, and the total amount of cells was delivered in five separate injections (200 000 cells per injection). Cells were then allowed to attach to the scaffold undisturbed for 30 min, and then culture medium was added to the constructs so that they remained completely submerged in culture medium. The medium was replaced every second or third day during the 14-day long culture. Each recellularised ovarian construct was then processed for histology, immunohistochemistry, and/or scanning electron microscopy (SEM). The cell density in the cultured constructs was established by counting haematoxylin$^+$ cells from sectioned ovaries, and the results were presented as cells per mm^2.

Scanning electron microscopy (Paper II)

Scanning electron microscopy samples were dehydrated and processed using standard SEM methods. The specimen was cut in half so that a cross section could be viewed. This work was conducted at the Centre for Cellular Imaging at the Core Facility at Sahlgrenska Academy, University of Gothenburg.

Immunohistochemistry (Papers I – IV)

For Papers I and II, DNA was fluorescently labelled by 4´,6-diamidino-2-phenylindole (DAPI; labelled blue). Since paraffin denatures the RFP protein and consequently inactivates the fluorescence, an antibody for RFP was applied to label the cells used for the mouse ovary scaffold after recellularisation. These cells were also labelled with the apoptotic marker cleaved caspase-3 and a marker for cell proliferation (Ki67) to evaluate whether the cells were undergoing cell death or still proliferating 14 days after recellularisation.

For Papers III and IV, non-fluorescent immunohistochemistry was applied using the sensitive Mach 3 and vulcan fast red kits (alkaline phosphatase based; Biocare Medical, California, USA). Instead of a fluorescently tagged secondary Ig-G antibody, the Mach 3 and vulcan fast red kits label the primary antibody with a red polymer, and since it is a 3-step staining protocol, the staining is very sensitive and gives little background staining. Antibodies used to identify infiltrated host immune cells in isolated grafts from Papers III and IV were specific towards leucocytes (cluster of differentiation, CD45; used in paper III only), T-cells (CD4), cytotoxic T-cells (CD8a), B-cells (CD22), NK-cells (NCR1), pan-macrophages (CD68) and M2-macrophages (CD163).

Gene expression analysis with digital droplet PCR (Papers III and IV)

Messenger RNA was extracted from the uterus grafts that were kept in RNALater using an mRNA extraction kit (Qiagen). The isolated mRNA was reversed transcribed to coding DNA (cDNA) using standard molecular biology kits, and gene expression was then quantified by digital droplet polymerase chain reaction (ddPCR). Briefly, a small cDNA quantity from each sample was added to the PCR master mix. This sample was then further divided into 16000–20000 droplets using a droplet generator (BioRad, Stockholm, Sweden). A PCR was then conducted on each individual droplet, and positive and negative

droplets were then sorted in BioRad's QX200 ddPCR system. The absolute value in copies per µl was established using Poisson's mathematics. The major advantages compared to standard quantitative reverse transcriptase PCR analysis are that the ddPCR is a more sensitive analysis, gives absolute copies of cDNA (not relative), and generates thousands of individual measurements per sample (generating parametric data for statistical evaluation).

The genes investigated using this system included the proinflammatory cytokines interferon γ (IFN-γ), IL-1β, IL-2, IL-6, and tumour necrosis factor (TNF). In Paper III, the gene expression of the additional chemokines was also investigated: eotaxin-2, RANTES (regulated on activation, normal T cell expressed and secreted), monocyte chemoattractant protein 1 (MCP1), macrophage inflammatory protein 1-alpha (MIP-1α), macrophage inflammatory protein-3 (MIP-3α) and IL-8. Each gene expression was normalised to the expression of the housekeeping gene peptidyl-prolyl cis-trans isomerase H (PPIH) and was transformed to fold change for comparison between experimental groups.

Statistical analyses (Papers I – IV)

The GraphPad Prism software (GraphPad, CA, USA) was used for all statistical analyses in this thesis. The distribution of the obtained datasets was all assessed using the Shapiro–Wilk test. If the data passed the normality test, Welch's t-test was applied for two group comparisons. For multiple group comparisons of normally distributed data, one-way ANOVA with Tukey's corrections was used. Non-parametric data (that did not pass the normality test) were assessed using the Mann–Whitney U-test for two-group comparisons, and for non-parametric multiple group comparisons, the Kruskal–Wallis test and Dunn's post-hoc test that corrects for multiple group comparison were used.

RESULTS AND COMMENTS

Paper I

Three different mouse ovarian decellularisation protocols were successfully developed. The SDC and SDS based protocols generated promising ovarian scaffolds, while a protocol that combined the two detergents caused excessive damage to the ECM and did not seem to generate good quality scaffolds. During the protocol optimisation process, it also became evident that there was no benefit of perfusing the ovaries during the decellularisation process. Hence, the decellularisation process was conducted by keeping the ovaries submerged into the decellularisation solution, which was equally efficient as perfusion. Furthermore, the mild detergent Triton X-100 and the organic compound DMSO were ineffective for mouse ovary decellularisation, which was surprising considering that this combination was effective for the rat uterus (Papers III and IV).

Histological evaluation of the remaining ECM based scaffolds revealed that the SDC derived protocol preserved the sGAGs, collagens, elastin, and the overall total weight better than the more aggressive SDS derived protocol. However, there was more DNA remnants of a considerable long base pair size in the SDC-derived scaffolds. These lingering DNA fragments may act detrimentally after engraftment since they could potentially activate proinflammatory mechanisms. This should be considered in future Tx studies of bioengineered ovaries. However, this study reports on two interesting scaffold types for future ovarian bioengineering studies for the mouse model. Further assessments should include a more detailed characterisation of the scaffolds and investigate whether they are biocompatible and can support cellular reconstruction.

Paper II

The same ovarian scaffold types as of Paper 1 were analysed at greater depth in Paper II, to distinguish a principle decellularisation protocol that could be used for future primary follicle growth studies and subsequent Tx studies.

The quantification of the most common ECM molecules that remained in the ovarian scaffolds after decellularisation revealed that the SDS protocol reduced the quantity of these important molecules more than the SDC protocol. Hence, also in this study, the milder SDC treatment seemed to generate a better scaffold type. However, all three scaffold types were found to be biocompatible using the MTT assay and proved that no toxic remnants from the decellularisation process lingered in the scaffolds. All scaffold types could also support the growth of RFP-labelled MSCs for two weeks in vitro, which showed a continued active proliferative state ($Ki67^+$). Statistically, there was no significant difference in cell densities between the scaffolds. However, there was a tendency that more cells could be found in the SDC derived scaffolds. If the group sample sizes were larger, this observation may have become statistically significant. In general, the cells were located in the cortex regions of all scaffold types, and the cell density was sparse in the medulla region of all scaffolds. This suggests that there were technical issues during the recellularisation and that there are room for improvements concerning the methods for seeding cells into the scaffold and to maintain their growth and division. It also suggests that the cells may not be prone to migrate into the deeper tissue compartments unless they are stimulated or forced to do so. Hence, additional recellularisation optimisation studies would be advantageous to improve the cell densities of the recellularised scaffolds. MSCs have been shown to provide important therapeutic effects after engraftment, and future studies should also include adding immature follicles to the recellularised ovaries and assessing the folliculogenesis after transplantation and whether the endocrine function can be restored. This is naturally the primary goal of a bioengineered ovary to be able to maintain an environment that is suitable for growth of a primordial follicle all the way to a large Graafian follicle, which is ready to ovulate.

It may well be that initially, in such a development, larger secondary follicles will be needed to be inserted for further folliculogenesis to take place. However, the niche for primordial follicles should be better in a bioengineered ovary than in a standard in vitro setting. In summary, the results indicated that scaffolds derived from the SDC based protocol was particularly vital. This protocol should be further explored in future studies. Yet, the immunogenicity of such constructs should also be monitored.

Paper III

Like the ovarian scaffold studies presented in Papers I and II, three decellularisation protocols were established for the rat uterus that generated scaffolds with varying degrees of donor DNA and ECM components. It is unclear whether these components are immunogenic and/or whether the decellularisation process itself causes new antigens that can provoke a response. Damaged and fragmented endogenous molecules may act as DAMPs and induce a detrimental proinflammatory environment which could induce rapid scaffold degradation and block regeneration mechanism rather than potentiate them. To investigate this, three types of rat uterus scaffolds were Tx to a syngeneic (inbreed) rat model, and the infiltration of immune cells and the local expressions of proinflammatory cytokine and chemokines were investigated.

The first two decellularisation protocols (P1 and P2) were based on the mild detergent Triton-X100, but because of the more isotonic buffered decellularisation process in P1, this specific protocol generated scaffolds with a significantly higher donor DNA content compared with P2. P3 was based on the slightly more aggressive detergent SDC and generated low DNA-containing scaffolds.

At five days after engraftment, it was clear that scaffolds with a high concentration of remaining donor DNA (P1 scaffolds) induced large cell infiltration (haematoxylin^{+} cells). Further analysis showed that many of

these cells were leukocytes ($CD45^+$). We also found an early infiltration of leukocytes into grafted P3 scaffolds of which there was a high proportion of $CD4^+$ T-cells. Interestingly, the general immunological profile during the 30-day observation time showed that P1 scaffolds generated an early transient immune reaction, while scaffolds from P3 induced a more prolonged activation of the immune system, which seemed to remain throughout the study period. However, scaffolds derived from P2 were tolerated better by the recipient's immune system and presented an immunological profile similar to autologous tissue (the gold standard).

Hence, this study showed that there was a decellularisation protocol-dependent immunologic response and that the decellularisation process can produce DAMPs that can reduce scaffold quality and be detrimental to scaffold survival after engraftment, even in a syngeneic situation. The study also suggests that rat uterus scaffolds produced by a mild, yet efficient protocol (P2; Triton-X100, DMSO and dH_2O) limit the formation of DAMPs. Thus, using a mild decellularisation protocol for scaffold production seems advantageous for future in vivo bioengineering studies.

Paper IV

The study presented in Paper IV investigated the potential allogeneic immunological properties of the uterine scaffolds. This represents a similar situation to how it would be in a clinical setting using decellularised scaffolds, where there would be an allogeneic donor organ from which the scaffold is produced from. It is generally believed that the close homology of ECM molecules between species should prevent a detrimental immune reaction after the Tx of scaffolds derived from decellularised tissue. However, as seen in Paper III, the decellularisation process can cause damaged and fragmented molecules that can act as DAMPs. Newly exposed epitopes in the ECM may also act immunogenic, and this response may be potentiated in an allogenic Tx setting. This needs to be explored further to ensure the

safety of the graft and for the recipient after scaffold engraftment. Hence, a similar experimental setup as seen in Paper III was used in Paper IV, using the same scaffold types but including allogeneic recipient animals as the Tx model.

Interestingly, the P2-derived rat uterus scaffolds were also more inert to the recipient's immune response in the allogeneic setting compared with the P1 and P3 scaffolds. Scaffolds derived from P1 (with the highest amount of donor DNA) induced an elevated immune response soon after engraftment, while P3 scaffolds induced a delayed reaction. The majority of the identified macrophage populations in the grafts were not of the regeneration-related class M2 phenotype (the ratio of $CD163^{+}/CD68^{+}$ cells was low in all groups). However, the proinflammatory cytokine gene expression in the grafts was comparably low, which suggests that there was only modest proinflammatory class M1 macrophage activation. To decipher the exact immunological mechanisms following the engraftment of decellularised tissue, more cell markers and genes should be assessed (e.g., chemokines, anti-inflammatory cytokines, and cell markers for T-regulatory cells and a greater diversity of macrophage markers). However, this study focused on assessing potential detrimental effects (i.e., proinflammatory signals) that could cause bioengineered tissue engraftment failure.

The results confirmed that the amount of remnant donor DNA after the decellularisation process is important from an immunological perspective and that the acceptable limit for decellularised rat uterus seems to be around 1% of the remaining donor DNA in this allogeneic transplantation model. Evident by the lower immunological response quantified in recipient animals of P2 derived grafts compared with the response seen in animals receiving grafts of the other scaffold types.

DISCUSSION

Paper I

The most common types of cancer during childhood are ALL, followed by neuroblastoma, Hodgkin's lymphoma, non-Hodgkin's lymphoma, Wilm's tumour, Ewing's sarcoma, and osteosarcoma of the pelvis (146). Surgery, chemotherapy, and radiation therapies, alone or in combination, can cure most cancers diagnosed. For example, the five-year childhood cancer survival rate is about 80% (147) and approximately 70% of patients are still cured 10 years after diagnosis (148, 149). However, fertility can be significantly compromised due to gonadotoxic treatment side-effects (22, 150). Radiation is particularly toxic to the ovaries. Concerning chemotherapy, differences in toxicity to the ovaries exist based on the mechanisms of action for a specific agent and whether combination drugs are used. In general, chemotherapeutic agents and their effects on female fertility are classified as mild, moderate, and severe, with alkylating agents such as cyclophosphamide having potent gonadotoxic effects.

Fertility preservation methods such as ovarian cortex cryopreservation may allow these women to become biological mothers after treatment (24). However, fertility preservation for prepubertal women with lymphoma or leukaemia is particularly challenging because there is a risk of reintroduction of cancer cells during the ovarian cortex re-transplantation procedure (25). For these reasons, ovarian bioengineering research has explored the idea of culturing isolated immature follicles in-vitro (33-35) or in various biomaterials (42, 47, 151) that can support follicular growth during folliculogenesis. These concepts have been extended to include transplantation of bioengineered ovaries, with the aim of allowing the follicles to mature after engraftment and, at the same time, re-establish the endocrine function (94). These principles show promising results but have thus far failed to support the growth of immature follicles to a mature stage in large animal models. Hence, more dynamic biomaterials need to be developed.

As a first step in the development of more suitable biomaterials for growing follicles, we used a mouse model to evaluate different decellularisation protocols to generate novel ovarian ECM scaffolds. Small variations in the decellularisation protocols can significantly modify the scaffold properties and may thus have meaningful consequences for follicular growth. Mouse and rat models are valuable for this type of research, as tissue is more readily accessible from these species than from larger mammals, and decellularisation protocols may therefore be evaluated more rapidly. Additionally, standard protocols exist for the isolation of primordial follicles in the mouse and many mechanisms behind the follicular activation and early growth have been characterised. For example, in vitro activation of primordial follicles includes the activation of the phosphoinositide 3-kinase (PI3K)-AKT-mTOR signalling pathway (152) and tensin homolog deleted on chromosome 10 (PTEN) suppression induces primordial follicle activation (153).

The experiments described in Paper I represent the first step in the creation of a bioengineered ovary which potentially can solve the issue of the risk of introducing malignant cells during the restoration of fertility after cancer treatment. It is important to conduct such studies in a systematic way and gradually develop protocols toward larger animal models, including non-human primates, before any clinical introduction.

The three different detergents used for the removal of donor cells from mouse ovaries included: a) SDS (a strong detergent), b) SDC (an intermediate detergent), and c) Triton-X100 (a mild detergent). Like many other groups, we found that the SDS treatment generated ovarian scaffolds with very low donor DNA levels (94, 113, 128, 154). This scaffold type fulfilled the criteria stated in a highly impactful review article (less than 50 ng donor DNA per mg dry tissue and DNA strands less than 200 base pairs in size) (111). The SDC treatment was somewhat less effective than the SDS treatment in removing the DNA but the ECM morphology seemed better preserved. On the other hand, treatment with the mild Triton-X100 detergent was found to be ineffective. This was surprising because Triton-X100 was effective

during a five-day perfusion protocol for rat uterus decellularisation (95). Nonetheless, these findings signify the importance of developing organ-specific decellularisation protocols for future bioengineering applications, and that protocols may not be easily transferred to different organ types.

A protocol using a combination of SDS and SDC was also evaluated. This approach was designed to determine whether reducing the exposure time to each detergent would result in better ECM preservation and maintain the beneficial DNA removal properties of the SDS treatment. Indeed, this led to scaffolds with very low DNA levels. However, the treatment also disrupted much more of the ECM structures than anticipated. Hence, we concluded that two of the three evaluated scaffold types were sufficiently promising to warrant further investigation (the SDS-based and SDC-based protocols).

Naturally, the detergent concentration also plays a critical role in the protocol's decellularisation capacity. For example, we used 0.5% SDS in the protocols presented herein. However, small pieces of human ovarian tissue (cortex and medulla) were successfully decellularised using a protocol that included 0.1% SDS with a slightly longer exposure time to compensate for the lower concentration (18–24 h) (113, 154). The resulting scaffolds showed a well-preserved ovarian ECM that could support the growth of immature follicles. In a different study, a concentration of 1% SDS was used to decellularise mouse, sheep, and human ovarian tissue strips in an "overnight treatment" (112). This protocol did not result in beneficial scaffolds and was even worse than scaffolds derived from another aggressive decellularisation protocol based on sodium hydroxide.

Temperature is another variable that may affect the efficacy of decellularisation protocols. For example, low temperature seems to be beneficial preventing endogenous enzymatic digestion of the ECM during the decellularisation procedure for pancreatic tissue (155).

Paper II

The more profound analysis conducted in Paper II on the same mouse ovarian scaffold types as in Paper 1 found that the SDS-based protocol (P1) reduced the quantity of ECM molecules to a greater extent than the other two protocols. Similar observations were reported in a porcine heart valve decellularisation study, where it was concluded that more favourable scaffold qualities resulted from the use of SDC than SDS (156). However, treatment of some tougher tissues (e.g., joint discs) may require the use of relatively aggressive detergent protocols rather than weaker detergents (157). Again, these conclusions underscore the importance of developing organ- and species-specific decellularisation protocols for bioengineering applications.

Nevertheless, SDS is by far the most used detergent to decellularise tissues, including ovarian tissue. However, there are several reports indicating that scaffolds produced by SDS based protocols might be more difficult to recellularise (158, 159). This may be due to the difficulties associated with removing toxic SDS residues from the scaffolds after decellularisation (160), or due to the inflicted collagen fibre microstructure rearrangement (161) which may modulate cell behaviour during the recellularisation phase.

The scaffold types used in Paper II had good recellularisation properties, and there was no significant difference between the cell densities after recellularisation when SDS and SDC based protocols were used. However, a few of the recellularised ovaries from the SDC derived scaffolds contained much higher cell densities than those from the other two protocols. This may be a sign that SDC based scaffolds could be more beneficial during recellularisation compared with scaffolds derived from SDS based protocols. It must be noted that technical factors also play an important role in obtaining a successful recellularisation. For example, it is challenging to consistently inject a large number of cells into small scaffolds without accidently penetrating the needle through the scaffold. It is also difficult to prevent cells from leaking out of the injection site upon needle withdrawal. Pors et al. also highlighted these issues and evaluated the use of Matrigel to seal the

cavity caused by the needle incision, thereby improving the recellularisation efficiency (113). Further studies on how to best recellularise decellularised tissue, with extended culturing periods or with culturing methods that facilitate media diffusion during in vitro culture (e.g., using spinner flasks) (162) will likely lead to better treatment outcomes.

The continuation of this research project will include the addition of immature mouse follicles of different developmental stages to the produced ovarian scaffolds and the study of their growth capabilities in vitro. Transplantation studies would also be valuable in determining whether such bioengineered ovaries could stimulate follicular growth in vivo, hormone production, and oocyte maturation.

Papers III

In Paper III, decellularised rat uterus tissue was subcutaneously transplanted to syngeneic rats to evaluate whether the decellularisation process generated allo-independent immunoreactive elements (i.e., DAMPs) which may act detrimental after engraftment. DAMPs can be formed from damaged nucleotides, or molecular fragments of collagen, hyaluronic acid, fibronectin, elastin, and other cellular and ECM components (142, 143). The protein denaturing properties of the detergents used during the decellularisation process may be responsible for the generation of such molecules. Yet, to our knowledge, no previous study has investigated this in detail.

Briefly, our results indicated that there were decellularisation protocol-dependent differences in the activation of the immune system after engraftment. The Tx were conducted in a syngeneic animal model; therefore, these immunological effects resulted from allo-independent DAMPs. There was an early infiltration of $CD45^+$ leukocytes in two of the evaluated scaffold types (P1 and P3). The high density of immune cells declined over time in the P1 scaffolds but remained high in the P3 scaffolds for the duration of the experiment. As there was no dramatic

increase in T-cell or macrophage density in the P1 or P2 scaffolds, the results suggest that there was a much lower presence of DAMPs in these scaffold types compared with the P3 type. Generally, P3 scaffolds caused a greater infiltration of $CD4^+$ T cells and $CD22^+$ B cells. This may indicate a negative milieu for tissue regeneration and ongoing mechanisms of scaffold destruction (163). These results were further supported by gene expression analysis, which showed a slightly elevated pro-inflammatory effect in P3 derived scaffolds.

The three decellularisation protocols used were based on a mild detergent for P1 and P2 (Triton-X100) and a medium strength detergent for P3 (SDC). Yet, there was a significant difference in donor DNA levels between P1 and P2 scaffolds. Hence, the initial immune response that was detected in P1 scaffolds was caused by nucleotide-dependent DAMPs that were more abundant in P1 scaffolds. Interestingly, this type of DAMPs seems to be of lesser concern than the types present in P3 scaffolds because the pro-inflammatory immune reaction diminished over time in P1 scaffolds but remained in P3 scaffolds. It is likely that the DAMPs in P3 scaffolds were of intracellular or ECM origin, as these scaffolds contained a low amount of donor DNA. These scaffolds were treated with a more aggressive detergent than P1 or P2 scaffolds, which probably caused greater damage to structural molecular components, resulting in more DAMPs.

This study focused on pro-inflammatory responses after engraftment, but additional information on anti-inflammatory mechanisms would provide a more complete picture of immunological events (164). Additionally, identifying the ratio of infiltrated class I and class II macrophages would provide a better understanding of how these cell types affect scaffold degradation (M1) or tissue regeneration (M2) (165). More specific identification of the DAMPs responsible for the immune response would also facilitate decellularisation protocol development by providing information about what molecules need to be spared during the decellularisation process.

However, our results clearly show that scaffolds produced by the mild, yet highly effective P2 caused fewer DAMPs than P1 and P3. This

important knowledge will aid in the development of immune-inert scaffolds for future bioengineering applications.

Paper IV

Paper IV had a similar experimental design to the study presented in Paper III. The focus of this study was to evaluate any potential immune response after scaffold Tx in an allogeneic setting. This represents a more clinically relevant situation because the preference is to have scaffolds ready on demand. The donor material would thus be collected from a deceased subject in advance, without prior tissue matching, as the decellularisation process should optimally remove all immune-provoking molecules, including major histocompatibility complex class I and class II. However, very few studies have examined the general immune response following decellularised tissue engraftment. Instead, most scientist seem to rely on a highly cited review article that, without using any relevant references, concluded that scaffolds with less than 50 ng of donor DNA per mg of scaffold will not invoke an immune response after engraftment, as long as the DNA strands are shorter than 200 base pairs (111). Yet, several recent studies have suggested that scaffolds with a greater amount of lingering donor DNA work rather well after engraftment (134, 140, 144). It is therefore likely that the criteria set by Crapo et al. (2011) are not relevant to all organs, and that more forgiving donor DNA levels can be tolerated by the recipient without inducing an immune response. This is not a trivial topic since less aggressive decellularisation protocols can then be used, which will reduce ECM damage and lead to fewer DAMPs in the scaffolds (Paper III). For a successful translation of promising protocols from rodent studies to larger animal models and eventually to human material, it is crucial to ensure that graft and patient safety is considered, and that the recipient's immune system remains favourable.

Interestingly, we noted a similar response in the recipients to that observed in the syngeneic setting in Paper III. Allogeneic transplantation of P1 derived scaffolds activated a modest and

potentially detrimental early response that had ceased after 30 days. As this scaffold type contained the highest amount of allogeneic donor DNA, it is likely that this was the cause of the rapid cellular infiltration seen on day five after engraftment (166, 167). It is plausible that when these foreign DNA fragments were cleared from the tissue, the immune response began to accept the graft since we did not see an activation of T-cells or macrophages in this group. Conversely, P3 induced a more delayed and prolonged immune response after engraftment which could be a sign of an ongoing negative inflammatory mechanism and graft destruction (168).

Also, as mentioned for Paper III, this study would have benefited from a more in-depth investigation of the immunological response that included both the pro-inflammatory and the anti-inflammatory pathways. Additionally, quantifying the infiltrating fibroblast and endothelial cells that play active roles in tissue remodelling and regeneration may also have led to a greater understanding of the overall processes that take place after the Tx of decellularised tissue.

However, this study focused on deciphering any pro-inflammatory events that could be detrimental to the graft and the regenerative processes we wish to stimulate. Our results clearly show that, in the allogenic Tx setting, P2 scaffolds were the least immunogenic among the tested scaffolds. They generated a similar immunological profile as autologous tissue grafts and should therefore be considered safe to use and a promising material for uterus bioengineering applicants in the rat. Conclusively, scaffolds resulting from the use of a mild detergent during decellularisation was more favourable after Tx than scaffolds developed using aggressive detergents. This knowledge should be useful when developing future decellularisation protocols for novel tissues and organs from large animals or of human origin.

CONCLUSION

Paper I Three mouse ovarian decellularisation protocols were successfully developed. An SDC based and an SDS based protocol generated promising ovarian scaffolds.

Paper II When assessed further, these two scaffold types continued to prove relevant. The SDS based protocol reduced the quantity of the ECM more than the SDC based protocol, but all scaffolds were biocompatible and the recellularisation of the scaffolds was successful, particularly in scaffolds derived from the SDC protocol. Hence future ovarian bioengineering experiments in the mouse model should focus on using SDC protocols for scaffold development.

Paper III Transplantation studies using syngeneic rat uterus scaffolds showed a protocol-dependent immune response. Hence, the decellularisation process produce DAMPs that can reduce scaffold quality and be detrimental to scaffold survival after engraftment, even if the tissue is syngeneic. However, one of the rat uterus scaffold types that were developed using a mild yet efficient protocol (Triton-X100, DMSO and deionized water) did not provoke a large leucocyte infiltration or a high pro-inflammatory response; it seemed to be tolerated by the recipient's immune system.

Paper IV Interestingly, the same rat uterus scaffold type remained inert to the recipient's immune system following an allogeneic transplantation. Our results also confirmed earlier studies that showed that the amount of donor DNA that remains after the decellularisation process is important from an immunological perspective. Our results suggest that the acceptable limit of remaining donor DNA in decellularised rat uterus is around 1% in an allogeneic transplantation model.

FUTURE ASPECTS

Papers I-II

The stem cell recellularised ovarian scaffold produced by the SDC based protocol in Papers I and II should be evaluated as a biomaterial for primordial mouse and human follicles during in vitro follicular growth experiments. Additionally, such bioengineered ovarian tissue should be transplanted into an ovariectomised mouse model to evaluate in vivo follicular growth and to determine if such a transplant also could restore the endocrine function of the ovary with replenished estradiol and progesterone serum levels. Naturally, these experiments should also include attempts to obtain mature oocytes and assess their functionality in subsequent IVF and embryo transfer techniques to produce live offspring in the mouse. If human follicles should be used this way, a xenograft mouse model would need to include an immune-deficient mouse strain.

Once such results have been established, it would be interesting to continue to assess these principles using larger animal models, e.g., the sheep model.

Papers III-IV

The scaffold type developed by the mild decellularisation protocol in Papers III and IV should be used to construct large grafting materials with various types of cells and assess their regenerative properties to restore fertility in a uterine wall injury in the rat model. Additionally, similar decellularisation principles should be translated to the sheep model with subsequent Tx studies; both to evaluate if these biomaterials have regenerative properties, and to investigate the immunological response after engraftment to ensure that safe biomaterials can be constructed by decellularisation protocols.

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