Progesterone's effect on gamete transport in the fallopian tube

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Gothenburg 2014

Cover illustration: Anna Bylander

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ISBN 978-91-628-9178-7

Printed in Gothenburg, Sweden 2014
Ineko AB

To all animals that give their lives to research

ABSTRACT

The fallopian tube plays an important role in successful female reproduction because it transport gametes to the fertilisation site, nourishes the developing embryo and transports the embryo to the uterus at a suitable time for implantation. The overall aim of this thesis was to investigate the role of progesterone in the control of gamete transport.

Ciliary cells are important for the transport of gametes and embryos through the fallopian tube. In paper **I**, we developed a method to measure ciliary movement in mouse fallopian tubes. Using a high-speed camera connected to a microscope, we were able to document the rapid effects of progesterone on the ciliary beat frequency *in vitro*. In ciliary cells from a mouse fallopian tube treated with progesterone at concentrations of 20 µM and a more physiologically relevant concentration of 100 nM, we found a rapid reduction in the ciliary beat frequency by 10 % and 15 %, respectively, within 30 minutes of the addition of progesterone.

In paper **II**, we investigated the possible involvement of the classical progesterone receptor in mediating the rapid effect of progesterone. The ciliary beat frequency was significantly reduced within 10-30 minutes by low concentrations of progesterone (10-100 nM) and by another more specific agonist. Co-exposure to an antagonist completely blocked the effect of progesterone. In mice lacking a functional progesterone receptor, we found no effect of progesterone. These findings strongly indicate that progesterone reduces the ciliary beat frequency by acting on the classical progesterone receptor. The rapid onset of the effects suggests that a non-genomic mechanism is involved.

In paper **III**, we used a microarray to investigate possible changes in the gene expression in mouse fallopian tubes after *in vitro* exposure to progesterone for 20 minutes or 2 hours. We could not detect any change in the gene expression with the microarray after 20 minutes of exposure to progesterone, which is consistent with the hypothesis that the rapid reduction in the ciliary beat frequency is not dependent on transcription. In fallopian tubes exposed to progesterone for 2 hours, 11 genes were differentially expressed compared with the controls. This change was confirmed by quantitative PCR at 2 h and 8 h. The most interesting gene regulated by progesterone was endothelin-1, a signal peptide known to induce muscle contraction in the fallopian tube. In this paper, we also studied the role of the progesterone receptor in the transport of the oocyte-cumulus complex. Gonadotropin-treated mice were given a single injection of one of the progesterone receptor antagonists Org

31710 or CDB2194 or vehicle 6 hours before ovulation. In the mice treated with both antagonists, the oocyte-cumulus complex travelled faster through the fallopian tube than in the mice only given vehicle.

In conclusion, we found that progesterone rapidly reduces the ciliary beat frequency in ciliary cells from mouse fallopian tubes and that this reduction is mediated through the classical progesterone receptor, most likely via a non-transcriptional mechanism. We show that progesterone regulates endothelin-1, a peptide known to induce muscle contractions in the fallopian tube. This finding suggests that endothelin-1 is a mediator of the previously shown effects of progesterone on tubal contractility. We confirm earlier studies by demonstrating that progesterone and the progesterone receptor are important for normal tubal transport. Taken together, the results presented in this thesis contribute to a deeper understanding of the role of progesterone and the progesterone receptor in the regulation of gamete and embryo transport along the fallopian tube.

Keywords: Fallopian tube, progesterone, ciliary beat frequency, gamete transport.

ISBN: 978-91-628-9178-7

SAMMANFATTNING PÅ SVENSKA

Äggledarna är en del av de kvinnliga reproduktionsorganen. De är tunna rörformade organ som sträcker sig, en på varje sida, från livmodern upp mot äggstockarna. Det är i äggledarna som befruktningen sker och ett embryo börjar utvecklas. Äggledarens funktion är att transportera ägg och spermier till platsen för befruktning, att ge näring åt det befruktade embryot och att sedan transportera embryot i precis rätt tid till livmodern för implantation. För en lyckad befruktning på naturlig sätt är äggledaren väsentlig och defekter i äggledaren är en orsak till infertilitet hos kvinnor och kan vara en orsak till utomkvedshavandeskap. Syftet med studierna i den här avhandlingen har varit att undersöka det kvinnliga könshormonet progesterons snabba effekter på flimmerhårens slagfrekvens och om dessa effekter sker via den klassiska receptorn för progesteron. Vi har också undersökt hur genuttrycket i äggledaren påverkas av progesteron och vilken roll progesteronreceptorn har för äggtransporten.

I den första studien utvecklade vi en metod för att kunna mäta frekvensen på flimmerhår i äggledare från möss. Från icke könsmogna honor isolerade vi äggledaren, klippte upp den först på längden och sedan i små bitar. Med hjälp av en höghastighetskamera kopplad till mikroskop och en dator kunde vi mäta med vilken frekvens flimmerhåren viftade. Vi undersökte därefter om progesteron sänkte flimmerhårens slagfrekvens. När en basfrekvens var fastställd tillsatte vi progesteron i höga till måttliga koncentrationer eller enbart lösningsmedel och mätte frekvensen. Båda koncentrationerna av progesteron sänkte flimmerhårens slagfrekvens inom 30 minuter.

I den andra studien undersökte vi om progesteron orsakar den snabba sänkningen i flimmerhårens slagfrekvens genom att binda till den klassiska receptorn för progesteron. Detta gjorde vi genom att tillsätta speciella molekyler som antingen förväntas ge samma effekt som progesteron, agonister, eller blockerar receptorn så att progesteron inte kan binda till den, antagonister. Agonisterna sänkte slagfrekvensen inom 10-30 minuter, och vi såg en effekt av progesteron redan vid en mycket låg koncentration. Samtidig behandling med antagonist tog helt bort effekten av progesteron. Vi visade också att hos möss som saknar en fungerande progesteronreceptor så påverkar inte progesteron slagfrekvensen.

I det tredje arbetet undersökte vi med hjälp av teknikerna microarray och kvantitativ PCR, (qPCR), vilka gener i äggledaren som påverkas av progesteron. Vi studerade genuttrycket efter 20 minuters exponering av

progesteron för att identifiera eventuella gener som kunde vara involverade i regleringen av flimmerhårens slagfrekvens. Efter 20 minuter kunde vi inte finna några gener som påverkats av progesteronbehandlingen, vilket är i linje med hypotesen att progesterons snabba sänkning av flimmerhårens slagfrekvens sker oberoende av förändringar i genuttryck. Efter 2 timmar kunde vi emellertid påvisa 11 gener vars aktivitet påverkades av progesteron. Microarray ger en övergripande bild men det krävs ytterligare analyser med andra metoder för att verifiera resultaten. Av de 11 gener vi sett var påverkade av progesteron valde vi därför att vidare analysera några av generna med qPCR. Resultaten från dessa studier visade att progesteron ökade uttrycket för genen endothelin-1, vilket ingen annan studie visat förut. Vi visade också att en receptor för endothelin-1, endothelin receptor A hade minskad genaktivitet. Endothelin-1 produceras av epitelceller i äggledaren och från tidigare studier är det känt att denna peptid påverkar muskelkontraktioner i äggledaren.

I den tredje studien undersökte vi också den klassiska progesteronreceptorns roll i äggtransporten. Icke könsmogna möss behandlades med hormoner för att inducera ägglossning. Några timmar innan ägglossning fick de en injektion av endera av två antagonister som blockerar progesteronreceptorn. En grupp av möss fick bara en injektion av olja. När progesteronreceptorn blockerades transporterades äggen snabbare genom äggledaren.

Sammanfattningsvis har studierna i avhandlingen visat att progesteron sänker flimmerhårens slagfrekvens redan efter 10-30 minuter genom att binda till den klassiska kärnreceptorn för progesteron. Effekten är sannolikt för snabbt för att vara beroende av förändringar i genuttryck, vilket pekar mot att en alternativ intracellulär signalväg är involverad än den för progesteronreceptorn klassiska regleringen av genuttryck. Vi visar också att progesteron reglerar genuttrycket av endothelin-1 efter två timmar, en peptid som påverkar muskelkontraktionen. Det är således möjligt att progesteron påverkar muskelkontration i äggledaren via effekter på endothelin-systemet. Vi bekräftar också resultat från tidigare studier som visar att progesteron och progesteron receptorn är nödvändiga för en fungerande transport. Konsekvenser av en för snabb transport kan vara att ägget inte blir befruktat eller att det befruktade embryot når livmodern för tidigt och inte kan implanteras. Våra resultat bidrar till en ökad förståelse för de hormonella mekanismer som reglerar transporten av könsceller och det befruktade embryot genom äggledaren.

LIST OF PAPERS

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

I. ***Rapid effects of progesterone on ciliary beat frequency in the mouse fallopian tube.***

Bylander A, Nutu M, Wellander R, Goksör M, Billig H, Larsson DGJ.
Reprod Biol Endocrinol 2010, 8:48

II. ***The classical progesterone receptor mediates the rapid reduction of fallopian tube ciliary beat frequency by progesterone.***

Bylander A, Lind K, Goksör M, Billig H, Larsson DGJ.
Reprod Biol Endocrinol 2013, 11:33.

III. ***Progesterone-mediated effects on gene expression and egg transport in the mouse fallopian tube.***

Bylander A, Gunnarsson L, Shao R, Billig H, Larsson DGJ.
Submitted manuscript

CONTENTS

ABBREVIATIONS

AIJ	Ampullary isthmic junction
AF	Activation function
Amigo2	Adhesion molecule with Ig-like domain 2
Arfl4	ADP-ribosylation factor like 4D
ATP	Adenosine triphosphate
Ca^{2+}	Calcium
DBD	DNA-binding domain
CBF	Ciliary beat frequency
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
Edn1	Endothelin-1
Edn2	Endothelin-2
Edn3	Endothelin-3
Ednra	Endothelin receptor A
Ednrb	Endothelin receptor B
FSH	Follicle stimulating hormone
GnRH	Gonadotropin releasing hormone
hCG	Human chorionic gonadotropin
HRE	Hormone response element
Hprt1	Hypoxanthine phosphoribosyltransferase 1

IVF	In vitro fertilisation
LBD	Ligand binding domain
LH	Lutenising hormone
MAPK	Mitogen-activated protein kinase
mPR	Membrane progesterone receptor
OCC	Oocyte-cumulus complex
PBS	Phosphate Buffer Saline
PGR	Progesterone receptor
PGRMC	Progesterone receptor membrane component
PI3K	phosphatidylinositol 3-kinase
PMSG	Pregnant mare's serum gonadotropin
qPCR	Quantitative polymerase chain reaction
Rasd1	RAS, dexamethasone-induced 1
Rpl19	Ribosomal protein L19
R5020	Promegestone
RU486	Mifepristone
STAT	Signal transducer and activator of transcription
Src	Src tyrosine kinase
TRPV4	Transient receptor potential vanilloid
UTJ	Utero-tubal junction

INTRODUCTION

Successful natural reproduction in humans involves several crucial steps. For example, there must be a successful and well-timed transport of gametes to the site of fertilisation. Then, after fertilisation, it is important to provide a suitable environment to nourish the newly fertilised and developing embryo during its transport to the uterus. Finally, reproduction requires successful implantation. Fertilisation and the transport of both gametes and the embryo take place in the fallopian tubes, and the mechanisms involved are regulated by ovarian hormones.

The fallopian tube

The fallopian tubes are some of the female reproduction organs and were first described by Gabriel Falloppio in the sixteenth century. He observed the ducts connecting the ovaries and uterus and considered them to be sperm-transporting vessels. Today, we know that apart from transporting sperm to the oocyte-cumulus complex (OCC), the fallopian tubes also capture and transport the OCC during ovulation, provide the site for fertilisation, nourish the developing embryos, and transport them to the uterus [1, 2]. From the success of in vitro fertilisation (IVF), it is clear that exposure to the milieu of the fallopian tube is not absolutely necessary for fertilisation or implantation to occur and succeed. Therefore, the importance of a well-functioning fallopian tube for successful reproduction might be neglected in modern medicine. However, for natural reproduction in vivo, the fallopian tubes are essential, and it is important to understand their functions. Such knowledge can be used to improve the current approaches for assisted reproduction, to better understand and manage ectopic pregnancies and to better understand the mechanism of action of current existing contraceptive methods and to develop new, more effective contraceptive drugs.

Anatomy and Histology

The fallopian tubes are paired muscular tubes attached to either side of the upper part of the uterus and leading to the sides of the ovaries. In humans, they are not attached to the ovaries; instead, they open into the peritoneal cavity. In women they measure approximately 7-14 cm in length [3, 4].

A cross-section of a fallopian tube from the edge to the centre shows the serosa, the myosalpinx, the endosalpinx and the lumen. The serosa is the outer surface facing the peritoneal cavity. The myosalpinx is composed of smooth muscle bundles. There are three smooth muscle layers, and the most dominant layers are the longitudinal outer layer and the middle circular layer. There is also a thin inner longitudinal layer in the isthmic region. The endosalpinx is a mucosa that is folded out into the lumen and lined with epithelium, which consists of four cell types, namely ciliary cells, secretory cells, peg cells and basal cells [5]. The size of the lumen, the shape of the mucosa and its distribution of cells, as well as the thickness of the endosalpinx, varies along the tube [3,6,7].

The human fallopian tube consists of different anatomical segments; from the ovary to the uterus, we have the *infundibulum* with its fimbriae, the *ampulla*, the *isthmus* and the *intramural* or *utero-tubal junction* (UTJ).

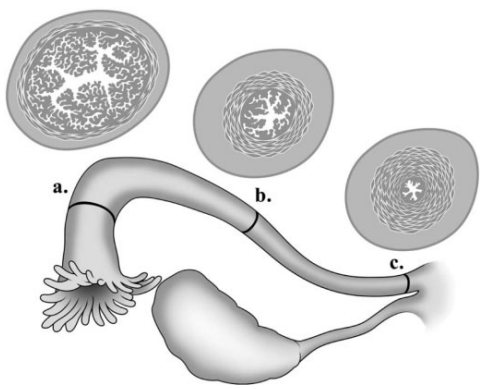

Figure 1. Illustration of the human fallopian tube showing A, the infundibulum region, B, the ampulla region and C, the isthmus region. The immunohistochemistry pictures show the muscle layers, the mucosa and the lumen in each region. Lyons et al. Human reproduction 2006:363-372

The infundibulum is the part closest to the ovary, and it ends with a fringe-like structure called *fimbriae*. The fimbriae are covered with millions of ciliated cells and sweep over the ovary, capture the ovulated OCC and draw the OCC into the fallopian tube. The ampulla, which comprises more than half the length of the tube, is the site of fertilisation. In this part, the

myosalpinx is thin, and the mucosa is rich in folds that fill the lumen and form a labyrinth-like structure. The ciliated cells are more abundant than the secretory cells. The isthmus is a narrow part of the fallopian tube near the uterus. In humans, this part is approximately 3 cm long. It has three developed muscle layers, the secretory cells dominate, and the mucosa has fewer folds than those of the ampulla. The intramural part is found in the uterine wall, measures approximately 1 cm in length and consists of three muscle layers [3, 4, 6, 7].

Blood supply and lymphatic drainage

The fallopian tubes are supplied with arterial blood from both the ovarian and uterine side. The ovarian artery originates directly from the aorta, whereas the uterine artery is a branch from the internal iliac artery, which is formed by a bifurcation of the aorta. The fallopian tube venous drainage system follows a similar pattern as the arteries: through the uterine veins into the internal iliac vein and through the pampiniform plexus to the ovarian vein. The lymphatic plexus in the fallopian tube is drained by ovarian and uterine lymphatic vessels [4, 8].

The arteries from the ovaries leading to the fallopian tube are in close contact with the corresponding veins and lymph vessels from the ovaries and uterus, allowing for a direct local exchange of substances, such as steroid hormones, from the veins and lymph vessels to the arteries. This counter-current transfer mechanism can facilitate local hormonal regulation within or between organs through local increases in the arterial concentrations of substances [8, 9].

The hormones produced in the ovary are released to the ovarian venous blood flow, and by counter-current transfer, some fractions of the hormones are transferred to the ovarian arterial blood and will reach not only the ovary but also the fallopian tube and the uterus. In women, this counter-current mechanism is proposed to be important for the pregnancy/non-pregnancy signal from the uterus and fallopian tube to the ovary and for the influence of the ovarian hormones on the function of ovarian, tubal, and possibly, uterine tissues [10]

Nerve supply

The fallopian tube is provided with both sympathetic and parasympathetic nerve fibres. Sympathetic fibres from T10 through L2 reach the inferior mesenteric plexus, and postganglionic fibres then pass to the fallopian tube. The parasympathetic supply occurs via vagal fibres from the ovarian plexus supplying the distal portion of the tube. Part of the isthmus receives its

parasympathetic supply from S2, S3, and S4 via the pelvic nerve and the pelvic plexuses [1, 11].

Ciliary cells

In the epithelium lining the mucosa in the fallopian tube, four different cell types has been identified; the most common and abundant are secretory cells (60%) and ciliary cells (25%), and the less common are intercalated peg cells and basal cells. The ciliary cells are generally situated on the apex of mucosal folds and are most common in the fimbriae (50%); their number is reduced along the fallopian tube to less than 35% of that in the isthmus [5, 12, 13]. The secretory cells contain apical granules that produce tubal fluid [12-14] while the function of peg cells is debated [15]. Both ciliary cells and secretory cells bear cilia; the ciliary cells have motile cilia, and the secretory cells have non-motile cilia. Cilia are microscopic, hair-like organelles that project from a cell's surface. In humans, cilia have been found on a large variety of cells in the body, and there are at least eight categories of cilia or cilia-derived organelles ranging from approximately 2 μm to 50 μm [16]. The non-motile cilia or primary cilia are found on nearly every cell type in the body, providing important sensory functions and play a key role in the development and homeostasis of cell proliferation [17]. Motile cilia are only present in the female reproductive tract, the respiratory tract, the brain and sperm tails [18] and are important for the gamete/embryo transport in the fallopian tube, the transport of mucus across the respiratory epithelia, the cerebrospinal fluid movement and the sperm movement. The motile cilia are present at the cell surface in large numbers, whereas cells with non-motile cilia only have one cilium [19].

A cilium consists of a basal body and an axonemal core enclosed by a plasma membrane. The axoneme is composed of nine sets of microtubule doublets arranged in a cylinder. In the animal kingdom, axonemes appear in two patterns: the motile 9+2 configuration, in which the nine microtubule doublets surround a central pair of single microtubules, and the non-motile 9+0 configuration, where the central pair is missing [18]. Apart from the two central microtubules, the 9+2 structure cilia also have thousands of dynein molecular motors distributed along the length and circumference of the axoneme; each motor is attached permanently at one end to an outer doublet and is able to attach to and detach from the neighbouring doublet. These dynein arms are responsible for ciliary movement, which is achieved by ATP-driven dynein activation cycles that cause neighbouring microtubule doublets to slide relative to each other [19]

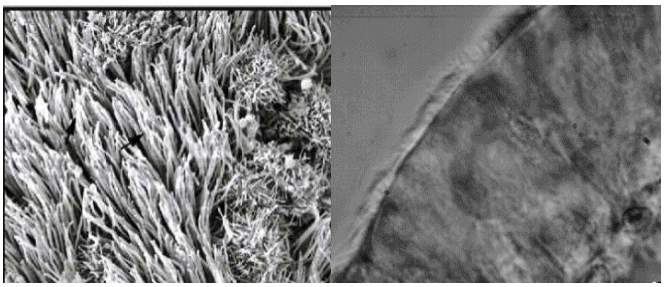

Figure 2. The left image shows a scanning electron micrograph of the epithelium of a hamster infundibulum with ciliary cells (arrows) and secretory cells (image from talbotcentral.ucr.edu). To the right is a light micrograph of ciliary cells on the edge of a piece of tissue from a mouse fallopian tube (image taken by Anna Bylander).

Hormonal regulation of female reproduction

Female reproductive organs are regulated by follicle stimulating hormone (FSH) and luteinising hormone (LH). These hormones are synthesised and secreted by the anterior lobe of the pituitary gland. The secretion of FSH and LH is regulated by the pulsatile secretion of gonadotropin releasing hormone (GnRH) that is produced in the hypothalamus. FSH acts on the ovary and stimulates the growth of follicles, and the follicle cells starts to produce oestrogen. LH acts on the mature follicle to induce ovulation and to transform the ruptured follicle to the corpus luteum after ovulation. After ovulation, the corpus luteum starts to produce progesterone. The secretion of FSH and LH is regulated by a feed-back system involving oestrogen and progesterone. Both oestrogen and progesterone act on the fallopian tube and uterus to prepare the tissues for fertilisation and implantation [20].

The cyclic changes in hormone levels and their effect on reproductive tissue can be divided into different phases based on the events in the ovary and the uterus. The ovarian cycle is divided into three phases, the follicular phase before ovulation, ovulation and the luteal phase after ovulation. The uterus cycle is divided into the menstrual phase, the proliferation phase and the secretory phase.

Endocrine response in the fallopian tube

During a reproductive cycle, the mucosa of the fallopian tube undergoes morphological changes due to increases and decreases in oestrogen and progesterone levels [1]. In the beginning of the menstrual cycle, the epithelial cells of the mucosa are low in height. Their thickness increases during the follicular phase, and a maximal height is reached at approximately the time of ovulation. At this time, the secretory and ciliated cells have the same size.

During the periovulatory period, the secretory cells peak in activity and release their contents into the lumen, after which their height is reduced compared with that of the ciliated cells. This change makes the cilia protrude and may permit them to assist in transporting the ovum and sperm. In the luteal phase, the heights of both cell types decrease, and there is a partial deciliation [21-24].

The serum oestrogen level increase during the follicular phase and peak around ovulation. The serum level of progesterone is low before ovulation and starts increasing after ovulation with a peak during the luteal phase. Oestrogen stimulates epithelial cell hypertrophy, secretion and ciliogenesis, whereas progesterone is associated with atrophy and deciliation [21, 25, 26].

Morphological changes are not observed in the myosalpinx during the menstrual cycle, but the contractile activity of the tubal muscle displays cyclic variations due to the change in the sex steroid levels. During the follicular phase and under the influence of high levels of oestrogen, the frequency of the spontaneous contractions in the circular muscle increase reaching a maximum around the time of ovulation. In the luteal phase under the influence of rising levels of progesterone, the frequency of muscle contraction is lower again [27].

Steroid hormones

All steroid hormones are synthesised from cholesterol and contain the characteristic arrangement of four cycloalkane rings joined to one another. Their main sites of production are the gonads and adrenal glands. Steroid hormones are fat soluble and can diffuse from the blood through the cell membrane into the cytoplasm of the cell. According to the classical action of steroids, a steroid binds to a specific receptor in the cytoplasm or the nucleus, and upon binding, most of the hormone-receptor complexes dimerise before entering the nucleus, if the receptor was not already located in the nucleus. In the nucleus, the hormone-receptor complex binds to specific DNA sequences and induces transcription of its target genes. Steroid hormones are divided into five groups depending on the type of receptor they bind to: glucocorticoids, mineralocorticoids, androgens, oestrogens and progestogens. Steroid hormones are often carried in the blood while bound to specific carrier proteins, such as sex hormone-binding globulin or corticosteroid-binding globulin [28-30].

Progesterone

Progesterone (pregn-4-ene-3,20-dione) belongs to the class of steroids called progestogens. Progesterone is essential for mammalian reproduction because it is involved in ovulation, gamete transport and implantation [31]. If pregnancy occurs, progesterone maintains the pregnancy by enabling uterine growth and suppressing the contractility of the uterus. Progesterone is also required for the lobular-alveolar development in mammary glands and the inhibition of milk production during pregnancy. In the brain, progesterone affects sexual response behaviour. The main site for progesterone production during the menstrual cycle is the ovaries and more specifically, the corpus luteum. If pregnancy occurs, the production is shifted to the placenta [32, 33].

Classical progesterone receptors

The classical progesterone receptor (PGR) is a ligand-activated transcription factor, and it belongs to the nuclear receptor superfamily. The basic structure of PGR includes five functional domains. At the C-terminal, there is the ligand–binding domain (LBD) that performs the progesterone-binding function. The DNA-binding domain (DBD) is located centrally, and the hinge region is found between the LBD and DBD. There are transactivation domains (AF for activation function), one located N-terminally to the DBD (AF-1) and one located in the LBD (AF-2) [34]. The transactivation domains regulate the level and promoter specificity of the target gene activation [35]. Inactive PGR is found in the nucleus or cytoplasm and is bound to a heat shock protein. Upon progesterone binding, the receptor separates from the heat shock protein, two ligand-receptor complexes then homodimerise. The homodimers are then translocated into the nucleus, and the active PGR binds to a hormone response element (HRE) in the promoter of the target genes. The AFs recruit different co-regulators that are responsible for the transcription of DNA to mRNA [30, 36].

From the PGR gene, two isoforms are transcribed, PGRA and PGRB. The difference between the two isoforms is that at the N-terminal, PGRB has an additional sequence of 125-164 amino acids, depending on the species. This sequence contains a third transactivation domain (AF-3), and as a result, the two isoforms can regulate different target genes in response to progesterone [35, 37, 38].

PGR expression has been defined in progesterone-responsive tissue, and the expression is controlled by oestrogen and progesterone, which increase and decrease, respectively, the expression of PGR in most target tissues [32]. The crucial role of PGR in reproduction has been confirmed using knockout mice.

Studies in mice expressing only one isoform demonstrated that PGRA and PGRB mediated mostly distinct, but to some degree, overlapping reproductive responses to progesterone. PGRA knockout mice show a normal response to progesterone in the mammary gland but display severe uterine hyperplasia and ovarian abnormalities. Mice lacking PGRA also fail to ovulate. In PGRB knockout mice, the response to progesterone in the ovary and the uterus is not affected, but the lack of PGRB results in reduced pregnancy-associated mammary gland morphogenesis [39].

Gamete transport

One of the functions of fallopian tubes is to transport gametes to the site of fertilisation and then transport the embryo to the uterus for implantation. The mechanism of tubal transport is complex and can be affected by several different factors and conditions that may diminish fertility [25].

Sperm migration

In mammals, the site of spermatozoa insemination is the uterus or vagina, the latter for women. When spermatozoa are released, they must travel through different anatomical regions of the female reproductive tract, such as the cervix, uterus, and uterotubal junction, and then travel through the isthmus region of the fallopian tube before reaching the fertilisation site. Within minutes after intravaginal placement, the spermatozoa reach the isthmus region in the fallopian tube; this process is much faster than would be possible with only the motility of spermatozoa. It is likely this transport is enhanced by uterine contractility [40]. In all mammals except for humans and some non-humans primates, coitus precedes ovulation whether or not ovulation occurs spontaneously or is induced by mating. The time interval between coitus and ovulation is constant. Therefore, spermatozoa always find the same biological environment when they enter the reproductive tract, resulting in a consistent migration pattern [3]. In many mammals spermatozoa are stored in a reservoir in the isthmus until ovulation, this maintain the viability and fertility of spermatozoa. . The reservoirs are created when spermatozoa bind to the tubal epithelium. This binding delays capacitation and prolongs the fertile life of spermatozoa. The storage of spermatozoa may also serve to prevent polyspermic fertilisation by allowing only a few sperm at a time to reach the OCC in the ampulla [40]. In humans, coitus may occur at any time during the menstrual cycle, and the spermatozoa will encounter a different biological environment depending on when coitus occurs and different patterns of sperm migration are expected. On the other hand if only considering sperm migration during the fertile period, when

coitus might result in pregnancy, this variability is reduced [3, 41]. The distinct spermatozoa reservoir found in other mammals has not been observed in women. However, spermatozoa interact with the epithelium in the isthmus, and their heads have been shown to bind to the epithelium in vitro, prolonging fertility [42]. The adhesion to the epithelium would also slow the migration of spermatozoa to the ampulla. The migration would also be slowed by the mucus folds in the lumen. Before fertilisation, sperm undergo two changes, capacitation and hyperactivation. Capacitation involves changes in the plasma membrane that prepares the sperm to undergo the acrosome reaction and fertilise the oocyte, whereas hyperactivation is a change in the movement in which sperm start to swim more vigorously and in a circular pattern. This change in movement is necessary for the sperm to navigate to the oocyte through the many folds in the mucosa of the ampulla [40]. Progesterone has been shown to induce capacitation, hyperactivated motility and the acrosome reaction in spermatozoa [43] and has also been suggested to be a chemoattractant that guides spermatozoa to the oocyte [44].

Transport of the oocyte-cumulus complex and embryo along the fallopian tube

Transport of the oocyte-cumulus complex (OCC) through the fallopian tube begins at the time of ovulation, when the ciliated cells at the fimbriated end of the oviduct come into contact with the OCC, and ends when the OCC or developing embryo passes into the uterus [45]. The duration of the transport varies from species to species. In opossums, the transport is as short as 24 h, whereas in dogs, it is as long as 8-10 days. In humans, the transport takes approximately 80 h [46]. In individuals of the same species and the same physiological condition, the transport time is quite stable, but when comparing pregnant individuals with cycling individuals, the transport time may be different, suggesting that the transport is subject to physiological regulation [3].

The contact between the developing embryo and the uterine environment must be initiated at the right stages of embryo development and endometrial maturation. The manner in which this occurs determines the pattern of transport through the fallopian tube [45]. The OCC movement has been observed in rabbit and mouse fallopian tubes, and these observations showed that the net forward movement is a result of back and forth movements of the OCCs rather than a smooth continuous forward movement [47]. The pattern of transport seems to be that the OCC travels at different speeds through various parts of the fallopian tube and that there are periods of non-forward

movement where the OCC/embryo remains at the same part of the oviduct for hours [45]. Most studies on gamete transport are performed in rabbits and mice because it is more problematic to study egg and embryo transport in women; however, some examinations have been performed on patients undergoing salpingectomy for sterilisations. When the ovum is released, it reaches the site for fertilisation after approximately 8 hours. The developing embryo then lingers in the ampulla for approximately 72 h before passing through the isthmus in less than 10 h. The portion of the time that the eggs stay in the ampulla and isthmus varies between species. In women, the egg spends 90% of the time in the ampulla, whereas in mice, only 25% of the time is spent in this region. A long time within the ampulla seems to be typical for primates [48].

Mechanism and regulation of transport

The transport of gametes through the fallopian tube is a result of ciliary movement, muscle contractions and the flow of secretions. These processes are influenced and regulated by ovarian hormones. The picture of the regulation of gamete transport is complicated due to the structural differences between the different fallopian tube segments. If fertilisation occurs, the object being transported is very different both physiologically and biologically at the time it reach the uterus compared with when it entered the fallopian tube; this difference also contributes to the complexity. The relative importance of the ciliary beat frequency (CBF), muscular contractility and tubal fluid is debateable, but the mechanism involved in the transport may differ before and after fertilisation and from one segment to another [45, 49].

Ciliary beat frequency

Several biological, chemical and hormonal agents affect the ciliary beating in the fallopian tube. CBF regulation involves intracellular second messengers, such as cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP), as well as calcium (Ca^{2+}) and pH. The CBF regulation by cAMP and cGMP involves protein kinase A and most likely protein kinase C. Protein kinase A has a stimulatory effect, whereas protein kinase C has an inhibitory effect on the CBF. The mechanism behind the calcium regulation of the CBF is not completely clear, but elevated levels of calcium increase the CBF, whereas a decrease in calcium levels usually decreases CBF [50]. The CBF also requires the hydrolysis of adenosine triphosphate (ATP), and in vitro, ATP increases the CBF in a dose-dependent manner [51]. Other factors affecting the CBF are angiotensin II, interleukin-

6, prostaglandins, adrenomedullin and ovarian hormones. Angiotensin II increases the CBF at nanomolar concentrations through the angiotensin II receptor [52]. Interleukin-6 has an inhibitory effect on the CBF [53], and prostaglandins stimulates the CBF [54]. Adrenomedullin is a peptide hormone that has been shown to increase the CBF in a dose-dependent manner [55, 56]. The role of ovarian steroids in CBF regulation has been well studied [57-61] Oestrogen has no effect on the CBF, while exposure to high levels of progesterone for 24 h reduces the CBF by nearly 40-50% [59, 62]. Peritoneal, follicular and tubal fluid also affect the regulation of the CBF [58]. Whether there is a cyclic change in the CBF during the menstrual cycle or whether anatomical differences affect the CBF is not clear. There are conflicting results and further investigations are necessary. In humans, a frequency between 5-20 Hz has been measured in vitro for ciliary cells from the fallopian tube [58, 59, 63] The ciliary beating seems to be coordinated; the cilia on one cell evidently beat together, and the cilia on different cells in close contact with each other also seem to synchronised [64]. Effectively, the beating of the cilia at least around ovulation is towards the uterine end of the fallopian tube [63, 65].

Muscle contractions

Two types of muscle contractions have been described in the fallopian tube: continuous tonic contractions and short-lived frequent periodic contractions [66, 67]. The tonic contractions take place at the ampulla-isthmic junction and the utero-tubal junction, and at these sites, the transport is known to be temporarily arrested. A proposed function for the tonic contractions is to serve as a sphincter mechanism to momentarily stop the transport at these sites. The periodic contractions are responsible for the pendular movement of the OCC and might increase the interaction between gametes and the tubal fluid rather than cause a transport in any direction [3, 68]. There are three muscle layers in the fallopian tube, one outer longitudinal, one middle circular and one inner longitudinal layer, and the frequency of contractions are higher in the circular layer than in the longitudinal layer. The circular muscle displays cyclic changes in contraction pattern during the menstrual cycle in response to changes in oestrogen and progesterone levels [27]. The regulation of muscle contraction is complicated and affected by several factors, such as hormones, neurotransmitters, prostaglandins and endothelins. To make the regulation even more complicated, circular and longitudinal muscles respond differently or in the opposite manner to the same agent [3]. Endogenous oestrogen increases the contractility, whereas endogenous progesterone decreases it [27, 69]. Before ovulation, the contractions are gentle. As the levels of oestrogen increase, the contractions become more

vigorous and reach a maximum around ovulation [27]. The muscle contractions bring the fallopian tube closer to the ovary and the fimbria sweep over the ovarian surface to pick up the ovulating OCC. The increasing progesterone levels after ovulation inhibit the motility of the fallopian tube, which may lead to relaxation of the tubal musculature to allow the passage of the embryo into the uterus [49].The E-series of prostaglandins relaxes muscular activity, whereas the F-series of prostaglandins stimulates it. The muscle response to prostaglandins is affected by progesterone; progesterone increases the response to the E-series and decreases the response to the F-series [70-72]. Two isoforms of endothelin, endothelin-1 (EDN1) and endothelin-2 (EDN2) have been shown to increase muscle contraction [27, 70, 73-76].

Adrenergic nerves are thought to be involved in regulating the muscular contractions of the fallopian tube, particularly in the isthmic region [77]. Both adrenaline and noradrenaline have a stimulatory effect [78], and both α and β adrenoreceptors are present in the musculature in the isthmus region. The stimulatory α receptors are present in the outer longitudinal muscular layer, whereas the inhibitory β receptors are present in the middle circular and inner longitudinal muscle layers [79]. During the follicular phase, oestrogen potentiates the activation of α receptors, and the muscle layers in the fallopian tube are more sensitive to α-adrenergic compounds, such as norepinephrine which leads to isthmic contraction. After ovulation, during the luteal phase, progesterone potentiates the activation of β receptors in the inner muscle layer leading to isthmic relaxation [78, 80].

It is possible to detect electrical activity in the form of slow waves and action potentials along the fallopian tube, and this activity is thought to be involved the gamete transport [81, 82]. The frequency of electrical activity and the shape and direction of the waves vary during the menstrual cycle [82]. In the follicular phase, the electrical activity consists of a single slow spike lasting 3-6 seconds. The waves spread from both ends of the oviduct towards the AIJ. The activity directed from the ampulla towards the AIJ assists the transport of the OCC, and the waves initiated at the uterine end and spreading towards the AIJ facilitate the transport of spermatozoa [82]. In the luteal phase, the activity changes from smooth waves to a series of spikes lasting up to 10 seconds. This change should result in an increase in the duration and force of contraction during the important period when the developing embryo is transported through the fallopian tube. The spread of the waves is directed towards the uterus [82].

Tubal fluid

The epithelium of the fallopian tube secretes a variety of bioactive compounds forming the tubal fluid that provide the environment in which fertilisation and early embryo development occurs [83]. The production of tubal fluid varies during the menstrual cycle, and the maximal production of tubal fluid occurs around midcycle [83, 84]. The tubal fluid is very rich in potassium (K^+) and bicarbonate (HCO_3^-) ions and has a high concentration of the amino acids arginine, alanine and glutamate. The main energy substrate is glucose, and the concentration of glucose varies through the menstrual cycle. Prostaglandins, steroid hormones, and growth factors have also been found in the tubal fluid, and specific tubal glycoproteins produced by the epithelial cells have been identified in several species [84, 85]. After ovulation and follicular rupture, the follicular fluid becomes the major component of the tubal fluid. Contractions of the muscle layer push the tubal fluid along the lumen, possibly causing movements of gametes and the embryo. Components of the fluid also exert effects on the CBF and muscular contractility [2]. Before ovulation, the isthmic fluid becomes a mucous. This might have dual effects and affect the behaviour of spermatozoa and the transport of the OCC. The isthmic mucus provides a medium for sperm transport around ovulation, and this medium protects the sperm from ciliary activity in the direction of the uterus [22, 63, 86]. The mucus, together with isthmic muscle contractions, may act to lock the OCC in the ampulla. As the progesterone levels increase in the post-ovulatory period, the mucous disappears, and the muscles are relaxed to allow transport through the isthmic region [22, 86].

Endothelins

Endothelins are vasoconstrictive peptides of 21 amino acids that are important in vascular physiology. Three isoforms of endothelin have been identified: endothelin-1 (Edn1), endothelin-2 (Edn2) and endothelin-3 (Edn3). Endothelins are produced in a variety of tissues and cells, including endothelial and smooth muscle cells, and they perform a wide variety of physiological functions. Endothelins produce their effects by activating two G-coupled receptor subtypes: endothelin receptor A (Ednra) and endothelin receptor B (Ednrb). Ednra has higher affinity for Edn-1 and Edn-2 than for Edn-3, while Ednrb has the same affinity for all the isoforms [87, 88].

Endothelins in the fallopian tube

The role of endothelin in female reproduction is important. Both EDN1 and EDN2 have been shown to affect the contractions of the fallopian tube in different species, including humans [74-76, 89]. Studies in the bovine oviduct

revealed that EDN1 significantly increases the amplitude of oviductal contraction in vitro during the periovulatory period of the oestrous cycle. This increase was further enhanced if EDN1 was combined with oestrogen, progesterone and LH. The expression of *EDN1* mRNA and the mRNA for *EDNRA* and *EDNRB* was highest in the periovulatory period; this finding suggests that the endothelin system in the bovine oviduct is up-regulated during this phase of the oestrous cycle and may have a central role in the control of local oviduct contraction during the period of gamete and embryo transport [89]. Moreover, EDN1 significantly increases the production and secretion of prostaglandins in bovine oviduct epithelial cells in culture. This result indicates that at the time around ovulation, EDN1 induces the highest contractility while it simultaneously induces a stimulation of prostaglandin production [90]. The production and secretion of EDN1 seems to be regulated by hormones. Oestrogen alone, oestrogen in combination with LH or oestrogen in combination with both LH and progesterone can significantly increase the production of EDN1 in epithelial cells from the bovine oviduct. Meanwhile, neither LH nor progesterone alone showed any significant effect on EDN1 production [90, 91].

There is no evidence for the production of EDN2 in the fallopian tube; instead, it is suggested that the EDN2 that is produced and secreted from the granulosa cells would induce contractions in the fallopian tube. It is possible that granulosa cells in the OCC continue to produce EDN2 while in the oviduct and that EDN2 regulates the transport in a local manner [74]. The role of EDN3 in the fallopian tube is not as well studied, but EDN3 might play an important role during fertilisation and the early development of the embryo [92].

Genomic vs non-genomic actions of progesterone

The classical model of steroid actions involves binding of the steroid to a specific receptor present either in the nucleus or the cytosol. This binding is followed by the translocation of the receptor-ligand complex to the nucleus, if the complex is not already in the nucleoplasm; once the complex enters the nucleus, transcription and protein translation are modulated. These steps cause a delay between the binding of the hormone to its receptor and the detection of the effect of the steroid. Apart from these classical transcriptional effects, all steroid hormones also mediate rapid actions that are not dependent on gene transcription or protein synthesis [93, 94]. Non-transcriptional effects have been described for all classes of steroid sex

hormones, and evidently, the mechanisms of rapid steroid signalling are not identical [95]. Criteria for a response to be classified as a non-transcriptional are; the response must occur within a few minutes, the response should not be affected by transcription and translation inhibitors, and the response ought to occur at physiological concentrations of steroids since higher concentrations of steroids can have non-specific effects, for example, perturbation of cell membrane [93]. The speed of the response of the steroid is system-dependent, and variations from a few seconds (opening of ion channels) up to an hour (inhibition of apoptosis) have been found [96-100]. The non-transcriptional responses to steroids occur via second messenger cascades, and steroid hormones have been shown to activate different signalling pathways, including the adenyl cyclase, tyrosine kinase (Src), mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K) and signal transducer and activator of transcription (STAT) pathways [94, 96, 101]. Receptors that mediate these effects have been thought to be membrane-associated or integrated in to the membrane [102]. In recent years, however, it has become clear that the classical steroid receptors are involved in the rapid signalling for all steroid hormones [103]. For progesterone, several different rapid responses in reproductive tissue have been reported, and the two most analysed systems for studying these non-transcriptional effects are the acrosome reaction in human spermatozoa and the oocyte maturation in Xenopus laevis. Wessel et al reported a reduction in the CBF in ciliated cells from a bovine oviduct within 15 minutes after exposure to a high concentration (20 μM) of progesterone. This effect could not be blocked by equimolar concentrations of mifepristone (RU486), an antagonist to the classical PGR. The authors interpreted this finding as strong support for the involvement of a different receptor than PGR in mediating the response [104]. There are several progestin receptor candidates for such non-transcriptional effects, including membrane progesterone receptors (mPRs), progesterone receptor membrane components (PGRMCs), and the classical nuclear progesterone receptor [105].

Membrane progesterone receptors

Membrane progesterone receptors belongs to the large family of proteins termed progestin and adipoQ receptors (PAQRs) [106, 107]. Three isoforms (mPRα, mPRβ and mPRγ) were initially cloned from fish ovaries, and they were later found in variety of species, including humans [108, 109]. The mPRs mediate rapid progesterone signalling and functions in several different cell types and animal models, e.g., the induction of oocyte maturation in fish and amphibians and sperm motility in fish. Progesterone signalling through mPRs has also been confirmed in human breast cancer cells, myometrial cells and lymphocytes [109-113]. Both mPRβ and mPRγ are present in the

fallopian tube of mice and humans, and they are regulated by progesterone. Both mPRβ and mPRγ have been found on ciliated cells. Although mPRβ was located on the cilia, mPRγ was located at the base of the cilia [114, 115]. This finding makes the mPRs a highly interesting candidate for mediating the rapid reduction of the CBF by progesterone.

PGR and non-genomic signalling

Apart from transcriptional effects, progestins can rapidly activate different signalling pathways, such as the Src/Ras/MAPK pathway, in breast cancer cells and mammary epithelial cells [116-119]. These effects are dependent on the classical PGR, suggesting that PGR functions both as a mediator of nuclear transcription and a modulator of cell signalling pathways [120]. The human *PGR* contains a polyproline motif within the N-terminal domain that interacts with the SH3 domain of tyrosine kinase (Src). [116]. Via this proline motif, liganded PGR binds to the SH domain of Src The activation of Src results in the recruitment and activation of downstream ERK1/2MAPK modules [121]. This polyproline motif is present in both isoforms of PGR, but the activation of Src and downstream MAPK is only mediated via the PGRB isoform [122]. The intracellular locations of PGRA and PGRB differ; PGRA is predominantly located in the nucleus, whereas PGRB is distributed between the cytoplasm and the nucleus [122, 123]. It is likely that progestin activation of Src is mediated by the PGRB located outside the nucleus [120]. A biological role for this rapid activation of Src/MAPK might be an alternative way for progesterone to influence gene transcription independently of the direct nuclear transcription activity of PGR [122].

AIM

The overall aim with this thesis was to investigate the effect of progesterone and the classical progesterone receptor on different factors influencing the transport of gametes, particularly oocytes. The rapid effects of progesterone on the ciliary beat frequency were investigated. The roles of progesterone and its receptor in oocyte transport and in the progesterone regulation of EDN1 in the fallopian tube were studied.

Specific aims

Paper I

The aim of this paper was to establish a method for studying the CBF in the mouse fallopian tube and to investigate whether progesterone at physiological concentrations has any rapid effects on the CBF in the mouse fallopian tube.

Paper II

The aim of the second paper was to investigate the mechanisms behind the observed rapid effect of progesterone on the, specifically whether the PGR is involved in this regulation.

Paper III

The aim of the third paper was to investigate progesterone's effects on gene expression at early and later time points in the mouse fallopian tube. An exploratory analysis of what genes are regulated may provide information about the mechanisms mediating the effects of progesterone on ciliary beating and muscular contractions, which eventually control gamete transport.

METHODOLOGICAL CONSIDERATIONS

Animal models

In this thesis, we used mice as an animal model to study the role of progesterone in the transport of gametes. Mice have the most genes in common with humans, and therefore, they are a very useful model for studying physiological functions and human diseases. Regarding the reproductive system, there are both similarities and differences between mice and humans. The mice fallopian tube is curly with a bursa that surrounds both the ovary and the fallopian tube. During ovulation, ova are released into the bursal cavity and are transported to the fallopian tube through the bursal fluid. Thus, in mice, the ova never enter the peritoneal cavity, as is the case for humans. The hormonal regulation of gamete transport and the duration of the egg/embryo transport to the uterus are, however, thought to be quite similar in mice and humans [45, 48].

Immature mouse model (papers I, II and III)

In all our in vitro studies, we have worked with immature C57BL/6N mice (3.5-5 weeks old). Immature mice were chosen to avoid the involvement and effects of endogenous gonadotropins on reproductive tissue and to obtain mice at a similar developmental stage. Because the endogenous levels of steroid hormones are low in these mice, the effects of added hormones are easier to interpret.

PgrLacZ mice (paper II)

In paper II, we used PgrLacZ mice to investigate the role of the PGR in mediating the rapid responses of progesterone on the CBF. In the PgrLacZ mice (C57BL6/129SvEv background), the LacZ reporter and neomycin resistance genes were inserted into exon 1 of the murine *Pgr* gene. The lacZ reporter contains its own start codon and nuclear localisation signal. It was inserted 120 aa downstream of the initiating methionine for the PGR-B isoform, and a short region of the N-terminal domain containing the initiating methionine for the PGR-A isoform was deleted. This strategy disrupted the transcription of both PGR isoforms [124]. The mice that are homozygous for the PgrLacZ insertion are a phenocopy of the PRKO mice previously described by Lydon et al [31]. The mice that are heterozygous for the PgrLacZ insertion are a phenocopy of the wild type [124].

Gonadotropin-induced mouse model (paper III)

To study egg transport, we induced ovulation in immature mice with equine chorionic gonadotropins (eCGs), also known as pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG). The mice were first treated with PMSG (5 IU, i.p.), which resulted in follicular growth and preovulatory maturation. Next, 48 hours after the PMSG injection, the mice were treated with hCG (5 UI, i.p.). In rodents, ovulation occurs 12-14 hours after hCG treatment [125].

In vitro studies

In vitro studies make it easier to control the exposure of hormones or other substances to the cell of interest without any involvement of endogenous hormones, other organs or substances. The main disadvantage with in vitro studies is the difficulty of completely mimicking the in vivo conditions. The process of removing tissue from the body may stress the tissue, which might not behave and respond exactly as it does in vivo.

Measuring ciliary beat frequency

Isolation of ciliated cells (Papers I and II)

The theory of dissection was simple. First, the fallopian tube should be dissected out, and the blood, fat and connective tissue should be removed. Then, the fallopian tube should be opened longitudinally and chopped into small pieces. Although the procedure seemed straightforward, it was challenging to perform practically, mainly because of the small size, the curly shape and the fragility of the tissue. The fallopian tube had a tendency to return to its original curly shape although the connective tissue that keeps the tube coiled was removed; this tendency made it difficult to open the fallopian tube longitudinally. The next challenge was to cut the tissue into small enough pieces to obtain clusters of ciliated cells without causing too much damage to the cells. During the dissection, the tissue was first washed in PBS (phosphate buffered saline, pH 7.0) and then kept and handled in G-mops^{TM} Plus medium (Vitrogen, Gothenburg, Sweden). This medium is used during in vitro fertilisation and is designed especially for the manipulation and handling of oocytes and embryos in ambient atmosphere. We chose to work with this medium because after the dissection, the tissue samples had to be transported approximately 1 km from our animal house to the Department of Physics for the measurements. Because the proportion of ciliated cells decreases from approximately 50% in the fimbria to less than 35% in the isthmus [25], only the upper half (infundibulum and ampulla) of the fallopian

tubes was used for the measurements. The pieces of tissue were kept in G-mops at 37°C overnight, and the measurements of the CBF were performed the day after dissection. The ciliary cells were kept overnight because the measurements were quite time consuming, and completed measurements on two, or in some cases more, tissue pieces from each mouse were desirable.

Detection system

To measure the CBF, an inverted bright field microscope equipped with a 100x oil immersion objective was used. We wanted to perform the measurement at the same temperature as Wessel et al did in their study [104]; thus, the microscope objective was heated using an adjustable heater loop attached to the objective. The temperature was measured continuously during every CBF measurement. Using a 12-bit high-speed camera the ciliary movement was recorded within a region of interest of approximately 50 × 50 pixels and with a speed of 100 frames per second. From the recorded images the spatial positions of cilia were determined by a center-of-mass algorithm using LabVIEW (National Instruments, Austin, TX). Finally the CBF was calculated through a fast Fourier transform (FFT) in which the recorded ciliary movements are decomposed into components of different frequencies. If possible, the CBFs of two ciliated cells were measured at the same time, and the mean frequency of the two cells was used in the calculations.

Measurement of the ciliated beat frequency

The measurements were performed in petri dishes (35 mm Ø) with glass bottom microwells (14 mm Ø) (MatTek Corporation, Ashland, USA) in pre-warmed G-mops medium. The first problem to solve before we could measure the CBF was how to minimise the movements of the small pieces of tissue during the measurement and how to maintain the position of the piece while adding the drug. The best solution we found was to keep the pieces of tissue still with a micromanipulator, which consisted of a sterilised needle (TransferTip®, iD 15 µm, oD 20 µm, Eppendorf AG, Hamburg, Germany) connected to a joystick (TransferMan, Eppendorf). Thus, when a piece of tissue with beating cilia was found, it was held against the bottom of the petri dish using the needle.

Experimental setup

Wessel et al had observed a reduction in the CBF 15 minutes after the administration of progesterone to cow fallopian tubes in vitro [104]. We wanted to establish a mouse model to study the effects of progesterone on ciliary beating. First, we wanted to measure the baseline frequency of the ciliated cells and then add the drug and measure the frequency over time. Before we could start measuring the effects of progesterone, however, any

possible effect of the incubation time on the CBF needed to be investigated. Therefore, the CBF was measured on 10 ciliated cells from 4 individual mice for 2 hours. Considering our pilot experiments, we decided to measure the frequency every 5 minutes for 60 minutes after the addition of the drug and compare the baseline frequency with the mean frequency for the last 30 minutes of the measurement for statistical comparisons..

Hormonal treatment in vitro

In papers I and II, we studied the rapid effect of progesterone on the ciliary beat frequency and the possible role of PGR in mediating this effect. In paper I, ciliary cells were first exposed to 20 μM progesterone, a concentration also used by Wessel et al [104]. Then, we used a more physiologically relevant concentration of 100 nM, which was based on the maximum serum levels in the luteal phase of normal cycling females [59, 126, 127]. In paper II, we used 30 nM and 10 nM progesterone, in addition to 100 nM to determine whether the reduction in the CBF also occurs at lower concentrations of progesterone. In paper II, we used a PGR agonist, promegestone (R5020), and a PGR antagonist, mifepristone (RU486), to evaluate the role of PGR in mediating the rapid response of the CBF. R5020 is a synthetic progestin that binds to PGR with a higher association constant than that of progesterone, and it has low affinity to the mPRs [128]. RU486 was the first clinical PGR antagonist; it binds to PGR with higher affinity than that of progesterone but displays low affinity for the mPRs [128, 129]. In addition to being a PGR antagonist, RU486 also has antiglucocorticoid activity [130]. The ciliary cells were exposed to 100 nM R5020 and for the antagonist experiment, ciliary cells were pre-treated with 1 μM RU486 before the addition of 100 nM progesterone. In the presence of progesterone, RU486 is a competitive antagonist; therefore, we used a higher concentration of RU486 than the progesterone concentration to ensure complete blockage of Pgr. Ciliary cells from the fallopian tube of PgrLacZ mice were exposed to 100 nM progesterone.

Administration of drugs

To add progesterone, R5020, RU486 or control medium to the ciliated cells, we removed half the medium in the petri dish and then added the same amount of medium containing the drug.

Changes in the experimental setup in paper II

One problem we faced in this experimental setup was locating a piece of tissue with beating ciliated cells in the periphery of the sample piece, where it was possible to measure the CBF. Although we acquired many tissue pieces from the fallopian tubes of one mouse, only a fraction of the samples had the

potential for use in the experiments both due to the position of the ciliary cells and the fact that many cells were damaged by the dissection. To determine whether we could increase the number of cells with beating cilia, we tried to keep the tissue samples in F10 medium with calf serum in a CO_2 (5%) incubator overnight. Comparing the pieces of tissue kept in G-mops with the pieces of tissue kept in F10 medium revealed that the amount of beating cells was higher when the tissue was kept in F10 and CO_2 overnight. Therefore, the tissue samples were stored differently in paper II compared with paper I. In paper II we changed the temperature to more resemble the body temperature and measured the CBF at 37°C instead of 35 °C, the temperature during the measurements in paper I and by Wessel et al [104].

In paper I, the measurements were performed in 2 mL G-mops, but in paper II we reduced that volume to 1 mL. The small pieces of tissue tended to float in the medium, and it was difficult to attach them to the bottom of the dish. In the smaller amount of medium, it was easier to fasten the pieces. Another problem was that the removal and addition of medium sometimes made the tissue piece detach from the bottom although it was held down with the needle. Unfortunately, this problem increased when the volume of medium was changed.

Breeding and genotyping of PgrLacZ mice

In our lab, we received six PgrLacZ mice, three heterozygote females, two heterozygote males (PgrLacZ (+/-)) and one homozygote male (PgrLacZ (-/-)) as a kind gift from Dr Michael Schumacher from University Paris-Sud, France. From these animals, we composed three breeding pairs. When the offspring were three weeks of age, they were sorted, and an ear biopsy was taken for genotyping. To obtain genomic DNA, the ear tissue was mixed with DirectPCR lysis buffer for the ear (Viagen Biotech, Los Angeles, USA,), and the system was used according to the manufacturer's protocol.

Touchdown PCR

The genotyping was performed using the touchdown polymerase chain reaction (PCR). The principle of touchdown PCR is that the initial annealing temperature is higher than the optimal Tm of the primers, and the temperature is then reduced by 1 °C every cycle until the Tm temperature or touchdown temperature is reached. When the final annealing temperature is reached, general PCR amplification of up to 20-25 cycles is performed. The primers we used for the genotyping all had different melting temperatures; therefore, it was difficult to find an optimal annealing temperature for the PCR reaction. Using touchdown PCR solved that problem, and we could use all primers in the same reaction. Only female knock-out mice, PgrLacZ(-/-), were used in

the CBF measurements. PgrLacZ (+/-) females and males and PgrLacZ (-/-) males were used for further breeding.

LacZ staining

To investigate the possible location of PGR in the mouse fallopian tube, we used fallopian tubes from PgrLacZ (+/-) mice and a staining kit (InvivoGen, San Diego, USA) to determine the β-galactosidase activity in the tissue. Fallopian tubes were frozen in OTC medium (Tissue-Tek O.T.C. Sakura Finetek Europe B.V), then cut into pieces (5-10 µM) in a cryostat (Leica CM3050S, Leica Microsystems AB) and placed on glass slides. The slides were then prepared according to the manufacturer's protocol. In cells expressing the LacZ gene, β-galactosidase catalyse the hydrolysis of X-Gal, producing a blue precipitate that can be visualised under a microscope.

Statistical analysis

Changes in the CBF

To analyse the CBF data, we calculated the Δ frequency before and after the treatment. The baseline CBF for different mice and even different cells from the same mouse varies considerably; thus, the CBF after the treatment and the CBF for each cell were always normalised against its own baseline CBF. For each cell, the difference between the mean CBF during the last 30 minutes of the measurement and the mean baseline CBF was calculated. If the CBF was measured on more than one cell from a piece of tissue, the average Δ frequency of two cell replicates was used in the statistical analysis.

In paper I, the difference in ΔCBF between control cells and the cells exposed to progesterone was calculated using Student's t-test. There was a predefined hypothesis that progesterone lowers the CBF; thus, a one-sided Student's t-test was applied.

In paper II, we investigated whether progesterone at low concentrations – also induced a reduction in the CBF and if this was a dose-dependent effect. The ciliary cells exposed to 10, 30 and 100 nM progesterone were analysed using a one-way ANOVA and then Dunnett's post hoc test. For experiments with the R5020, RU486, and progesterone treatments of PgrLacZ mice, a paired two-sided Student's t-test was applied.

Gene expression studies

Microarray

Microarrays are widely used and have opened up great possibilities in the biology research field as microarrays provide the ability to analyse the mRNA levels of thousands of expressed genes across the genome in one sample.. The principle of a microarray is that mRNA is extracted from the studied tissue or cells, converted to biotin-labelled cRNA and hybridised to a microarray chip. When a specific transcript binds to its matching probe on the chip, a fluorescence signal is detected. The light intensity of the signal depends on the amount of mRNA in the sample and thus, gives a picture of the degree of gene transcription. There are several different types of microarray platforms based on the same principle but with a few differences between them. For this thesis, the MouseRef-8 v2 Expression BeadChip from Illumina was chosen to study the change in gene expression in the mouse fallopian tube after exposure to progesterone for 20 minutes and 2 hours. The experiments were run at the Swegen Centre for Integrative Biology at Lund University.

The huge amount of data generated from a microarray experiment must be processed before it is possible to perform statistical analysis and generate biological interpretations. Background correction is performed to remove noise and artefacts originating from the synthesis/hybridisation process. To enable comparisons between different samples, the data must be normalised. In the experiment run in this thesis, the data were normalised using robust multi-array analysis (RMA) to compensate for the overall signal difference due to unequal concentrations of the added RNA and variations in the hybridisation efficiency. A SAM analysis (statistical analysis of microarray) was performed to identify significantly differentially expressed genes between groups. When analysing thousands of potential responses in parallel and analysing the responses one by one, there is an apparent risk that some responses appear to be regulated just by chance and not because of a treatment-induced. Therefore, one must estimate the proportion of false positives, the false discovery rate (FDR). When applying an FDR approach, the q-value is defined to be the analogue of the p-value but is adapted to multiple-testing. In the SAM analysis the q-value for each gene, is the lowest false discovery rate for which that gene is called significant [131]. In our study, genes with a q-value $\leq$ 20% were considered significantly differentially expressed.

The output from the microarray analysis is a list of genes whose expression changes between differently treated groups. Often, this list is too complex for immediate interpretation. To aid in identifying patterns in the data, several bioinformatics tools are available. Some of these tools classify genes and gene products depending on their cellular location, molecular function and association with specific biological processes and pathways. Our array data only showed 11 differentially expressed genes between the fallopian tubes exposed to progesterone and the control fallopian tubes after 2 hours (after taking into account the risk of obtaining false positives). We used AmiGO (http://amigo.geneontology.org/amigo) to identify the GO terms for the biological processes for each of the 11 genes.

Quantitative polymerase chain reaction

The data and hypothesis generated from a microarray are ideally further validated using alternative approaches. In this thesis, quantitative polymerase chain reaction (qPCR) was used to further investigate a select number of genes from the microarray experiment. This procedure is a very sensitive and relatively fast technique that is used to amplify small amounts of DNA. The principle of qPCR or real-time PCR is that data are collected throughout the PCR progress, and the reactions are characterised by the point in time during cycling when the amplification of a target gene is first detected rather than the amount of target gene found after a fixed number of cycles. In this thesis, we used the Taqman qPCR technology with probes with a fluorescent reporter dye at the 5' end and a quencher at the 3' end and specific primers designed to match the genes in question. During the annealing phase, both the probes and the primers anneal to the target DNA. The primers initiate the polymerisation of a new DNA strand, and when the polymerase reaches the probe, it is cleaved and separated from the quencher, which results in an increase in fluorescence that can be quantified. The PCR cycle threshold (C_T) is used to detect the real signal above the noise and to compare the relative starting amounts of complimentary DNA between samples. To adjust for differences between samples that are not caused by the treatment or exposure, the qPCR data must be normalised; therefore, the use of housekeeping genes is crucial. A good housekeeping gene should vary little between the samples and should not be affected by the treatment.

In paper III, we used qPCR to analyse the expression of 9 genes *(Edn1, Edn2, Edn3, Ednra, Ednrb, PR, Amigo2, Arlf4* and *Rasd1*) in the fallopian tubes after the exposure to progesterone for 2 hours or 8 hours. We selected the ribosomal protein L19 (*Rpl19*) and hypoxanthine phosphoribosyltransferase 1 (*Hprt1*) as housekeeping genes. The median C_T values were normalised to the average of the median C_T for the reference genes. The resulting ΔC_T values

were used to test for differential expression between fallopian tubes exposed to progesterone for 2 hours or 8 hours and control fallopian tubes. For each gene, a two-sided Student's t-test was used.

The reason we choose to expose fallopian tubes to progesterone in vitro instead of in vivo was to exclude possible indirect effects on gene expression caused by the effects on the secretion of other hormones of the hypothalamus-pituitary-gonadal axis.

In vivo studies

Effect of PGR antagonists on OCC transport

To investigate the role of the classical PGR in the transport of OCC, PMSG/hCG-treated mice were injected i.p. with two different PGR antagonists (Org31710 (8 µg/g) or CDB2194 (8 µg/g)) 6 h after the hCG injection, whereas the control mice were given vehicle only. Mice from each treatment group were killed at different time points after the hCG injection, and the presence of OCC in the fallopian tubes was determined by flushing the fallopian tubes and observing whether any OCCs were recovered under the microscope. In this experiment, we choose to use two PGR antagonists different from those in the in vitro study. Both Org31710 and CDB2194 have reduced antiglucocorticoid activity and increased antiprogestin activity compared with RU486 [132, 133]. For each time point, the proportion of mice having OCC present in the fallopian tube was calculated. Fisher´s exact test were used to test whether the proportion of mice with OCCs present in the fallopian tube differed between the mice given vehicle and the mice given either of the antagonists.

RESULTS AND DISCUSSION

Progesterone rapidly reduces the CBF in mouse fallopian tubes through the classical progesterone receptor

The aims in papers I and II were to investigate whether progesterone rapidly reduces the frequency of mouse fallopian tube cilia, as previously observed in cow fallopian tubes [104] and to investigate the role of the classical progesterone receptor in mediating this rapid response. We therefore developed a method to measure the CBF from small pieces of mouse fallopian tube tissue in vitro. Two-hour recordings of the CBF showed that the CBF did not change over that time period; thus, our experimental setup, the establishment of a baseline frequency, the addition of drug and the subsequent measurements of the CBF for the next 60 minutes were possible.

Variation in the baseline ciliary beat frequency

In both papers I and II, we found a large variance in the CBF between individual mice. The lowest CBF observed was 10 Hz, and the highest was 41 Hz. The variability in baseline frequency could be caused by several factors. We used immature mice that only differ in age by a few days, thus excluding the possibility that the difference is because the mice are in different phases of the oestrous cycle. For the measurements, we used segments from the upper part of the fallopian tube, the infundibulum and ampulla regions; however, mouse fallopian tubes are small, so it was hard to determine the margins between different regions. Some measurements may have been performed on ciliary cells from the isthmus region as well. We did not separate the pieces from the regions, so the variations in the CBF could be due to regional differences. However, in a study on human fallopian tube cilia, no significant difference in CBF values between the different regions was observed [134], but that might not be the case in mice. Each ciliary cell was compared with itself before and after hormone/control treatment; thus, these individual variations are not crucial for the interpretation of the results.

In the first paper, we found a mean CBF of 23.3±3.8 Hz (average ± standard deviation) prior to any treatment, and in paper II, the mean CBF was 27.3 ± 7.2 Hz for the wild-type mice and 21.6 ±4.7 Hz for the PgrLacZ mice. This finding is consistent with the results in cows, where the mean CBF prior to treatment was 23.1 ± 4.7 Hz [104]. From ciliary cells in human fallopian tubes, CBF values ranging from 4-20 Hz have been reported [25, 58, 59, 63].

Measurements in guinea pigs show CBF values between 13-18 Hz [60, 61]. Other studies on ciliary cells from mice reveal frequencies of 21±1 Hz [135] and 8.3±2.5 Hz in the oestrous phase and 10.9±3.3 Hz in the diestrous phase of the oestrous cycle [136]. These variations in the CBF between species are not unexpected and might be just due to species differences in anatomy, reproduction cycle and gamete transport pattern. The variation in the CBF observed within a species is more difficult to explain, but the use of different methodologies might be one explanation.

Effect of progesterone on the CBF

In ciliary cells from mouse fallopian tubes, we found a reduction in the CBF of 2.28 Hz, which corresponds to approximately 10%, within 30 minutes after the addition of 20 µM progesterone, which is very similar to the result presented by Wessel et al [104]. Steroid hormones at high concentrations can exert nonspecific effects without the involvement of receptors; these effects occur through modifications of protein function that reflect changes in the physicochemical properties of membrane [93]. Therefore, we also exposed ciliary cells to a lower, more physiological concentration of progesterone (100 nM). Within 30 minutes, there was a significant reduction of 3.74 Hz in the CBF compared with the value for the controls; this decrease corresponds to a reduction of 15% on average. There was no significant difference between the magnitudes of the effects observed after the exposure to 20 µM versus 100 nM progesterone.

Several previous studies have investigated ciliary cells in the fallopian tube and the influence of ovarian hormones on the CBF, as well as possible CBF changes during the menstrual cycle [58, 59, 134]. Many of these studies were performed on ciliary cells from human fallopian tubes and investigated the effects on the CBF 24 hours after the addition of hormones. Exposure to progesterone at a concentration of 10 µM reduces the CBF by 40 to 50%. This concentration is rather high compared with the maximum serum levels of progesterone (~100 nM) that have been measured during the luteal phase in humans [59, 126, 127], and in mice, the serum levels normally range from 25-50 nM [137]. However, in follicular fluid, the concentration of progesterone is much higher than in the serum; thus, after ovulation, the fallopian tube is likely exposed to a higher concentration than that in the serum [138]. Oestrogen has no effect on the CBF but prevented the reduction in the CBF by progesterone when ciliary cells were incubated with oestrogen and progesterone together at equal concentrations (10 µM).
The physiological role of a reduction in the CBF could be to slow down the speed of the traveling OCC to make it linger in the ampulla to await

fertilisation and if fertilisation takes place, to ensure that the developing embryo does not arrive too early to the uterus [59].

Reduction of the CBF by very low concentrations of progesterone

In paper II, we investigated whether the reduction in the CBF also occurs after exposure to much lower concentrations of progesterone. The results from paper I showed that progesterone at concentrations of 20 µM and 100 nM reduced the CBF by 2.28 Hz and 3.74 Hz, respectively. In paper II, ciliary cells were treated with 100 nM, 30 nM and 10 nM progesterone. At all tested concentrations, progesterone significantly reduced the CBF by a similar amount: 2.10 Hz (10 nM), 1.7 Hz (30 nM) and 1.9 Hz (100 nM). This finding suggests that a maximal effect of progesterone is reached already at 10 nM, further supporting the specificity of the effect. The average reduction in the CBF induced by 10-100 nM progesterone was somewhat smaller than the reduction observed for 100-2000 nM in paper I; however, these were two separate studies that were not performed at the exact same conditions, so it is difficult to determine whether this difference was meaningful.

The rapid reduction in the CBF is mediated through the classical progesterone receptor

To investigate the role of the classical nuclear progesterone receptor in regulating the CBF, we performed four experimental series. First, ciliary cells were treated with 100 nM of the PGR antagonist R5020, which binds with high affinity to the PGR and has low affinity to the mPRs [128]. The exposure to R5020 caused a significant reduction in the CBF by 3.12 Hz, which is very similar to the effect on CBF caused by progesterone. After using an agonist, we used a PGR antagonist to block the receptor. First, we had to make sure that the antagonist, RU486, itself did not have any effect on CBF. For this purpose, ciliary cells were exposed to 1 µM RU486 for 90 minutes, and the CBF was measured continuously. The reason for the 90 minute measurement was that in the following experiment, we wanted to incubate ciliary cells with RU486 for 30 minutes before adding progesterone and then measure for 60 minutes as in previous experiments. RU486 alone did not have any effect on the CBF. When ciliary cells were pre-treated with RU486 and then exposed to progesterone in combination with RU486, the expected reduction in the CBF was blocked. It should be noted that RU486 has low affinity to the mPRs [111, 128]. We also treated ciliary cells from the fallopian tubes of mice lacking a functional progesterone receptor, PgrLacZ mice, with 100 nM progesterone. There was no significant change in the CBF in the mice lacking the PGR, and the tendency was, if anything, an increase in the CBF rather than a decrease. Taken together, these findings strongly

indicate an involvement of the PGR in the rapid reduction of the CBF by progesterone.

Apart from the slow response in the CBF caused by progesterone, Wessel et al showed that in the fallopian tube from cow, progesterone reduces the CBF within 15 minutes after progesterone exposure [104]. In papers I and II, we observed a reduction already 5 minutes after the progesterone administration, although the decrease was not significant. This time period is too fast to be a classic effect involving transcription and translation because changes in gene expression are not usually observed until $\geq$ 30 minutes after hormone stimulation [93, 139, 140]. Progesterone displays rapid non-genomic effects in other tissues, and the most well-known examples are the induction of sperm hyperactive motility and the acrosome reaction in human spermatozoa [141] and the induction of oocyte maturation in amphibians and fish [142].

The results from the experiment in paper II showed that the rapid reduction in the CBF in the mouse fallopian tube by progesterone could be completely blocked by a PGR antagonist. This finding is not in accordance with the results of Wessel et al, who were not able to inhibit the progesterone effect on the CBF using a PGR antagonist. Wessel et al pre-treated ciliary cells with 20 µM RU486 for 2 hours, and then the cells were directly exposed to 20 µM progesterone. Because equimolar concentrations of progesterone and RU486 were used, it is quite possible that RU486 did not provide enough competition for progesterone. Indeed, in our study 10-fold higher concentrations of RU486 (1000 nM) completely blocked the effect of 100 nM progesterone.

Progesterone regulation of the muscular contractility versus the CBF

The study by Wånggren et al demonstrates the effects of 100 nM progesterone on muscle contractions in the fallopian tube in vitro, and these effects were already detected after 20 min [73]. The short time course suggests that progesterone regulates muscular activity through both transcriptional and non-transcriptional pathways. Interestingly, in the study by Wånggren et al on tubal contractility, RU486 had a significant and rapid effect on contractility that was similar to that of progesterone, and when both were added together, the effect was additive rather than antagonistic. This finding is in stark contrast to our studies on the CBF, where RU486 by itself had no effect, and where RU486 was able to completely block the effect of progesterone. This result would suggest that the rapid effects of progesterone on muscular contractility are mediated through a somewhat different molecular mechanism than that controlling the CBF.

Location of the PGR in the fallopian tube

The classical PGR is found in the fallopian tube, but its exact location is still debated. There is immunohistochemistry data suggesting that the PGR is located in the lower half of the cilia in both human and mouse fallopian tubes [143], whereas another study, again based on antibodies, suggest that the PGR is located in the nuclear compartments of luminal, epithelial and smooth muscle cells in the mouse fallopian tube [144]. For a completely independent method to investigate the location of PGR, we used a staining kit to determine the β-galactosidase activity in intact fallopian tube tissues from PgrLacZ mice and wild-type mice. Only the cells expressing the PGR produce the enzyme. Using this method, we found support for locations of the PGR in both epithelial cells and muscle cells (Fig 3). This finding is in agreement with the observation that progesterone regulates both the CBF and muscle contractions, and it would be consistent with an involvement of the PGR in both processes. It is important to stress that the enzymatic method we have used is not dependent on antibodies and thus provides evidence that is completely independent of previous studies. One disadvantage of the enzymatic method is that it is not possible to pinpoint the location of the receptor to a cellular compartment.

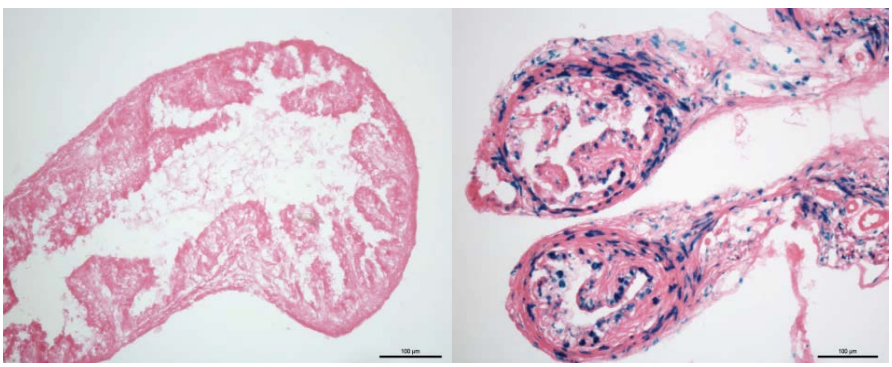

Figure 3. LacZ staining to detect the β-galactosidase activity in the mouse fallopian tube of cycling wild-type mice and PgrLacZ *mice. Positive staining (blue) was observed in the epithelial and muscle cells of* PgrLacZ *(+/-) mice. Bylander et al unpublished.*

Effects of progesterone on the gene expression in the fallopian tube

Whether the rapid reduction in the CBF is a transcriptional or non-transcriptional response is not known. For the response to a steroid to be classified as non-transcriptional, it must occur within a few minutes, should occur at physiological concentrations of the steroid and should not be affected by inhibitors of transcription and translation [93]. The rapid reduction in the CBF induced by progesterone meets two of these three criteria. We measured the CBF 5 minutes after the addition of progesterone, and at this time point, the CBF is reduced. Five minutes is most likely too fast to be caused by a change in mRNA expression [139]. We used physiologically relevant concentrations in the range of 10 nM to 100 nM and observed effects at all concentrations. Together, these results suggest that the rapid reduction in the CBF is a receptor-mediated, non- transcriptional response, but further investigations are necessary to clarify the nature of the response. Inhibitors for both transcription and translation are available, and performing experiments using some of these substances would be optimal. However, these substances are quite toxic to cells and would inhibit all transcription and translation, which might cause many effects in the cells. Therefore, we chose to not perform such experiments; instead, in paper III, we investigate the changes in gene expression in the fallopian tube after the progesterone treatment to identify possible genes involved in the reduction of the CBF and gamete transport.

In paper III, we examined the change in the gene expression in the fallopian tube after exposure to progesterone using a microarray and qPCR. For the microarray experiment, fallopian tubes were exposed in vitro to 100 nM progesterone for 20 minutes or 2 hours. There was no apparent change in the gene expression between the fallopian tubes exposed to progesterone and the control fallopian tubes after 20 minutes. After 2 hours, 11 genes were differentially expressed between the control and exposed fallopian tubes, as assessed by microarray. Although we could not detect any change in gene expression after 20 minutes, we cannot exclude the possibility that some genes are regulated after 20 minutes and were not detected by the microarray analysis. However, these results together with the results in paper II strengthen the suggestion that the rapid reduction in the CBF is mediated through the PGR via a non-genomic mechanism. Progesterone is known to rapidly activate the Src/Ras/MAPK, PI3 kinase/Akt and JAK2/Stat3 signalling pathways in breast cancer cells and mammary epithelial cells [116-118]. One or several of these pathways could potentially be involved in

regulating the CBF, but we have not pursued this possibility in detail so far. To confirm the observed change and further analyse the gene expression in the fallopian tube, 9 selected genes from the microarray result were analysed using Taqman quantitative RT-PCR. The most interesting of the regulated genes was *Edn1*, which is known to induce muscular contractions in the fallopian tube [145]. There are three isoforms of endothelin, *Edn1*, *Edn2* and *Edn3* [87]. EDN2 has also been reported to induce muscle contractility in the fallopian tube [74]. In the fallopian tubes of gonadotropin-treated mice, the *Edn3* mRNA expression is increased around the time of ovulation, suggesting an important role for EDN3 during fertilisation and early embryo development [92]. Although neither *Edn2* nor *Edn3* appeared to be regulated, as indicated by the microarray results, they were included in the qPCR analysis together with the endothelin receptors *Ednra* and *Ednrb*. We also included *Pgr*, which we thought might be regulated by progesterone but not detected by the microarray analysis. Then, we selected 3 additional genes (*Amigo2*, *Arlf4* and *Rasd1*) based on a high fold change indicated by the microarray results and a connection to progesterone in the literature. The qPCR analysis was performed on a new set of fallopian tubes exposed to progesterone in vitro for 2 hours or 8 hours.

Progesterone regulates *Edn1* and *Ednra*

The qPCR analysis confirmed the significant up-regulation of *Edn1*, *Amigo2* and *Rasd1* from the array results. The Taqman assay for *Arlf4* did not result in any amplicon; thus, we did not obtain any results for that gene. There were no changes in gene expression between the control and exposed fallopian tubes for *Edn2* and *Edn3*, and the expression of these two genes was low compared with that of *Edn1*. The expression of *Pgr* was down-regulated after the progesterone treatment, as was the expression of *Ednra*, but there was no change in the expression of *Ednrb*.

Our result shows for the first time that the mRNA levels of *Edn1* and its receptor *Ednra* are regulated by progesterone in mouse fallopian tubes. Previously, studies have shown that oestrogen alone, oestrogen in combination with progesterone, and oestrogen together with both progesterone and LH increase the production and secretion of EDN1 in bovine oviduct epithelial cells. Progesterone alone did not have any effect on the EDN1 production or secretion [90, 91]. In the bovine fallopian tube, EDN1 induces muscle contractions in the periovulatory period. The expression of *Edn1* mRNA and *Ednra* and *Ednrb* mRNA is also highest during this period. In the periovulatory period, EDN1 also stimulates the production and secretion of prostaglandins from the bovine fallopian tube

[89]. Prostaglandins are well known stimulators of muscle contractility in the fallopian tube, the E-series of prostaglandins relaxes the muscle activity, and the F-series has a stimulatory effect on muscle activity [70]. Together, these findings suggest that in the bovine fallopian tube, locally produced EDN1 plays a major role in regulating the local muscle contraction and controlling the transport of gametes around the time of ovulation [89].

In the human fallopian tube, EDN1 produces a concentration-dependent tonic contraction in both the follicular and luteal phases, and at least in the luteal phase, EDN1 inhibited spontaneous rhythmic contractions. The use of an antagonist for both endothelin receptors showed that EDN1 acting through EDNRA affected both the tonic and rhythmic contractions, but EDN1 acting through EDNRB only affected the rhythmic contractions [75, 76, 146]. Immunohistochemical studies showed that the dominant location of EDN1 and EDNRB is in the epithelium of the fallopian tube and that EDNRA is dominant in the muscle layer [146]. From previous studies, it is clear that EDN1 has an important role in controlling gamete transport in a local manner. In paper III, we demonstrate that the mRNA levels of *Edn1* in turn are regulated by progesterone. Because a change in mRNA level does not always correspond to a change in protein translation and secretion, it is necessary to examine the expression of EDN1 at the protein level after the exposure to progesterone to further establish the role of progesterone in the EDN1 regulation in the fallopian tube.

Progesterone did not regulate the expression of *Edn2* or *Edn3*. In the human fallopian tube, EDN2 induces a concentration-dependent tonic contraction in the luteal phase but does not have any effect on spontaneous rhythmic contractions. [75, 76]. This result is in agreement with a study in rats [74]. EDN2 is produced by the granulosa cells surrounding the follicle and is known to stimulate follicle rupture. As the OCC enters the fallopian tube, the EDN2 produced by the granulosa cells may locally regulate the muscle contraction [74]. EDN3 does not affect muscle contractions, but a role for EDN3 during fertilisation and early embryo development has been suggested [92]. In mice treated with gonadotropin, the mRNA levels of *Edn3* are highly increased 12 hours after hCG treatment [92]. In mice lacking *Pgr*, this increase was inhibited, suggesting that the regulation of *Edn3* might be PGR dependent [147].

Our qPCR results also showed that in the fallopian tube, the expression of *Pgr* was down-regulated by progesterone, which is in agreement with other studies [32, 144]. The expression of *Ednra* was also down-regulated by progesterone. In other reproductive tissue from mice, the uterus and placenta,

progesterone up-regulates *Ednra*, suggesting that *Ednra* might be differently regulated in different tissue. Exposure to progesterone changed the gene expression of *Amigo2* and *Rasd1*; there is no obvious role of these genes in the regulation of gamete transport, but further investigations are necessary to clarify this change.

The progesterone receptor regulates gamete transport

In paper III, we also investigated whether a single dose of a PGR antagonist given before ovulation can alter the tubal transport of the OCC. Earlier studies have shown that endogenous progesterone has a role in regulating the tubal transport of the OCC and embryos [148, 149]. In mice continuously treated with the PGR antagonists RU486 or ZK98734, the embryos arrived earlier to the uterus. [149]. In our study, gonadotropin-treated mice were given a single i.p. injection of Org 31710 (8 µg/g), CDB2194 (8 µg/g) or vehicle 6 hours after the hCG injection. The mice were killed, and the fallopian tubes were flushed at different time points after the hCG injection. At each time point, the number of mice with OCC present in the fallopian tube was counted. Our results show that in the mice treated with an antagonist, the OCC travelled faster through the fallopian tube compared with the travel in mice that were only given vehicle, which is in agreement with earlier studies and confirms that progesterone is essential for normal transport.

CONCLUSION

The investigations in this thesis further support the assertion that progesterone and the progesterone receptor play an important role in regulating the transport of gametes and embryos through both transcriptional and non-transcriptional mechanisms.

We have found that the mouse provides a sufficient model for studying the ciliary beat frequency (CBF) in the fallopian tube *in vitro*. We have demonstrated that progesterone, at a low nanomolar concentration, rapidly reduces the CBF. We show that this effect is mediated via the nuclear progesterone receptor, most likely through a non-transcriptional mechanism. The function of the rapid reduced CBF is most likely to reduce the speed of the traveling oocyte-cumulus complex to increase the probability that fertilisation takes place in the ampulla and that the embryo does not arrive to the uterus to early. The granulosa cells in the traveling oocyte-cumulus complex produce and secrete progesterone that might locally reduce the ciliary beat frequency.

We show that the mRNA levels of endothelin-1 (*Edn1)* and endothelin receptor A (*Ednra)* are regulated by progesterone in the mouse fallopian tube. In the fallopian tube, EDN1 is believed to play a role in the regulation of gamete transport through its ability to affect muscle contractions. We also show that a single dose of a PGR antagonist accelerates the transport of the oocyte-cumulus complex through the fallopian tube, confirming the evidence from earlier studies that a functional PGR is important for normal tubal transport.

Because functional transport through the fallopian tube is essential for the success of natural reproduction *in vivo*, it is important to understand the mechanisms and regulation of gamete transport. This knowledge can be used to better understand the causes of infertility and ectopic pregnancies in women and also to develop new and improved contraceptives.

FUTURE PERSPECTIVES

The knowledge of the regulation of gamete and embryo transport in the fallopian tube is far from complete. The transport is aided by movement of ciliary cells, muscle contraction and tubal fluid. The regulation is complicated because several factors affect both ciliary movement and muscle contractions. The difference in anatomy along the fallopian tube and the biological and physiological differences between the gametes and the embryo contribute to the complexity of the transport.

The use of transcription and translation inhibitors would clarify whether the rapid reduction in the ciliary beat frequency caused by progesterone is a transcriptional effect. Studies on second messenger systems could provide information about possible non-transcriptional pathways involved in the regulation.

Further investigations on the regulation of endothelin in the fallopian tube are necessary. For instance, studies on the regulation of EDN1 by progesterone at the protein level would further indicate whether *Edn1* is regulated by progesterone. The role of endothelin in the fallopian tube and in regulating muscle contractions must be further clarified. Antagonists that block endothelin receptors would reveal information about the role and importance of endothelin systems in gamete/embryo transport.

Studies of the ciliary beat frequency and muscle contractions in the fallopian tube are performed *in vitro* and examine either muscle contractions or ciliary movement. The *in vivo* events may not be identical to the *in vitro* observations. The best knowledge would be provided by studying the transport *in vivo* and altering different factors. Therefore, trying to develop methods for *in vivo* studies of the fallopian tube would be of high interest, although very challenging.

ACKNOWLEDGEMENTS

Joakim Larsson min huvudhandledare, tack för all hjälp och stöttning under de här åren. Trots att du har många järn i elden har du har alltid haft tid när det behövts. Tack för all värdefull input i skrivandet av artiklar och avhandling. Jag är väldigt imponerad av ditt kunnande, din arbetsglädje och enorma entusiasm för det du gör.

Håkan Billig min biträdande handledare, tack för goda råd och den input i projektet som du bidragit med.

Mattias Goksör min andra biträdande handledare. Tack för hjälpen med den tekniska biten. Framför allt tack för stödet och omtanken du visat och för att du i stunder av panik och ångest påmint mig om vad det är som verkligen är viktigt i livet. Tack också för trevliga familjeträffar.

Lina Gunnarsson, Du kom som en räddande ängel och mycket tack vare dig gick det här vägen. Tusen tack allt, för att du alltid är glad och positiv och för att du peppat mig. Jag beundrar dig verkligen, din otroliga kunskap, din entusiasm och energi.

Magdalen Nutu, tack för att du tog så väl hand om mig när jag började på Endokrin och introducerade mig i projektet. Tack för trevligt rese-sällskap till Dalsjöfors och för alla trevliga lunchträffar.

Mina rumskamrater på berget, Carolin Rutgersson, Erik Kristiansson och Carl-Fredrik Flach, Tack för att ni gett mig många skratt och gjorde att det var en så trivsam stämning på kontoret.
Carolin, det var ett privilegium att få dela kontor med dig, två lika entusiastiska forskare i samma rum ☺ Tack för allt stöd och omtanke du visat genom åren, alla pratstunder, hjälpen med det sista musförsöket och för att du svarat på alla mina frågor om allt möjligt rörande disputation.
Erik, tack för hjälp med statistikfrågor, förtrevligt hockeysnack, även om vi kanske inte har samma åsikt i frågan om vilket lag som är bäst och trevliga besök i Scandinavium. Heja Frölunda ☺
Carl-Fredrik, tack för trevliga och roliga samtal om ditt och datt och för att du förgyllde luften på kontoret med doften av Elisabeth Arden ☺

Världens bästa Sara Karlsson, tack för hjälpen med allt från tips rörande PCR och SPSS till uppmuntran och peppning och trevliga lunchträffar. Framför allt tack för att du är min vän.

Julia Johansson, super kvinnan som kan allt, tack för sällskap under stora delar av doktorandresan på berget, alla luncher och fikapauser. Mest av allt tack för trevligt sällskap utanför jobbet. Kul att vi följts åt genom graviditetskrämpor och i livet som småbarnsföräldrar.

~~**Filip Cuklev Stern,** tidigare medlem i Joakims grupp, numera qPCR expert på Genomics, tack för hjälpen med qPCR körningarna, hjälp med beräkningar och att du tålmodigt förklarat och svarat på alla mina frågor.~~

Karin Lind, under ett år utökade du progesterongruppens medlemsantal till det dubbla ☺ Det var trevligt att ha någon att bolla idéer och lösa knepiga PCR problem med och trevligt med sällskap på EBM och Chalmers.

Filip Cuklev Stern, tidigare medlem i Joakims grupp, numera qPCR expert på Genomics, tack för hjälpen med qPCR körningarna, hjälp med beräkningar och att du tålmodigt förklarat och svarat på alla mina frågor.

Birgitta Weijdegård för all din fantastiska hjälp med diverse laborativt arbete. Du är en klippa!

Nuvarande och gamla medlemmar i Joakims grupp tack för att ni alla gör att det är så trevligt att komma till jobbet.

Före detta kollegor på Endokrin som gjorde att även om det känts motigt ibland, så var det alltid trevligt att komma till jobbet och träffa er. Ett speciellt tack till **Robert Jakubowicz** för att du sprider glädje och positiv energi omkring dig och för tips och råd längs vägen.

Mina vänner, tack för att ni alltid finns där.

Mina fantastiska föräldrar och syskon, tack för att ni finns och alltid ställer upp vad det än gäller, jag hade aldrig klarat mig utan er.

Peter Lundberg, min sambo tack för allt. För att du stöttat och peppat mig i detta. Uppmuntrat och tröstat mig när självförtroendet svikit. Snart är det äntligen över ☺

Wilma och Nowa, mina underbara döttrar, de bästa resultaten jag fått i livet och min största glädjekälla. Tack för att ni under den här resan, fått mig att tänka på något annat varje dag och att ni ständigt påminner mig om vad det är som verkligen är viktigt i livet.

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