THE ROLE OF PROGESTERONE IN THE REGULATION OF CILIARY ACTIVITY IN THE FALLOPIAN TUBE

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I may not have accomplished
all I intended, but I think
I have arrived where I wanted

Till min familj

ABSTRACT

The overall aim of this thesis was to investigate the distribution and regulation of membrane progesterone receptors (mPRs) that may be involved in regulating ciliary activity in the fallopian tube.

The fallopian tube serves to transport the egg and spermatozoa to achieve fertilization. Later, the formation of the pre-embryo is thought to result from the movement of cilia in the epithelium and the muscular activity in the wall of the fallopian tube. The environment in which the gametes exist and develop is greatly influenced by the action of ovarian hormones. Progesterone is essential for many aspects of female reproduction and is also an important regulator of gamete transport and ciliary activity in the fallopian tube. The effects of P_4 in the body are mediated predominantly through the activation of nuclear progesterone receptor (PGR) isoforms. Rapid effects of P_4 in cells and tissues lacking the nuclear receptors indicate that there are other also functional receptors for P_4 in addition to the classical nuclear receptors. In the last four to five years, evidence has been obtained that supports the involvement of mPRs in P_4 signaling in mammalian reproductive tissues and the brain. The mPRs comprise three subtypes (α, β and γ) and belong to the seven-transmembrane domain progesterone adiponectin Q receptor (PAQR) family.

Using antibodies designed to detect specific mPR sequences, we showed that mPRs are present in reproductive and non-reproductive tissues in mice of both sexes. Using mice as well as tissue from healthy fertile women, we have shown that mPRβ and γ are expressed in ciliary cells in the fallopian tube epithelium. While mPRβ was specifically localized on the cilia, mPRγ was found at the base of the cilia of the same cells. Immunohistochemistry (IHC), confocal microscopy, Western blot, reverse transcriptase PCR and real time PCR were used to detect and confirm the expression and specific cellular localization of the mPRs in the fallopian tube. Treatment with P_4 in a gonadotropin-primed mouse model reduced the expression of the mPRβ and γ genes in the fallopian tube, whereas treatment with estradiol rapidly down-regulated both the gene and protein expression of mPRβ in immature animals. In humans, the variation in receptor expression over the menstrual cycle showed similarities to the regulation observed in mice before, around and after ovulation. A method based on light reflectometry was designed to study possible rapid effects of P_4 on the tubal ciliary beat frequency (CBF) of mice ex vivo. We found a significant and rapid reduction of CBF in P_4-treated cells compared to controls.

In conclusion, this study demonstrates that mPRs are present in the ciliary cells of mouse and human fallopian tubes and that P_4 can regulate ciliary activity within the fallopian tube.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Äggledarna hos en kvinna sträcker sig som två armar från livmodern till äggstockarna. Med hjälp av den flikiga ända som påminner om en hand fångar äggledarna upp ägget som äggstockarna avger vid ägglossning en gång per månad. Äggledarna transporterar sedan ägget och spermierna mot varandra för att en eventuell befruktning skall äga rum. Befruktningen innebär att en spermie smälter samman med äggcellen och blir till en ny cell. Befruktningen sker normalt genom samlag och om kvinnan har ägglossning, men kan också ske på konstgjort väg. I det ögonblick då spermien befruktar äggcellen bildas en ny cell som får en unik genetisk egenskap. Denna befruktade äggcellen transporteras vidare mot livmodern, en resa som normalt tar 3-4 dagar. Under resans gång delar cellen sig, växer och mognar för att så småningom bli ett embryo (foster i tidigt utveckligsstadium) och sedan ett foster. När resan genom äggledarna tar slut har embryot kommit till livmodern där det fäster i dess näringsrika vägg för att under graviditetens långa period växa och utvecklas.

Alltså är en välfungerande äggledare grundläggande för att en kvinna skall bli gravid på ett naturligt sätt. Ofrivilig barnlöshet, eller oförmågan att uppnå graviditet, kan bero på många orsaker, dels hos mannen, dels hos kvinnan eller båda. I de fall där felet ligger hos kvinnan kan rubbningar i äggledarnas funktion vara orsaken till infertilitet. Äggledarna har en tub-liknande form som upprätthålls med hjälp av dels den vätska som rinner genom äggledarna, och dels av den muskel vägg som klär utsidan av äggledarna. Insidan av äggledaren består av ett skikt celler som huvudsakligen är av två typer: cilierade och sekretoriska celler. Ciliecellerna har utväxta hårliknande strukturer på sig, medan de sekretoriska cellerna saknar dessa. När ”håret” på cillieceller rör sig och viftar, skapas samordnade vågor som förflyttar dels ägg, spermier och embryot, men även avlägsnar bakterier eller virus som kan orsaka lokala infektioner. Exempel på en bakterieorsakad infektion är klamydia som kan leda till sammanväxningar i äggledarna. De signaler som gör att cilierna rör sig kommer från insidan av cellen och kan initieras till exempel av ämnen som rinner genom äggledarna eller av signalmolekyler i blodet (hormoner). Dessa signalpartiklar tas sig in i ciliecellen genom kanaler som finns i cellens hinna eller genom att hormonerna binder till mottagarstrukturer som sitter i cellen och ciliens hinna. De viktiga könshormonerna som finns i äggledaren är östrogen och progesteron. Koncentrationen av progesteron ökar efter ägglossning, och finns därmed i äggledaren i högre mängd efter ägglossning dvs. vid den tidpunkt då befruktningen kan ske. Progesteron är nödvändigt inte bara för äggledaren, utan för att ägglossning skall ske och för att förbereda livmodern att ta emot ett eventuellt befruktat ägg.

Det övergripande syftet med mina studier i den här avhandlingen, har varit att undersöka hur progesteron påverkar ciliernas rörelse. Progesteron är ett steroidhormon som är fettlöslig och kan ta sig genom cellens hinna; inne i cellen binder progesteron till sin mottagarstruktur (också känt som ” den klassiska receptorn”) som sedan aktiverar gener i cellkärnan så att nya proteiner bildas. Denna process som går via cellkärna är långsam, dvs. tar tid och kallas ”klassiskt”. För några år sedan upptäcktes en ny grupp av mottagarstrukturer som sitter i cellens hinna och som förmedlar

effekter av progesteron på bara några minuter och skiljer sig från den klassiska processen. Under några år har internationell forskning lagt fram bevis för att en liten grupp av proteiner fungerar som mottagarstrukturer och förmedlar effekter av progesteron inom framförallt fortplantnings processer. I arbete I och delar av arbete II i avhandlingen har vi undersökt om mottagarstrukturerna för progesteron förekommer hos människor och möss. Då vävnadsmaterial från människor är mycket begränsad har vi använt i vissa undersökningar en djurmodell där unga, sexuell omogna möss studerats. Vi har sedan isolerat olika vävnader inklusive äggledarna och studerat dessa med hjälp av olika molekylär biologiska tekniker. Vi har visat att två mottagarstrukturer, kallade beta-formen respektive gamma-formen, finns i fortplantnings organ hos både honor och hanar. Med en mer specifik teknik har vi visat att beta och gamma formen finns på distinkta platser i cilicellen, vilket kan tydda på att de uppfyller olika funktioner. En intressant observation var också att dessa mottagarstrukturer förekommer olika mycket i vissa organ. Gamma-formen finns till exempel mycket i lungan, där det också finns cilieceller, medan beta-formen finns mycket i äggstockar hos honor och testiklar hos hanar.

För att studera hur dessa strukturer regleras, så har vi behandlat mössen med de kvinnliga könshormonerna östrogen och progesteron. För att skilja mellan direkt och indirekt effekt av hormoner har vi behandlat både den omogna musmodellen och den sexuell mogna musmodellen med könshormoner. Vi har i arbete II sett att progesteron på ett snabbt sätt påverkar både beta som gamma-formen i den hormonell mogna musmodellen, medan östrogen påverkade receptorerna i den omogna modellen. Studier av humant material visade likheter med de resultaten från musen avseende hur receptorerna förekomst varierar kring ägglossningen.

I arbete III försökte vi beskriva effekten av progesteron på ciliecellen genom att mäta frekvensen med vilken cilierna slår med eller utan behandling med progesteron. Vi använde den omogna musmodellen från vilken vi isolerade cilieceller från äggledare. Med hjälp av en fysikalisk metod som baseras på ändringar i ljusets brytning (som också kallas ljus reflektometri) kunde vi fastsälla en slagfrekvens för cilieceller från mus. Relativt snabbt efter behandling med progesteron såg vi en minskning i slagaktivitet hos cilierna som tyder på en snabb och direkt påverkan av progesteron.

Sammanfattningsvis har vi visat att snabbt verkande mottagarmolekyler för progesteron finns i äggledaren hos möss och människor, samt att dessa finns på distinkta platser i ciliecellen. Progesteron och östrogen tycks reglera dessa mottagarmolekyler på ett snabbt sätt. Transporten av ägg och spermier är beroende av fungerande aktivitet hos cilierna. Denna rörelse påverkas av progesteron. Våra studier tyder på att icke-klassiska receptorerna kan vara inblandade i denna signaleringskedja. Resultaten bidrar således till en ökad förståelse kring de mekanismer som reglerar ägg och spermietransport.

LIST OF PUBLICATION

This thesis is based on the following papers, which will be referred to in the text by their Roman numerals:

I **Membrane progesterone receptor gamma: tissue distribution and expression in ciliated cells in the fallopian tube.**
Magdalena Nutu, Birgitta Weijdergård, Peter Thomas, Christina Bergh, Ann Thurin-Kjellberg, Yefei Pang, Håkan Billig, D.G. Joakim Larsson
Molecular Reproduction and Development 2007; 74: 843 - 850

II **Distribution and hormonal regulation of membrane progesterone receptors beta and gamma in ciliated epithelial cells of mouse and human fallopian tubes**
Magdalena Nutu, Birgitta Weijdergård, Peter Thomas, Ann Thurin-Kjellberg, Håkan Billig, D.G. Joakim Larsson
Reproductive Biology and Endocrinology 2009; 7:89

III **Rapid effects of progesterone on ciliary beat frequency in the mouse fallopian tube**
Anna Bylander*, Magdalena Nutu*, Rikard Wellander, Mattias Goksör, Håkan Billig, D.G. Joakim Larsson
Manuscript

* Both authors contributed equally to this manuscript

TABLE OF CONTENTS

ABBREVIATIONS

AC, mAC, sAC, tmAC	adenylyl cyclase, membrane; soluble, transmembrane
AIJ	ampullary isthmic junction
Akt	tymoma viral oncogene (protein family)
20β-S	17,20b, 21-trihydroxy-4-pregnen-3-one
cAMP	cyclic adenosine monophosphate
CBF	ciliary beat frequency
CD^8	cluster and differentiation 8
CDB	Contraceptive Development Branch
CNS	central nervous system
DBD	DNA-binding domain
17α,20β DHP	17α, 20β-dihydroxy-4-pregnen-3-one
E_2	estradiol-17β
E.coli	Escherichia coli
ER	endoplasmatic reticulum
ERE	estrogen response element
ESR	estrogen receptor
FC	flow cytometry
FSH	follicle stimulating hormone
G-protein	guanine nucleotide-binding protein
G_{olf}	olfactory G-protein
$GABA_A$	gamma-amminobutyric acid receptor A
GDP	guanosine diphosphate
GnRH	gonadotropin-releasing hormone
GPCR	G-protein coupled receptor
GRE	glucocorticoid response element
GTP	guanosine triphosphate
GVBD	germinal vesicle breakdown
hCG	human chorionic gonadotropin
HDL	high density lipoprotein
HRE	hormone response element
ICSI	intra-cytoplasmatic sperm injection
IDA	inner dynei arms
IF	immunoflourescence
IFT	intraflagellar transport
IHC	immunohistochemistry
i.p.	intraperitoneal injection
IVF	in vitro fertilisation
K_d	dissociation constant
kDa	kilo Dalton
KLH	keyhole limpet hemocyanin
LBD	ligand binding domain
LDL	low density lipoprotein

LH	luteinizing hormone
LHR	luteinizing hormone receptor
MAPK	mitogen activated protein kinase
MIS	maturation inducing steroid
MLC	myosin light chain
mPRα, β, γ	membrane progesterone receptors alpha, beta, gamma
NE	nuclear envelope
ODA	outer dynei arms
P_4	progesterone
P450	cytochrome P450 enzyme
PAQR	progestin and adipoQ receptor family
PCD	primary ciliary dyskinesia
Pgr	progesterone receptor gene
PGR A/B	progesterone receptor protein A & B
PGRKO	progesterone receptor knockout
PGRMC1	progesterone receptor membrane component 1
PIK	phosphatidylinositol kinase
PIP	phosphatidylinositol phosphate
PKA, PKG, PKC	protein kinase A, G, C
PLA, PLC, PLK	phospholipase A, C, K
PM	plasma membrane
PMSG	pregnant mare serum gonadotropin
POAH	preoptic anterior hypothalamus
PTX	pertussis toxin
qRT-PCR	qunatitative real time polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction
SB	surface biotinylation
shRNA	short hairpin RNA
SRC2	steroid receptor coactivator
TM	transmembrane
UTJ	uterojunction
WB	Western blot

INTRODUCTION

THE FALLOPIAN TUBE

"*Trumpets of the uterus*" was the first exact description of *fallopian tubes* given by Gabriele Falloppio (1523 - 1562) (Fig. 1), one of the most important anatomists of the sixteenth century. The anatomical description of the tubes was published in his first collected work in 1584, in Venice. He saw connecting ducts between the uterus and the ovaries and also considered these tubes to be semen-conveying vessels. Today several names, including *oviducts, uterine tubes* or *salpinges,* are used to describe the fallopian tubes.

FIGURE 1: Gabriele Falloppio

Role in reproduction

Besides serving as the normal site of fertilization, the fallopian tubes act as ducts for the transport of oocytes and spermatozoa. Tubal transit has been proven to depend on several physiological events, such as muscle contractility [1], ciliary activity [2] the composition of the follicular fluid [3]. There is still debate on which is the most important contributing mechanism in tubal transport, but it has been shown that ciliary activity plays a central role [4, 5].

Fallopian tubes are also essential for other purposes besides serving as a conduit between the gonads and the uterus; for instance, they enable the peritoneal fluid to come in contact with the uterine fluid. Keeping in mind the influence of P_4 on endometrial proliferation, one principal role of fallopian tubes might be to delay passage of the embryos until the uterus is suitably prepared [6]. In surgical studies with several animal models, the caudal part of the isthmus was observed to act as a sequestrating reservoir of viable spermatozoa in the pre-ovulatory period. The further progression of the spermatozoa to the site of fertilization might depend on factors present or introduced by ovulation in the fallopian tube [7].

Anatomy

The fallopian tubes are paired tubular organs whose anatomical shape differs somewhat between mice and humans (Fig. 2). The human fallopian tubes stretch from the uterus to the ovaries, measure about 7-14 cm in length and are not directly attached to the ovaries, but rather open into the peritoneal cavity [8]. Besides the curly anatomical form of fallopian tubes in mice, another difference is that mice have a bursa surrounding both the ovary and the fallopian tube. During ovulation, ova released into the bursal cavity travel through the bursal fluid and enter the oviduct without entering the peritoneal cavity [9]. In humans, the ends of the fallopian tubes lying next to the ovary feather into fringe-like structures called *Fimbriae (Latin for "fringe").* The *Fimbriae* sweep over the ovaries with their millions of tiny hair-like cilia and draw the newly released oocyte into the tube.

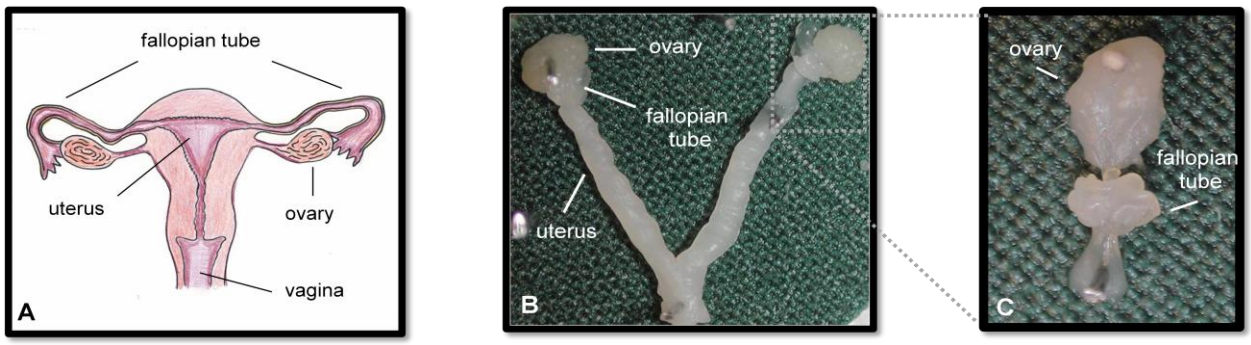

FIGURE 2: Anatomical illustration of the human (A) and mouse (B and C) female reproductive tracts.

Considering the fallopian tube in all its length, there are four anatomical segments that differ from each other in structure and function [8] (Fig. 3):

1. The *infundibulum* is a funnel shaped segment that contains the fimbriae. The majority of cells in this region are ciliated.
2. The *ampulla* comprises more than half the length of the tube and has an outer diameter of 1-2 cm in humans [10]. The meeting between the oocyte and the sperm takes place in this part of the fallopian tube. The mucosal folds here are large and almost fill the ampullary lumen.
3. The *isthmus* is a narrow part near the uterus and is about 3 cm long in humans. It has two well-developed muscular layers [10]. Secretory cells are predominant in this region, with smaller mucosal folds than in the ampulla.
4. The *intramural part* is located in the uterine wall and has three muscular layers. Its luminal diameter is only about 1 mm, and its length is about 1 cm. The mucosal folds are fewer and the ciliary cells are less abundant compared to the distal parts of the tube.

All anatomical segments are found in both humans and mice.

Histology

Moving from the center of the fallopian tube to the periphery, a cross-section shows: the internal *mucosa* (endosalpinx), the *muscular layer* (myosalpinx) and the *serosa*. The size of the lumen, the shape of the mucosa and the thickness of the muscular layer all vary along the length of the tube (Fig. 3).

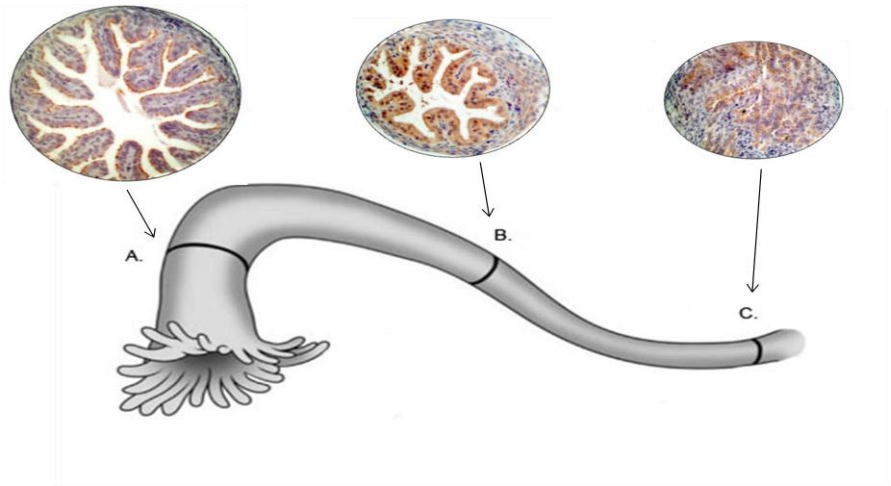

FIGURE 3: Schematic illustration of the human fallopian tube showing the folds in the A) infundibulum, B) ampulla and C) isthmus. The immunohistochemistry images are taken from a mouse fallopian tube in the same regions.

The *mucosa* is more complex in the ampulla, which features extensive folding. The lamina propria is a component of the mucosa that lies beneath the epithelium. The lamina propria contains a network of fibers, stroma cells, mast cells, capillaries and lymph vessels. The mucosal lining the mammalian fallopian tube is a simple columnar epithelium consisting of two cell types, ciliated cells and secretory cells, both of which bear cilia (Fig. 4). In mammals, cilia are membrane-bound hair-like processes that extend from the basal body. The ciliated cells of the fallopian tube have motile cilia, whereas the secretory cells have smaller, non-motile cilia (see below) [11].

Motile cilia are present in the female reproductive tract, in sperm tails and in the respiratory tract [11, 12]. The epithelial cells may possess several hundred 9 + 2 motile cilia that extend from the basal body (Fig. 4A and B). The cilia proper, including the basal body and its associated proteins, constitute the ciliary apparatus. The formation of the ciliary apparatus, called ciliogenesis, has been studied in many species, including humans [13], mice [14] and rabbits [15], and it is similar among different animals. Moreover, ciliogenesis in the reproductive and the respiratory tract is fundamentally the same [16]. Ciliary movement is generated by a connection between the outer and inner dynein arm and the doublet of the microtubule [17, 18]. The first true ciliary disease leading to male infertility has been shown to result from the lack of dynein arms [19]. Ciliogenic cells are frequently present in the tubal epithelium in the mid-follicular phase, whereas they lose most of their cilia in the luteal phase of the menstrual cycle [20]. In mammals, motile cilia are about 5-6 µm long and 0.25 µm in diameter. They comprise an axoneme composed of microtubules and microtubule-associated structures. The axoneme appears in two configurations: the 9 + 2 motile configuration and the non-motile 9 + 0 configuration [11] (Fig. 4C and D). The ciliary membrane is continuous with the cell membrane but is selectively different in its composition. The function of the motile cilia depends on specific receptors, and ion channes proteins, calcium ions (Ca^{2+}) channels and receptors involved in growth control pathways. Using cilia fractionation and proteomics, more than 1400 peptides, 200 axonemal proteins and peptide matches to over 200 expressed sequence tags have been identified in human cilia [21]. However, the distribution of certain receptors in primary versus motile cilia or in the ciliary versus apical membrane is not always the same [22].

The non-motile cilia, also named primary cilia, extend from the apical surface of secretory cells and are usually solitary (Fig. 4A and C). Whether the apical surface of the secretory cells is covered by domes, microvilli [23] or a single cilium [11] is still under debate. What is agreed upon, however, is that this type of cilium lacks a pair of singlet microtubules (9 + 0 configuration) and dyneic arms and is thus considered non-motile [24]. The physiological function of primary cilia has probably been underestimated for many years, but it has been shown that they may play sensory roles in many cellular systems [11]. The ciliary membrane of primary cilia is equipped with protein complexes and ion channels (e.g., Ca^{2+}) that potentially function as mechanoreceptors. Under the influence of fluid flow and when cilia bend, Ca^{2+} channels are activated, resulting in an increase in Ca^{2+} influx and the consequent activation of various subcellular events [25]. Primary cilia have a widespread distribution among the cells of the body, as much in epithelial cells in the fallopian tube [11, 26] as in non-epithelial cells like fibroblast and smooth muscle cells [12].

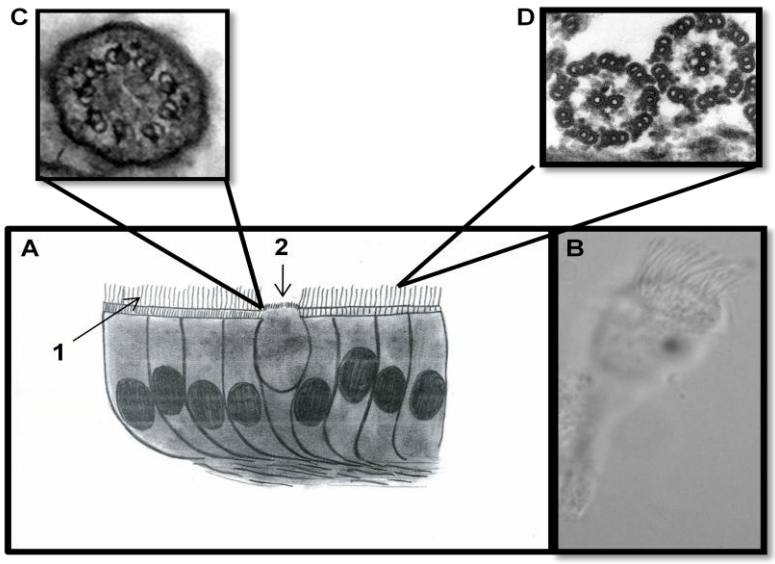

FIGURE 4: Illustration of the fallopian tube epithelium (A) containing ciliary cells (1) and a secretory cell (2). Microscopy image of a ciliary cell from a mouse fallopian tube (B) (100x). Electron microscopy images showing the structure of motile (D) and non-motile cilia (C).

Beside these two types of epithelial cells, the mucosa of the fallopian tube contains *intercalary cells* with a yet unknown function and *basal cells* attached to the basal membrane. The basal cells have been suggested to have dual functions: they may act as T-lymphocytes as a part of the immune system and also serve a regenerative function like stem cells [27, 28].

The *muscular layer* consists of an inner circular and outer longitudinal layer to which a third layer is added in the interstitial (intramural) region close to the uterus.

The *serosa* is the outer surface of the fallopian tube facing the peritoneal cavity [29].

Tubal innervation, vasculature and lymphatics

Both parasympathetic and sympathetic nerve fibers are found in fallopian tubes. The preganglionic fibers of the parasympathetic system come from spinal segments S2 and S4; they traverse the pelvic branches and end in ganglia close to the isthmus region. The other region of the fallopian tube, the ampulla, receives parasympathetic nerve fibers from terminal branches of the vagus nerve. The preganglionic sympathetic fibers in the fallopian tube originate from spinal segments Th11 and Th12 and contain both long and short adrenergic neurons [1].

Blood supply to the fallopian tube comes from the uterine and the ovarian arteries. The uterine artery comes from the internal iliac artery, which is formed by a bifurcation of the aorta. The ovarian artery originates directly from the aorta. The arterial system is in contact with the venous drainage system, which allows for a counter-counter mechanism for the exchange of substances such as P_4 and prostaglandins [30, 31].

The lymphatic plexus in the fallopian tube is drained by the uterine and ovarian lymphatic vessels. The lymph or interstitial fluid surrounding the cells enters the vessels by filtration. It has been

shown that particles introduced into the peritoneal cavity are transported via the peritoneal lymphatic flow, which is eventually transferred into the venous circulation system [8].

PHYSIOLOGY

Targets for progesterone

The steroid hormone P_4 is involved in a variety of physiological events in mammals, with target tissues throughout the body, from the brain, to the mammary glands, ovaries, uterus and even bone [32]. Broadly speaking, the best-known physiological roles of P_4 involve its effects on the uterus and ovaries for the release of the mature oocyte, facilitation of implantation and maintenance of pregnancy.

Patterns of gamete transport

The transport of the egg and spermatozoa towards the fertilization site and the further transport of the embryo to its implantation site in the uterus have been recognized as fundamental steps in the process of human procreation. One striking fact is that objects (egg, embryo or sperm) being transported in both directions (for fertilization or implantation) through the fallopian tube are subjected to a physically and chemically changing environment, reflecting regional differences and the complexity of the fallopian tube.

Sperm migration

In order to reach the site of fertilization after coitus, spermatozoa must pass through different anatomical regions of the female reproductive tract, such as the cervix, uterus and uterotubal junction. Within minutes, human sperm deposited in the vagina start their journey towards the cervical canal [33]; in mice, however, they are swept all the way through the cervix into the uterus [34]. Although sperm are exposed to several environmental impediments, such as the acidic pH of vaginal fluid [35], the defense mechanisms of the female immune system [36] and partly impenetrable mucus [37], this passage is regulated to maximize the chance of normal and vigorous sperm successfully completing the journey.

Compared to the vagina, cervix or uterus, the fallopian tube provides a haven-like environment for sperm. Immediately after entrance into the isthmus, the sperm appear to be trapped in a reservoir, which might contribute to prolonged sperm fertility properties [38] while reducing the risks of polyspermic fertilization [39]. However, a defined sperm reservoir has never been defined in humans. Instead, the sperm bind to carbohydrates moieties in the epithelium of the fallopian tube, a phenomenon that has been observed in vivo and vitro in both human and non-human species [40-42]. When sperm are incubated with epithelium, capacitation is delayed due to stabilization of the sperm membrane and reduced membrane fluidity, which prevents increases in cytoplasmatic Ca^{2+} [43]. Viability may also be preserved by mucus in the lumen and the complexity or regional density of mucosal folds, which must slow down the sperm's migration. Although the structure and composition of the flagella and motile cilia are identical, the beating patterns differ between

these organelles. The motion of flagella (e.g., in the tail of sperm) follows a propeller-like pattern. Thus, flagella serve in the propulsion of a single cell, such as in swimming in spermatozoa.

Oocyte and embryo transport along the fallopian tube

Transport of the egg through the fallopian tube starts with the rupture of the follicle at ovulation and ends when the egg or embryo passes into the uterus. The time taken by the oocyte to travel through the fallopian tube is somewhat constant within each species, but there is some variation among species. Time is needed for the egg to become fertilized during its journey and for the endometrium to prepare for implantation and allow post-fertilization development. It is obviously more difficult to study ovum transport in women because of ethical concerns. However, detailed observations made in other species such as mice and rabbits have been useful [44].

In humans, the fimbriae come in close contact with the ovary at ovulation and sweep the cumulus mass from the ruptured follicle into the fallopian tube with back-and-forth movements. Additionally, muscle contractions contribute to bringing the fimbria and ovary even closer together [45, 46]. If the egg falls into the peritoneal cavity, it is still possible for the fimbria to catch it, as was observed from human experiments in which microspheres were injected into the peritoneal cavity [9]. In humans, unlike in many other species, the egg spends most of its time in the ampullary segment. When the developing zygote gets to the ampullary isthmic junction (AIJ), passage through the isthmus takes hours or days and is induced by rapid back-and-forth movements suggested to occur due to muscular contractility and ciliary activity. However, isoproterenol treatment did not affect the CBF in rats, but abolished the undulatory movement caused by the muscle layer. When smooth muscle activity was blocked by isoproterenol, the transport rates remained unchanged. Furthermore, the ciliary beat frequency was not affected by isoproterenol, suggesting that ciliary movement can transport the egg in the absence of muscular activity [4].

In rodents, egg transport is stable and slow in the very beginning; however, it then starts to change, with more rapid forward and backward movements associated with muscular contractions. These rapid movements might ensure that gamete and tubal secretions are mixed [47]. In rabbits, the transport pattern has been worked out in detail and described to occur in several steps: the first step is a rapid contact between the fimbriated ends and the cumulus cell mass; the second step is a brief retention time near or at the AIJ, so that fertilization and the dislocation of cumulus cells can occur; the third step is the passage of the egg through the isthmus; and the fourth step is retention at the isthmic uterine junction, which accounts for over 40% of time spent by the egg in the fallopian tube in mice [48]. A noteworthy observation is that in mice, several eggs are transported and they tend to travel relatively close together.

Transportation time

The approximate length of the interval from ovulation to implantation varies from five days in rodents to 10 days in primates or 15 days in bats, but some intra-species differences were also observed when animals were studied under different physiological conditions [9, 44]. In mice and humans, the duration of tubal transport is similar, from 72 h in mice to 80 h in humans, despite the very different lengths of the fallopian tubes [9]. In pigs and rabbits, the transportation time is 48

hours and 55 hours respectively [49]. The egg travels at different speeds through the various portion of the fallopian tube, and there are marked differences among species in this process. In women, eggs appear to spend almost 60 h in the ampullary-isthmic junction after ovulation [44] . The passage through the isthmus takes less than 10 hours, meaning that the human egg spends 90% of the total duration of tubal transport in the ampulla. This prolonged time in the ampulla seems to be typical of primates and has pathophysiological implications as well. In humans, aberrations or dysfunction in tubal transport may result in implantation of the embryo in the fallopian tube (i.e., ectopic pregnancy). In mice and rabbits, there is a similar time passage pattern, but the egg is kept in the ampullary-isthmic junction for only 25% of the total duration of tubal transport, spending much more time instead at the isthmic-uterine junction. [47, 48].

Mechanisms of tubal transport

It is widely accepted that the physiological phenomenon of egg passage through the fallopian tube is the result of mechanical energy provided by the smooth muscle, ciliated and secretory epithelial cells [1]. The significance of muscular and ciliary activity or flow of mucus secretions is still arguable, but it is generally accepted that there are regional differences along the fallopian tube in terms of the contribution of each factor. There are also differences among species in the anatomy and distribution of cellular components of the fallopian tube that complicate the entire picture of gamete transport.

Ciliary movement

There is considerable evidence for the contribution of cilia to oocyte and embryo movement in the fallopian tube. First, the ciliated motile cilia are found predominantly in the ampullary region rather than in the isthmic region of the fallopian tube in many species [9]. Second, the pattern of ciliary movement changes at the time of ovulation. Despite the fact that cilia often beat out of phase with one another, at the time of ovulation this slatternly movement changes to a synchronized movement oriented toward the uterus, both in humans [50] and in rabbits [51]. When clusters of cilia become activated, they stimulate neighboring cilia and initiate a metachronal wave movement across the epithelium [52]. The mechanics of ciliary movement depend on the outer and inner dynein arms (ODA and IDA), which are force-producing molecular motors causing the slide of microtubules. Generally, the ODAs and IDAs function together, but the ODAs principally regulate beat frequency while the IDAs control beat form [53]. In both cilia and flagella, there is a motility process occurring between the ciliary/flagellar membrane and the axoneme. This process is called intraflagellar transport (IFT), and the proper function of cilia/flagella depends on the assembly of precursor molecules that are bound to IFT particles in order to be transported between the tip of the cilia and the cell body [54]. Clinical features of primary ciliary dyskinesia (PCD), which include, among other pathological conditions, rhinitis, sinusitis and male infertility, point to physiological processes in which ciliary motility is essential [55]. Mice defective in hydin, a component of the central pair of microtubules, develop hydrocephalus and die shortly after birth [56]. In humans, fluid flow produced by the motile cilia is disturbed because people diagnosed with PCD have *situs inversus totalis* [57]. Third, if muscular activity is inhibited by isoproterenol, a synthetic beta-adrenoreceptor antagonist, cilia alone are capable of transporting ova to the site of fertilization within a normal time frame [4, 5]. In contrast, women suffering from immotile cilia

syndrome are not always infertile [58, 59]. A number of studies have reported a significant increase in CBF after ovulation [2, 60], while others have found no such cyclical variations [61]. It is important to interpret the results with caution due to limitations of the techniques used and, most importantly, due to the *in vitro* nature of these experiments.

Contractile activity

Tubal smooth muscle exhibits brief contractions (episodic) and sustained contractions (tonic) that have been reported to occur in the proliferative and periovulatory periods and in vitro [62, 63]. The electrical properties of the tubal smooth muscle layer have been studied in humans [64] and intensively in rabbits [1]. Compared to the ampulla, the muscular coat of the isthmus is thick and tonic contractions appear to take place at the AIJ and the UTJ [65]. The spread of electrical and mechanical activity has been shown to take diverse directions on the tubalsegment and the muscular layer. In humans, the electrical activity spreads toward the uterus subsequent to menstruation, whereas prior to ovulation the activity in the AIJ is oriented from both ends of the oviduct, probably to facilitate the migration of the sperm and oocyte towards each other. After ovulation, the activity in the AIJ is more coordinated and oriented towards the uterus to facilitate further movement in the correct direction [64, 66]. These changes in contractile activity are thought to be controlled by systemic hormones, neurotransmitters and cytokines [67].

A counter-current transport system between blood vessels with flow in opposite directions is found in the testes and the kidney, and there is ample evidence that this mechanism is also present in the blood circulation between the fallopian tube, uterus and ovary. The ovary and uterus veins anastomose to form a network of blood vessels around the ovarian artery to facilitate the concentration of substances and communication within the female reproductive tract [68]. Many substances such as prostaglandins, oxytocin and steroids are transferred to tubal/ovarian blood; however, the concentration of free hormones is higher because protein-bound hormones do not pass across the vessel walls due to their much larger molecular size. In humans, it is not exactly known how the concentration of steroid hormones in tubal/ovarian blood compares to that in the peripheral blood, but in pigs, the concentrations of both P_4 and E_2 were higher in tubal blood compared to peripheral blood [69].

The secretory activity of the epithelial cells is pronounced in the isthmus, considering the decreased proportion of ciliated cells from 50% in the ampulla to approximately 27-35% in the isthmus [23]. After ovulation, due to decreased secretions, the cilia become visible and erect, but in the periovulatory period, the isthmic mucus is abundant and might play an important role in gamete transport. The migration of both the sperm and the ovum towards each other is prevented because the cilia are covered by the mucus; at the same time, E_2 stimulates isthmic contraction and the mucus locks the ovum to prolong its stay in the ampulla [70]. Relaxation of the muscular layer and the disappearance of the mucus begin with increases in P_4 in the postovulatory period [71].

Clinical interest

With the success of *In Vitro Fertilization* (IVF) in 1978, when the first “test tube baby” was born, and with the continuous improvements in IVF techniques, the incentives to study the fallopian

tubes probably decreased. However, in vivo fertilization, the fallopian tube plays an indispensable role in gamete transport, fertilization and early embryo development. It is evident that the mechanism of tubal transport is a complex process and those disorders or dysfunctions in these events can have far-reaching consequences for the fertility of a woman. Female infertility is often not due to one single factor. Many pathological conditions such as *infertility* and *ectopic pregnancy* are associated with ciliary damage, reductions in ciliary motion or both [72]. Some pathogens such as *Gonococci* destroy ciliated cells and reduce ciliary activity, whereas *Chlamydia*, for example, damages the entire mucosa [73].

REGULATION OF TRANSPORT

Progesterone synthesis, secretion and regulation

Progesterone (pregn-4-ene-3,20-dione), often abbreviated "P_4," belongs to a class of hormones called *progestogens* that have a basic 21-carbon skeleton (C-21). Progesterone is the major naturally occurring progestogen in mammals. Like other steroid hormones, it is synthesized from cholesterol by step-wise enzymatic conversions: 1) the conversion of cholesterol to pregnenolone by the enzyme P45011a1 ("cholesterol side-chain cleavage enzyme" or "cholesterol desmolase") and 2) the conversion of pregnenolone to P_4 by the enzyme 3β-hydroxysteroid dehydrogenase (Fig. 5). The major source of cholesterol for ovarian P_4 production is circulating lipoproteins. Humans, pigs and primates utilize low-density lipoproteins (LDL), whereas mice, rats and ruminants primarily utilize high-density lipoproteins (HDL) [74].

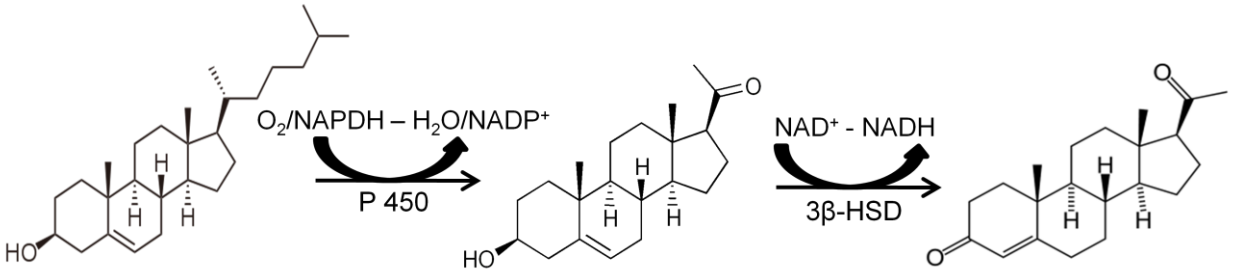

FIGURE 5: Chemical reactions in P_4 synthesis. P450 – cholesterol desmolase; 3β-HSD – 3-β-hydroxysteroid dehydrogenase; NAPDH – nicotinamide adenine dinucleotide phosphate; NADH – nicotinamide adenine dinucleotide

The main site of P_4 production in the non-pregnant female is the adrenal glands before ovulation and the corpus luteum after ovulation. Before ovulation, granulosa and theca cells help each other to produce estrogen under the influence of FSH and LH, according to the well-accepted two-cell, two-gonadotropin theory [75]. If pregnancy occurs, the production of P_4 is shifted to the placenta [76]. Progesterone is a central hormonal regulator of female reproductive processes. Its main functions are: 1) in the ovary and uterus: ovulation, facilitation of implantation, maintenance of pregnancy by facilitation of uterine growth and suppression of myometrial contractility; 2) in the mammary glands: lobular-alveolar development and inhibition of milk production during pregnancy; 3) in the brain: as a neuroactive steroid mediating signals for sexual response behavior

[32]. The effects of P_4 in the fallopian tube are still under investigation, but it has been shown to influence gamete transport [1, 71].

It is well established that the ovarian hormones E_2 and P_4 provide feedback to the hypothalamus and pituitary to trigger FSH-dependent follicular growth and LH-dependent follicle maturation and ovulation [77]. In vivo studies in humans indicate that P_4 stimulates its own production, a phenomenon known as "self-priming" [78]. During the preovulatory period, the serum level of P_4 is at a basal level, but these levels increase ten-fold in humans after LH-induced ovulation. This high level of P_4 is maintained during the first gestational period [79]. In mice, increased serum levels of P_4 were observed after hCG administration [80], comparable to the levels of P_4 in naturally cycling mice [81].

Hormonal changes in the fallopian tube

The fluid produced and secreted by the fallopian tube provides the environment in which important reproductive events take place. In many species, including humans, the rate of tubal fluid production ranges from 0.06 to 1.5 ml/day [82, 83]. Because of their anatomical position, the fallopian tubes are directly exposed to the steroids that ovaries produce around them. Therefore, in humans, follicular, peritoneal, seminal and uterine fluids might also contribute to the composition of tubal fluid [3]. Differently from the rest of the body, due to the counter-current exchange mechanism and other factors, the circulating levels of E_2 and P_4 throughout the fallopian tube are probably higher than the plasma levels. After ovulation, the concentration of E_2 and P_4 in peritoneal fluid increases markedly, and the levels of free circulating steroids are higher due to low levels of steroid-binding proteins in both the peritoneal and tubal fluids [84-86]. Studies in monkeys showed that tubal fluid concentrations of E_2 and P_4 were higher after ovulation [87], and studies from rodents have demonstrated differences in composition between ampullary and isthmic fluid [88]. Additional contributors to the steroid content of tubal fluid are the travelling oocyte-cumulus complex and the extruded granulosa cells in the lumen of post-ovulation fallopian tubes [89-91]. Tubal fluid also contains ions to maintain osmolarity and pH, as well as nutrients such as glucose, lactate, pyruvate and amino acids that are the source of energy for the early embryo [92].

The effects of steroid hormones on fallopian tubes

Endosalpinx

During the menstrual cycle, the fallopian tube undergoes morphological changes that are quantitatively different in the various parts of the tube. These cyclical morphological changes occur in the existing cells, since mitosis is rare in the fallopian tube [1]. Estradiol-dependent ciliogenesis occurs during the proliferative phase of the menstrual and estrous cycles [20, 93]. In both untreated and ovariectomized animals, E_2 administration induced ciliogenesis in the oviduct epithelium [94, 95]. Interestingly, ciliogenesis occurs during gestation in the human fetal oviduct, whereas in mice the ciliated cells differentiate in the first week after birth [14, 96]. Ciliogenesis in the human fetal oviduct must be regulated by mechanisms different from those in adults. The thickness of the epithelium increases in the follicular phase, reaching its maximum value (30 μm) in the late follicular phase before gradually decreasing in the luteal phase to a lowest point of 10-

15 µm. During menstruation, the epithelium thickness is uniformly low. Before ovulation, the heights of ciliated and non-ciliated cells are equal, whereas the cilia become more prominent after ovulation due to the decreased height of non-ciliated cells [97]. Progesterone has antiestrogenic activity and induces deciliation in the secretory phase of the menstrual cycle [98]. Secretory activity is more pronounced in the isthmus, and the secretion occurs in the late proliferative phase. The secretory cells atrophy after ovulation and some secretory granules have been observed in early ciliogenic cells, indicating that they might have differentiated from atrophied secretory cells [70, 99].

Ciliary beat frequency

The well-recognized changes that the fallopian tube undergoes during the menstrual cycle are not only restricted to morphological changes. The cyclical variation of ovarian steroid hormones affects the CBF of epithelial cells, with consequences for tubal transport. Inhibition of muscle activity by isoproterenol did not affect ovum transport [5]. The assessment of a baseline CBF is an important factor in the validation of ciliary activity. In vitro studies using human samples suggest a mean baseline CBF between 5 and 20 Hz [2, 61, 100, 101], whereas one in vivo study reported a baseline CBF of 5.5 Hz [102]. Several in vitro studies reported an increase in CBF after ovulation [2, 51, 60, 103]. This increased activity over the cycle was observed in the ampulla and the isthmus [2] and correlates to the number of ciliated cells present [102]. However, some studies did not support significant cyclical changes in CBF [61, 104] and others failed to register significant CBF variations between different tubal segments [51]. The effects of exogenous P_4 and E_2 on CBF have been studied by many, both in vivo and in vitro. Progesterone, when present at a high concentration (10 µM) with respect to the serum levels of P_4 in the luteal phase, causes a nearly 40% reduction in CBF [100]. This effect persists for 24 hours [100]. At the same time, E_2 alone did not affect CBF, but in combination with P_4, it did prevent the P_4-induced reduction in CBF as effectively as mifepristone, an antagonist of PGR [100]. This is difficult to explain because E_2 alone did not have any effect on CBF in any part of the tube or at any stage of the menstrual cycle [100]. Nevertheless, in vivo treatment with E_2 has been shown to affect ovum transport in a dose-dependent manner [105, 106]. Wessel et al. showed a rapid (15 min) decrease in CBF after treatment with P_4 at a concentration (20µM) relevant to those found in the placenta (> 20 µmol/l) [107, 108]. In both studies examining CBF reduction by P_4 [100, 107], treatment with RU486 pointed at a receptor-related effect rather than a toxic effect.

Contractile activity

The contractile activity of the tubal muscle displays cyclic variations, whereas morphological changes are not observed in the myosalpinx of the fallopian tube. During the periovulatory period, contractile activity increases and the orientation of activity depends on the day of menstrual cycle and location in the tube [67]. Ovarian hormones influence the way in which the fallopian tube myosalpinx responds to neurotransmitters, influencing adrenergic receptors and thus potentiating contraction or relaxation of smooth muscle cells [67]. Progesterone controls prostaglandin production, which increases in the luteal phase of the menstrual cycle [67]. The effects of prostaglandins E and F series on tubal motility appear to differ depending on the muscle type (longitudinal or circular) and different segments in the fallopian tube [67, 109, 110].

Nuclear Progesterone Receptors

The classical nuclear progesterone receptor (PGR), a member of the superfamily of ligand-activated transcription factors, was first cloned and characterized in the early 1970s [111]. Since then, PGR expression has been described in tissues known to be P_4-responsive, including reproductive tissues. However, PGR expression has been reported outside of the reproductive tract as well [32]. All members of the superfamily of nuclear receptors, to which PGR belongs, are organized into specific functional domains: 1) an amino terminal domain that is more variable and contains a transactivation domain (AF1); 2) a highly conserved DNA-binding domain (DBD) that gives the receptor its specificity depending on DNA sequences recognized by the DBD; and 3) a C-terminal ligand-binding domain (LBD) containing a second activation function domain (AF2). Between the DBD and LBD domains, there is a hinge region that is poorly conserved among members of this family and usually contains nuclear localization signals [112, 113]. The PGR is expressed as two major isoforms, PGR-A and PGR-B, encoded from the *Pgr* gene. In humans, the PGR-A and B proteins are 94 kDa and 114 kDa [114]. What makes PGR-B unique compared to PGR-A is an additional 164 amino acids at the N-terminal end [115], which contains a third activation function domain (AF3). As a result, the PGR isoforms recruit specific co-regulators in a cell- and promoter-specific manner and regulate different subsets of genes [116, 117]. Whereas PGR-B is a stronger activator of target genes, PGR-A can act as a dominant suppressor of PGR-B activity and other hormone receptors [118, 119]. The relative expression of PGR-A and -B in target tissues differs, and it is the ratio of PGR-A /-B that determines the cellular response to P_4 [32].

PGR is bound to heat shock proteins and is found in a free and inactive form in the cytoplasm and nucleus. Upon hormone binding, the receptor dissociates from the heat shock protein complex and translocate to the nucleus. Active PGR dimers bind to hormone-responsive elements (HREs) to facilitate transcription by coming into contact with components of the general transcription machinery (co-activators or co-repressors) or by promoting local chromatin remodeling. The consensus sequence of the HRE, common to PGR and other hormone receptors consists of a semi-palindromic half-site sequence, 5´-TGTTCT-3´, usually separated by three base pairs [120]. The recruitment of differential co-regulators affecting the ultimate receptor-specific response could be attributed to the low degree of sequence homology of the AF1 site in different nuclear receptors [112].

In addition, PGR contains a short proline-rich sequence in the N-terminal region that, upon P_4 binding, mediates direct interaction between cytoplasmic PGR, the Scr-homology 3 (SH3) domain of Scr (Rous sarcoma oncogene) and tyrosine kinases at the plasma membrane. This interaction leads to activation of the Ras/Raf-1/MAPK signaling pathway [121]. This kind of interaction with the Scr domains is specific to PGR. It is still debatable whether the PGR mediation of Scr activation requires co-regulators, but it appears that cyclin D1 is an important downstream target of the MAPK signaling pathway in breast cancer cell lines at least [122-124].

PGR expression has been described in P_4-responsive tissues, and studies in knockout mice have confirmed their indispensable role in reproductive physiology. The selective ablation of PGR-A or

-B helped to demonstrate that PGR-A is necessary and sufficient to mediate ovulatory response, whereas PGR-B is required for a normal proliferative P_4 response in the mammary gland [125]. Progesterone has a central role in reproduction, as it is involved in ovulation, implantation and pregnancy. In the female reproductive tract, PGR is present in the uterus, ovaries, cervix, vagina, fallopian tubes and breasts [32]. In the ovaries, PGR is expressed in theca cells, luteinizing granulosa cells, the corpus luteum and stroma and epithelial cells [32, 126]. The expression of PGR is under the control of E_2, which increases, and progesterone, which decreases PGR expression in most target tissues [127, 128]. However, in humans and primates, PGR expression persists in the corpus luteum [129], but its expression begins at earlier stages of follicular development [130]. More recent work by Clemens et al. has shown that E_2 induction of *Pgr* in granulosa cells appears to be indirect and that E_2 alone does not induce expression of Pgr in preovulatory rat granulosa cells [131]. In rats and mice, the expression of PGR has been shown to be transient [78, 132, 133]. Specific progestin binding has been described in other non-reproductive tissue, where the action of P_4 is less defined. For a review of the physiological role of P_4 in target tissues, see Graham & Clarke (1997).

Nuclear progesterone receptor in the fallopian tube

The lining epithelium of the fallopian tube and the uterus undergoes cyclical changes under the influence of P_4 and E_2. The important role of steroid receptors for fallopian tube structure and function was recognized in the late 1980s [134]. Specific monoclonal antibodies were developed and used to detect PGR in the fallopian tube [135]. Immunohistochemistry (IHC) methods and later Western blot and mRNA transcript detection by reverse transcriptase polymerase chain reaction (RT-PCR) have become standard methods for the detection of PGR in the fallopian tube [135-137]. The expression of PGR was demonstrated in the fallopian tube and uterus in the oviducts of humans [138, 139], rats [137, 140], and mice [80, 130, 139]. Because they are two functional distinct transcription factors, the investigation of a distinct cellular distribution and hormonal regulation of the two isoforms is relevant. The localization of PGR was observed in the nucleus of stroma, muscle and epithelial cells [80, 137], as well as in the lower part of cilia [139]. As commonly concluded by several studies, the expression of PGR increases during the follicular phase of the estrous/menstrual cycle and declines towards the luteal phase [80, 137-139]. Selective PGR investigation revealed PGR-A to be the predominant isoform throughout the cycle [130, 137]. Comparisons between cyclical PGR expressions in the fallopian tube with endometrial changes showed that levels of PGR are generally high in both tubal epithelia and endometrial glands during the follicular phase. In the luteal phase, however, PGR expression differs between these two organs [138]. The increased expression of PGR in the fallopian tubes and uterus appears to be important for the control of uterine and tubular responsiveness to E_2 in mediating early events such as cell proliferation for tissue development [80, 139, 140]. Furthermore, exogenous P_4 treatment led to a time-dependent decrease in PGR expression [80] in vivo, and upon ligand binding, the expression of PGR was rapidly down-regulated in vitro [141]. The implication of PGR in P_4-mediated reproductive processes in the fallopian tube has been demonstrated using both the PGR antagonist RU486 and E_2-induced receptor regulation [80, 100, 140].

Non-genomic action of progesterone

The fundamental characteristic of nuclear receptors is their ability to interact with DNA in response to hormone binding or other signal molecules and thus control gene expression. In addition to these genomic actions, steroid hormones exert rapid effects that happen too fast to be explained by the classical genomic mechanism. Typically these effects are: 1) rapid, taking place in seconds or minutes; 2) insensitive to translation/transcription inhibitors; 3) mimicked by steroids coupled to membrane-impermeable molecules; 4) insensitive to classical antagonists of genomic steroid action; 5) provable in isolated cell membrane fractions; and 6) demonstrable in cells or tissues lacking the nuclear steroid receptor, knockout models or in systems that are not producing or using the steroid hormone. Depending on the steroid hormone and the tissue/cell type-specific mediated response, four types of receptors have been proposed: a) transmembrane receptors that are unrelated to the corresponding nuclear receptor; b) modified nuclear receptors that localize to the plasma membrane; c) a subpopulation of the nuclear receptor that associates with various signaling complexes in the cytosol or at the plasma membrane; and d) transmembrane receptors for neurotransmitters or neuropeptides that are modulated by steroid hormones [142-145].

Progesterone receptor membrane component 1 (PGRMC1)

In an attempt to identify steroid-binding moieties that mediate effects in a rapid fashion, partial purification of liver microsomal membranes yielded two proteins with apparent molecular weights of 28 and 56 kDa, possessing P_4 binding affinity [146]. Further amino acid sequencing revealed that they are monomers and dimers of the same protein, now named *Progesterone receptor membrane component 1* (PGRMC1). Before the molecular structure of PGRMC1 was established, several homologue proteins were reported based on cellular context and function and were described in several reviews [147-150]. Furthermore, PGRMC1 has homologues in organisms that do not synthesize or bind P_4 [151], giving rise to the question of whether PGRMC1 is a functional P_4 receptor or not. The majority of studies report an intracellular localization of PGRMC1 to the ER, cytosol or nucleus [152-154], but a localization to the plasma membrane has also been reported [155, 156].

Progesterone has been proposed to be the ligand of PGRMC1, as the initially purified liver microsomal membrane fractions were shown to bind P_4 with both high (K_d 11 nM) and relatively low affinity (286 nM). The kinetics of association and dissociation of P_4 binding to the solubilized membrane fractions were rapid, with a $t_{½}$ value of 3-8 minutes. In the original report, P_4 displayed its highest affinity in a single point competition assay, but other effective competitors such as testosterone, cortisol and corticosterone bound to PGRMC1 with affinities in the same order of magnitude as P_4 did [146]. Furthermore, additional ligands including heme [157] and cholesterol [158] have been shown to bind to PGRMC1. Together, all these findings point to a limited hormonal specificity for PGRMC1. Correspondingly, a wide range of physiological roles has been proposed for PGRMC1, especially in functions mediated in association with other proteins. The protein-partner complexes that interact with PGRMC1 appear to be dependent on the cell type. The diversity of binding partners include: PAIRBP1 (formerly known as RDA288) in granulosa cells [159], SCAP and Insig-1, two proteins involved in sterol synthesis in COS-7 cells [160],

P450 proteins such as DAP1 [161] and an uncharacterized hormone/drug-binding protein [149]. The regulatory elements that control PGRMC1 expression are still unknown, but the promoter region of the human PGRMC1 gene has been shown to contain different response elements that target P_4 (via GRE), 2,3,7,8-tetrachlorodibenzodioxin (TCDD) or ligands such as omeprazol or cytochrome P450 enzymes (via AhR/Arnt) [161-163].

The multiple biological functions of PGRMC1 have been proposed based on protein associates, cell type, and level of expression. The proposed involvement of PGRMC1 in the regulation of cholesterol and steroid synthesis find support in that PGRMC1 has its highest expression in liver and cells or tissues like adrenal or granulosa and luteal cells that are highly steroidogenic. As PGRMC1 is expressed in many cell types involved in mammalian reproduction, it has been proposed that PGRMC1 may play a role in maintaining fertility. In the ovary, however, PGRMC1 is suggested to prevent the apoptosis of granulosa cell by mechanisms involving the regulation of Ca^{2+} levels and activation of kinase G-protein [164, 165]. At the same time, PGRMC1 may play a role in cancers associated with reproductive organs. Indeed, PGRMC1 is over-expressed in cancer cell lines from various tissues [166], and its increased expression is correlated with the stage of tumors in the ovary [167].

γ-aminobutyric acid type A ($GABA_A$) receptors

Steroid hormones like P_4 easily cross the blood–brain and the blood–nerve barriers, with the exception of their conjugated forms such as steroid sulfates. Many well-defined aspects of female sexual behavior in the brain are mediated by PGR in the hypothalamus and preoptic areas [168]. For example, in ovariectomized rats, treatment with P_4 induced lordosis, whereas in PGRKO mice P_4 has no such effect [169, 170].

The non-classic mechanisms of P_4 action in the brain also involve direct interactions of P_4 metabolites with the γ-aminobutyric ($GABA_A$) receptor. The native $GABA_A$ receptor is a transmembrane ion channel for which 16 different receptor subunit genes have been identified in mammals [171].

Its natural ligand is γ-aminobutyric acid, but the response can be modulated by different factors, including the reduced P_4 metabolite *Allopregnanolone (*3a-hydroxy-5a-pregnan-20-one). Circulating P_4, originating from the gonads and the adrenal cortex, is the major source of substrates for allopregnanolone synthesis in the brain [172]. Allopregnanolone acts as an agonist on the ($GABA_A$) receptor, modulating stress, mood and behavior [173]. The positive allosteric modulators of the $GABA_A$ receptor, including allopregnanolone, act by increasing the opening frequency of the ion channel. At concentrations higher than 100 nM, and thus physiologically relevant levels during pregnancy, positive neurosteroids can directly gate the $GABA_A$ receptor without the aid of γ-aminobutyric acid [174]. Negative allosteric modulators, such as *pregnanolone,* act as non-competitive antagonists at site(s) distinct from those that bind the positive neurosteroid modulators [171]. However, the focus is on finding the specific steroid-binding site on the $GABA_A$ receptor, and so far accumulated data show that steroid recognition sites reside on the receptor and not in the bilayer surrounding it [175].

Membrane Progesterone Receptors

The first strong evidence for a specific protein unrelated to PGR acting as a P_4 receptor via non-genomic mechanisms was published in 2003 [176]. The sequence of the mPR from spotted seatrout was then used to identify homologues in several species, including humans [177]. Using cDNA libraries from spotted seatrout ovaries and monoclonal antibodies, a membrane protein that bound P_4 was identified and named mPR. The putative mPRs were classified into three distinct subtypes named mPRα, β and γ [176] and they were subsequently found in a variety of animal models [147]. The mPR subfamily belongs to the seven-transmembrane progesterone and adiponectin Q receptor family (PAQR) [144].

In the human genome, there are 11 genes that encode proteins belonging to PAQR [178, 179]. The newly characterized family of proteins includes the yeast osmotin receptor (Izh2p), human adiponectin receptors (AdipoQ) and PAQR 5, PAQR7 and PAQR 8, also named mPRγ, mPRα and mPRβ respectively. Phylogenetic analysis showed that these proteins are highly conserved among vertebrates, and a subsequent classification into three distinct classes was established. Class I includes human PAQR 1-4 (where PAQR 1 and 2 are the corresponding hAdipoR1 and 2) and the yeast PAQR homologues (Izh1p and Izh2p). Class II was divided into two subgroups: one that contains mPRγ (PAQR 5) and two additional human genes named PAQR 6 and PAQR 9, so far unidentified in other species, and a second subgroup containing mPRα (PAQR 7) and mPRβ (PAQR 8). Class III contains the bacterial proteins earlier characterized as hemolysins and the human PAQR 10 and PAQR 11 [144]. From an evolutionary point of view, the PAQR genes are represented widely in eukaryotes and show a high degree of homology, particularly between humans and mice [178]. Phylogenetic analysis devided the common ancestor of the PAQR and GPCR families into two major subgroups: the hemolysin (HYL3) related proteins originating from Eubacteria to whom some PAQR belong, and the bacterierhodopsin originated from Archaebacteria which evolved to the GPCR classes. It appears like PAQR family in eukaryotes to share similarities to the prokaryotic HYL3 family, whereas proteins belonging to the HYL3 family have not been found in Archaebacteria. Thus, this suggests that PAQR and HYL3 families have same bacterial origin, in contrast to GPCR superfamily [180].

Several pieces of information suggest that proteins belonging to *Class II of PAQRs* function as G - proteins [180], but some studies failed to corroborate these findings [181, 182]. Interspecies sequence alignments and phylogenetic investigation of diverse PAQRs and GPCRs discovered a common core of seven transmembrane (TM) domains containing three unique and conserved motifs. Topological and hydropathy analysis predict an intracellular N-terminus and an extracellular C-terminus for proteins in Class I and II of the PAQR family. Furthermore, a structure with an additional eight transmembrane domains and an intracellular C-terminus has been proposed. The initially proposed mPR topology typical of GPCRs was recently reconfirmed by Thomas and co-workers, together with the involvement of the C-terminus and third intracellular loop domain in the activation of G-proteins [180]. There is now less controversy about human mPRs being bona fide steroid membrane receptors, but it is still debated whether they signal via G-proteins or not.

G-protein-coupled receptors are the largest group of membrane receptors that mediate the effects of many different hormones. The heptahelical GPCR receptor is composed of three subunits: α, β and γ. When a ligand interacts with GPCR at the cell membrane, the ligand either stabilizes or induces a conformational modification that activates the receptor. Upon activation, GDP is released, GTP binds to Gα, and the Gα-GTP complex dissociates from Gβγ and from the receptor. Both Gα-GTP and Gβγ are then free to activate downstream effects. The duration of the signal is determined by the rate of GTP hydrolysis and re-association of Gα-GDP with Gβγ. There are over 20 Gα subunits divided into four major families: $G\alpha_{q/11}$, which activates phospholipase C(PLC); $G\alpha_{s}$, which activates adenylyl cyclase (AC); $G\alpha_{i/o}$, which inhibits adenylyl cyclase; and $G\alpha_{12/13}$, which activates small GTPase [183, 184]. Furthermore, Gβγ can also mediate specific signals by interacting with several effectors such as: $PLC\beta_3$, β_3, AC, phosphoinositide 3-kinase (PI_3 kinase) or components of the MAPK cascade. It is also known that Gβγ activates K^+ and Ca^{2+} channels. At present, five different Gβ-subunits and 12 different Gγ-subunits have been identified and have been shown to be able to dimerize differently in vivo and in vitro depending on cell type or tissue specificity [185].

The three mPRs (α,β and γ) proteins vary in length from 330 to 354 amino acids, with an estimated molecular weight of around 40 kDa. The seatrout mPR (mPRα) fulfills all seven criteria listed by Zhu et. al. that, together, would argue for a role of the seatrout mPR as a true receptor [176]. These criteria/characteristics of the receptor are: 1) a seven-transmembrane domain, GPCR-like structure, 2) a dominant abundance of mPR mRNA in reproductive and endocrine tissues; 3) a localization at the plasma membrane of both oocyte and sperm; 4) high affinity (K_d 30 nM), low capacity (B_{max} = 0.49 nM), rapid and displaceable binding for P_4 with rates of association and dissociation of $t_{½}$ 7- 2 min when expressed in bacterial (*E. coli)* systems; 5) activation of pertussis–sensitive inhibitory G-protein with subsequent decrease in cAMP and adenylyl cyclase activity upon progestin binding to the mPR; 6) in vivo and in vitro progestin and gonadotropin up-regulation of both the seatrout mPR gene and protein; and 7) implication of mPRα in the oocyte maturation process, as confirmed both by changes in receptor levels during oocyte development and by inhibition of oocyte maturation upon microinjection of antisense nucleotides [176]. Initially, the proteins were expressed in an *E. coli* system that does not contain any known P_4 receptors. Later, culture of human breast cancer cells (MDA-MB-231 cells) was used instead, resulting in the expression of proteins in the plasma membrane and binding of both synthetic progestins and P_4 [176, 180]. Recombinant plasma membrane proteins from MDA-MB-231 cells transfected with the seatrout cDNA reconfirmed the characteristics of the seatrout mPR and its high affinity (K_d 4-8 nM), limited capacity (B_{max} = 0.03-0.3 nM) and rapid association and dissociation rate of progestin binding, with $t_{½}$ 2-5 min) [180]. Characterization of the mPRβ and γ proteins is still under investigation, but information available at present suggests that they present features similar to their protein family sibling mPRα. Saturation analysis of P_4 binding to the recombinant mouse mPRβ and human mPRγ in an *E. coli* expression system presented a single high affinity (K_d = 28-39 nM) and saturable P_4 binding (B_{max} = 03-0.5 nM). Competition binding of steroids to hmPRγ demonstrated that P_4 is the specific ligand, whereas other steroids such as E_2, androgen and corticosteroid had low or no affinity for hmPRγ [177]. Characterization of the Xenopus mPRβ in

CHO cells presented a high-affinity binding site with K_d = 200 nM, which is near the half-maximal effective concentration for induction of oocyte maturation by P_4 [186].

Sequence comparisons of the seatrout mPRα and β showed that these mPRs share a high degree of homology (49-60%) across species. The mPRγ form cloned from a kidney library, however, is more divergent and has less sequence homology (30%) with mPRα and mPRβ [177, 187]. Molecular characterization of the mPRs in channel catfish (*Ictalurus punctatus*) and other fish and mammalian forms showed a high degree of homology [187]. Characterization of the rat mPRs showed gene homology ranging from 27% to 49% between the mPRs [188]. Transmembrane and lipophilicity analysis identified seven potential transmembrane domains in many species except for zebrafish mPRβ, for which the analysis predicted five transmembrane domains [188]. The putative transcriptional regulatory elements located in the 5´-flanking region of the channel catfish mPR revealed the presence of several candidate regulatory elements for transcription factors, including CRE, SF-1, various steroid hormone regulatory elements (ERE and GRE/PRE/ARE) and elements involved in the control of cell growth and differentiation (Egfr, AhR/Arnt) [187].

Although some studies have failed to confirm the cellular localization of the mPRs at the plasma membrane as well as a function as a membrane P_4 receptor [189], a recent study by Smith et al. strongly supported the ability of the human PAQR (mPRs) to sense and respond to P_4. This study, however, did not provide evidence that the mPRs signal via G-proteins. The authors used the so-called yeast PAQR system that is equipped with two powerful tools: 1) a system that is devoid of the known P_4 binding/sensing proteins; and 2) a system with an intact signaling apparatus for monitoring signal transduction upon P_4 stimulation [144]. However, there are data supporting the notion that the mPRs are located at the plasma membrane and function by activating G-proteins [180], but that G-protein activation by other PAQR family members such as adiponectin receptors does not occur [179]. In contrast to PAQRs, both hmPRα and smPRα were demonstrated to possess an extracellular N-terminal domain and an intracellular C-domain that is involved together with the third intracellular loop in the activation of G-proteins [180]. Phylogenetic analysis showed a convergent ancestral origin for PAQR from HYL3 proteins found in *Eubacteria* and GPCR families from Bacteriorhodopsin found in *Archaebacteria*. Thus, this might explain the structural and functional GPCR-like characteristics stipulated for members of the PAQR family [180]. Since the initial report by Zhu et al. in 2003, the characteristics and possible functions of the mPRs have been investigated in several species, tissues and cell systems. These reports demonstrate gene expression in both reproductive and non-reproductive tissues (Table 1). The expression and cellular localization to the plasma membrane of the mPR proteins has been broadly demonstrated in many species and cell systems using different techniques and approaches, suggesting that they might be involved in diverse physiological functions in vertebrates (Table 2). The expression of the mPRs, however, varies among species and tissues and in relation to the reproductive cycle.

Table 1: Summary of published data characterizing *mPRs mRNA* level expression

SPECIES		mPRα	mPRβ	mPRγ
Spotted Seatrout	RT-PCR	Sperm [190]	ND	ND
	Northern blot	Ovaries, testes, brain, pituitary [176]	Brain, neuronal tissues [176]	
Zebrafish	RT-PCR	testes, ovaries [191]	Brain, pituitary, ovaries, testes [187, 191], liver, kidneys [192]	ND
Channel catfish	RT-PCR	Brain, pituitary, muscle, testes, aorta, gills, intestine, heart, spleen, liver, kidneys, ovaries [187]	Hypothalamus, ovary, pituitary, testes [187]	Intestine, gills, aorta, kidneys, ovaries, hypothalamus, testes [187]
Goldfish	RT-PCR	ovaries [193]	ND	ND
Xenopus laevis	RT-PCR	ND	Oocyte [186]	ND
Rat	RT-PCR	Corpus luteum, ovaries, adrenal, brain, kidneys, lungs [188]	Brain, ovaries, kidneys, lungs, heart, CL [188]	Kidneys, brain, CL [188]
Mouse	RT-PCR	Brain, testes, uterus [194]	POAH, pituitary, uterus, kidneys, Imm. neuronal cells [195] fallopian tubes, ovaries [196]	Kidneys [197], fallopian tubes, ovaries [196]
Sheep	RT-PCR	Hypothalamus, uterus, pituitary, CL [198]	ND	ND
Human	RT-PCR	Sperm, testes [199], myometrium [181, 200, 201]; lymphocytes [202]; breast tissues, placenta, amnion [181, 203]	T-lymphocytes, Jurkat cells, brain [186, 202]; myometrium, myometrial cells [181]; ovarian tumors [204]	Endometrium [181], ovarian tumors [204], fallopian tubes [205], HeLa cells [202]
	Northern blot	Ovaries, testes, brain, placenta, uterus, kidneys, adrenal [177]	ND	ND

ND, not determined; RT-PCR, real-time polymerase chain reaction; POAH, preoptic anterior hypothalamus; CL, corpus luteum

The expression and cellular localization of the mPRα protein has been broadly demonstrated in many species and cell systems, using different techniques and approaches. The expression and cellular localization has been demonstrated for mPRβ in several species and cell systems as well; however, less information is available for mPRγ. The table below summarizes the existing data regarding the expression and cellular localization of mPR proteins detected by different methods and in different species (Table 2).

Table 2: Summary of published data characterizing the expression and localization of *mPR proteins*

SPECIES		mPRα	mPRβ	mPRγ
Spotted Seatrout	WB	Sperm[a] (40+80 kDa) [190]; oocyte[a] (40 kDa) ovaries[a] [176]	ND	ND
	IHC	Sperm (PM-Flagella, head) [190]; oocyte (PM) [176]	ND	ND
Zebrafish	WB	Ovaries[a] (40 kDa) [206]	Ovaries, oocyte[a] (40 kDa) [206]	ND
	IF; SB	Oocyte (PM) [206]	ND	ND
Atlantic Croaker	WB	Sperm [a] ((80 kDa) [192]; sperm, testes[a] (40+80 kDa) [207]	ND	ND
	IHC	Sperm (PM mid piece flagella) [207, 208]	ND	ND
Goldfish	WB	Ovaries[a] (40 kDa) [193]	ND	ND
Xenopus laevis	WB	ND	Oocyte[a] (60 kDa; ortholog) [186]	ND
Red drum; Southern Flounder	WB	Sperm (80 kDa) [192]	ND	ND
Rat	WB	ND	Corpus luteum (microsomal fraction, 40 kDa) [188]	ND
Mouse	WB	Testes[a] (40+80 kDa) [199]; Brain, testes, uterus[a] (40 kDa) [194]	Brain, testes, uterus[a] (40 kDa) [194] Imm. neuronal cells[a] (40 + 80 kDa) [195]; fallopian tubes[a] (40 kDa) [196]	Lungs, liver, ovaries, testes fallopian tubes[a] (40 kDa) [205]
	IF	ND	Gonadotrophs PM; [195]	ND
	IHC	ND	PM-fallopian tube cilia [196]	Apical PM (ciliated cells; [205]
Human	WB	Sperm[a] (40+80 kDa) [199]; lymphocytes[a] [202]	Lymphocytes [a] (80 kDa) [202]; myometrial cells[a] (40+80 kDa) [201]; fallopian tubes[a] (40 kDa) [196]	Fallopian tubes[a] (40 kDa) [205]
	IF	Myometrium (PM muscle cell; [201]	Myometrial cell (PM; [201]	ND
	IHC	Sperm (Midpiece head and flagella) [199]	Fallopian tubes (PM cilia); [196], ovarian tumors (cytoplasmatic/nuclear) [204]	Fallopian tubes (PM ciliated cell) [205]
Transfected Cell system	WB	MDA-MB-231[a] (40 + 80 kDa) [180]; MDA-MB-231[a] (45 kDa tag) [206] *E. coli* [a] (45 kDa+tag) [193]	MDA-MB-231[a] (40 + 80 kDa) [201]; HEK 293 [a] (30 kDa) [182]	HEK 293[a] (30 kDa) [182]
	FC/ IHC/IF	MDA-MB-231 (N-term extracell./ C-term intracell) [180, 206] COS-1 (N-C-term. Intracell) [182]	COS-1 cells (ER) [182]	COS-1 cells (ER) [182]
	ELISA	MDA-MB-231 (N-term intracell.) [182]		

ND, not determined; [a], plasma protein preparation; ER, endoplasmatic reticulum; PM, plasma membrane; WB, western blot; SB, surface biotinylation; IHC, immunohistochemistry; IF, immunofluorescence; FC, flow cytometry; ELISA, enzyme-linked immunosorbent assay

Physiological functions of mPRα

Induction of oocyte maturation

The localization of mPRα at the plasma membrane of the oocyte is consistent among all investigated species [147]. Functional studies demonstrate that the receptor is involved in mediating oocyte maturation [190]. In fish and amphibian oocytes, a maturation-inducing steroid (MIS) is secreted from follicles upon gonadotropin stimulation; it acts on its own plasma membrane-located MIS-receptor and induces the resumption of meiosis followed by oocyte maturation. Two MISs have been identified in fish species: 17,20β,21-trihydroxy-4-pregnen-3-one (20β-S) and 17, 20b-dihydroxy-4-pregnen-3-one (17,20βDHP) [193, 209]. In spotted seatrout, the binding affinity of 20β-S to the MIS-receptor is higher than that of P_4 [210], whereas in other fish species, 17,20βDHP is the stronger MIS [211]. The abundance of the receptor protein is related to the hormone levels within the reproductive cycle. The concentration of mPRα in Xenopus oocytes was increased by hCG [212], and an in vitro study showed that there is a correlation between oocyte receptor abundance and steroid binding capacity [210]. The levels of MIS increased in female fish in response to the periovulatory LH-surge [213]. Furthermore, the increased in vivo ovarian receptor concentration correlates with high plasma levels of LH in spotted seatrout collected in the spawning season [214] and mPRα is up-regulated upon gonadotropin treatment in vitro [210, 215, 216]. Several lines of evidence support the notion that induced oocyte maturation by MIS is mediated via a rapid, non-genomic mechanism and specifically by the mPRα receptor [180] (Fig. 6). A particularly important finding was that microinjection of antisense nucleotides to the mPRα mRNA blocks the MIS-induced maturation of zebrafish oocytes [176]. The signal transduction pathway involves the activation of G-coupled proteins and is summarized in Fig. 6.

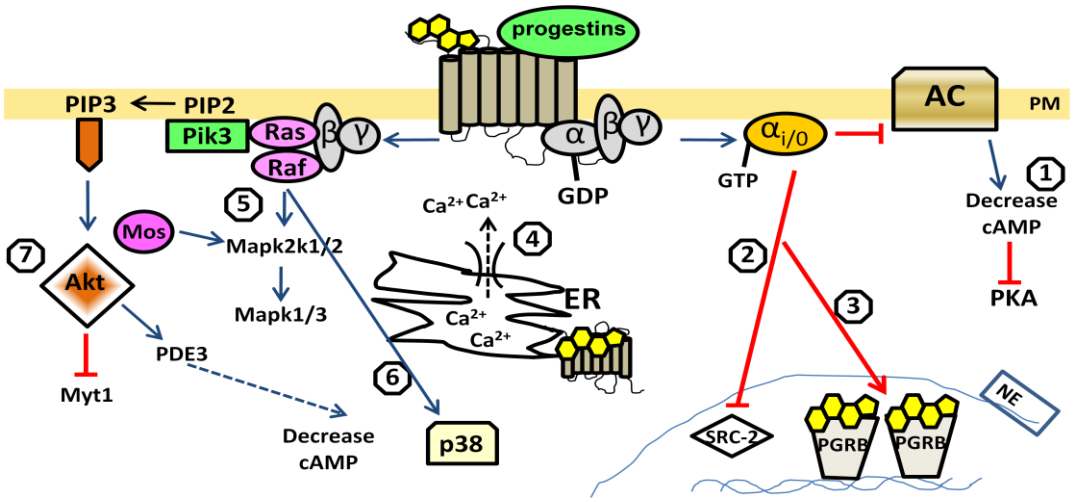

FIGURE 6: Summary of alternative signal transduction pathways activated in response to mPRα or mPRβ activation by P_4/progestins in different species. Pathways 1-7 are mediated through activation of the inhibitory G_i-protein. Pathway 1): decrease of cAMP levels (PTX-sensitive) in spotted seatrout oocytes during maturation [209], in MDA-MB-231 cells transfected with mPRα [176, 180], in human myocytes with mPRα and mPRβ [201], in Jurkat cells [202] and in GT1-7 cells [195]. Pathway 2): down-regulation of SRC-2 co-activator expression through mPRα in human myocytes [201]. Pathway 3): Activation of PGRB through mPRα and mPRβ in human myocytes [201]. Pathway 4): Increased intracellular Ca^{2+} levels through activation of ovine mPRα in ER [198]. Pathway 5): increased MAPK phosphorylation upon Mos activation in Atlantic croaker follicles [209], in MDA-MB-231 cells transfected with mPRα [176] and in zebrafish mPRα and mPRβ [206]; proposed signaling mechanism via mPRβ that controls resumption of meiosis in Xenopus oocyte maturation [186]. Pathway 6): activation of p38 resulting in phosphorylation of myosin light chain in human myocytes [201]. Pathway 7): activation of Akt via up-regulation of phosphodiesterase activity in Atlantic croaker oocytes during final maturation [209]. PM, plasma membrane; NE, nuclear envelope; ER, endoplasmic reticulum.

Stimulation of sperm motility

There is a plethora of studies demonstrating rapid, non-genomic effects of P_4 on the activation of spermatozoa of many species have been reported. Progesterone has been shown to induce the acrosomal reaction in human and stallion sperm [217, 218], a phenomenon that does not occur in fish, as they lack an acrosome [219]. Progesterone also stimulates hypermotility in mammalian sperm [220] as well as fish sperm [221, 222]. Furthermore, P_4 present in the egg microenvironment by the time of ovulation is a chemoattractant for mammalian spermatozoa [223]. Human spermatozoa chemotactically responded to a P_4 concentration gradient ranging from 1 to 100 pM [224]. Thus, the possibility of existing specific P_4 receptors and/or different receptors that independently mediate the effects of P_4 on the acrosomal reaction and sperm hypermotility exists. Indeed, a variety of receptors have been proposed as potential mediators of these effects [217, 225, 226]. The expression and localization of mPRα protein to the plasma membrane of sperm has been demonstrated in several species (see Table 2). Furthermore, the abundance of the mPRα protein was related to the extent of sperm motility in both fish and humans [208]. The fish

progestin (20β-S) possesses binding characteristics in spotted seatrout and croaker mPRα that are similar to those of recombined seatrout mPRα. These characteristics include a high affinity (K_d 20 nM) and low capacity (B_{max} 0.08 nM) [180, 207].

Several studies report the mPR activation of multiple classes of G-proteins, but only one study to date has demonstrated an activation of the G_{olf} through mPRα in fish sperm (Fig. 7). The increased cAMP levels resulting from the activation of membrane adenylyl cyclase (AC) was completely blocked by specific inhibitors of AC and the mPRα co-immunoprecipitated with the G_{olf} antibody [208]. The nuclear progestin receptor antagonist R5020 did not affect hypermotility in croaker sperm and did not activate the G_{olf} receptor, meaning that the genomic mechanisms are not involved in this process. Various members of the mACs and G_{olf} protein families are present in the germ cells of humans, rats and mice, and disruption of mAC resulted in reduced sperm motility and function in mice [227-230]. Furthermore, the associated G_{olf} and mAC signal transduction has been proposed to mediate sperm chemotaxis in response to odorants in mammals [231]. Under physiological conditions, calcium may activate phospholipases and modulate enzymes that interact with AC to increase cAMP; it is thus an important ion in the regulation of sperm motility [232]. However, the molecular mechanism of calcium-activated sperm motility is still not fully elucidated. Because the activation of the G_{olf} protein through mPRα has been reported only in fish sperm, further studies are necessary to elucidate whether this is a process occurring in other tissues or species. Interestingly, $G_{αi2}$ is expressed in the human endometrium and the ciliated cells of fallopian tubes [233, 234].

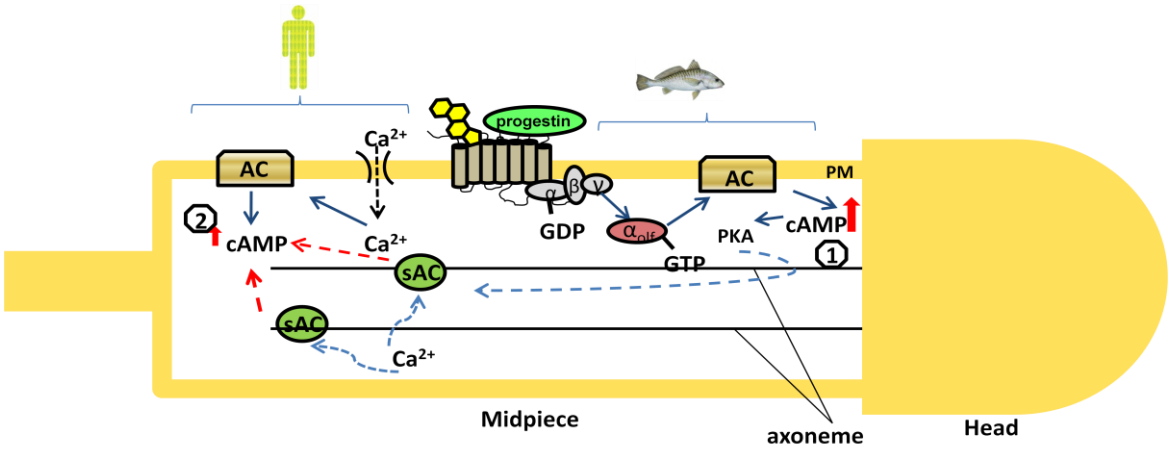

FIGURE 7: Summary of the signal transduction pathway in fish and human sperm upon progestin/P_4 stimulation of mPRα. Pathway 1): Increased cAMP levels in Atlantic croaker sperm by activation of a PTX-insensitive stimulatory G-protein (olfactory-G protein) and increased intracellular PKA [208]; Soluble adenylyl cyclase (sAC) in the axoneme can be activated to increase sperm motility [235]. Pathway 2): Increased Ca^{2+} levels interact with different ACs to increase motility; increased PKA can also diffuse to axonemal sAC and activate increased cAMP concentrations [223, 235].

Effects in human myometrium and T-lymphocytes

During pregnancy, the levels of P_4 produced by the placenta gradually increase in order to relax the myometrium; at labor, uniquely in humans and other primates, the levels of P_4 drop dramatically [236]. These effects of P_4 are thought to be mediated by PGR [237]. In 2006, however, Karteris and co-workers proposed a model for P_4-mediated actions in the human myometrium also involving the action of the mPRs [201] (Fig. 6). This report supported the possibility of P_4 initiation of myometrial contraction mediated via mPR through three mechanisms: 1) decreased PGRB transactivational activity by changes in the PGRB/PGRA ratio and SRC2 down-regulation; 2) G-protein-mediated inhibition of AC; and 3) G-protein activation of p38 and activation of MLC phosphorylation. This possible cross-talk mechanism between mPRs and the PGRs has been elegantly demonstrated in human pregnant myometria, in myometrial cell culture and in myocytes transfected with siRNA for mPRs. Expression of both the mPRα and mPRβ genes and proteins has been demonstrated, and the receptors exclusively coupled with the inhibitory G-protein demonstrated by pertussis toxin treatment to activate AC and decrease cAMP, which, together with activated p38 MAPK, increase the phosphorylation of MLC. In addition, the elevated levels of mPRs at term, upon P_4 stimulation, activate G_i-protein, which in turn initiates PGRB transactivation via a specific steroid receptor coactivator (SRC2). This dual action of P_4, however, is shared with the effect of P_4 on the PGRB/PGRA ratio because P_4 easily passed the plasma membrane and pertussis toxin only partially blocked the response to P_4. There is a shift in the PGRB and PGRA ratio during pregnancy and at term; during pregnancy, PGRA cannot act as a repressor of PGRB transactivation because its levels are decreased, whereas it does so at term when its expression increases. Thus, the transactivation of PGRB by mPR during pregnancy causes relaxation in the myometrium, whereas at term mPR cannot activate PGRB due to a changed PGR balance, and the contractile state begins as a result.

The immunomodulatory role of P_4 during pregnancy and during different phases of the menstrual cycle has been suggested before. The cellular immune response in the luteal phase of the menstrual cycle and pregnancy increases due to an increased risk of infections. The T-cell lymphocytes play an important and integral role in the body's defenses and CD^8 (cluster and differentiation 8) is a co-receptor for the T-cell receptor. The CD^8 antigen fulfills a cytotoxic effect, as it is capable of killing cells that are infected, damaged of dysfunctional. A protective role of CD^8 T lymphocytes in preventing abortion has been reported in mice [238]. Investigation of potential P_4 receptor mediators failed to detect the involvement of PGR [239]. Alternative investigations clearly demonstrated by several techniques the presence of both mPRα and mPRβ in the plasma membranes of human T-lymphocytes and Jurkat cells [202]. The characteristics of P_4 binding to mPR in Jurkat cells were similar to those of the recombined mPRα in breast cancer cells (K_d of 4.6 nM) [180]. Progesterone activation of G-protein was concentration-dependent, and additional immunoprecipitation experiments demonstrated the involvement of the $G_{\alpha i}$ subunit (Fig. 6). Treatment with R5020 and dexamethasone did not activate the G-protein signal transduction, thus demonstrating that PGR is not involved. However, this finding is not surprising because PGR was not detected in these cells. Importantly, because P_4 activation of mPR does occur in T

lymphocytes, future studies must elucidate the functional implication of this activation for the human immune system.

Effects of progesterone on GnRH release

Progesterone feeds back on the hypothalamic-pituitary axis to control GnRH and gonadotropin release. This has been considered to be mediated by binding of P_4 to its cognate receptor PGR in the hypothalamus and pituitary [240-243]. However, studies of PGRKO clearly demonstrated that P_4 inhibits the release of GnRH by a mechanism independent of PGR. The presence of mPRs at both gene and protein levels has been demonstrated in mice gonadotrophs, and the saturation binding analyses showed high affinity binding (K_d 5.85 nM) and a limited capacity (B_{max} 62.2 nM) binding for P_4. The signal transduction mechanism mediated by the P_4 activation of mPRα involved coupling to the inhibitory G_i-protein and decreased cAMP accumulation [195] (Fig. 6). In addition, the mPRα mRNA and protein were present in fish brains [147, 176], and a rapid negative feedback effect of 20β-S on GnRH secretion at the beginning of the reproductive cycle has been detected in the preoptic anterior hypothalamus (POAH) in Atlantic croakers [221]. However, several studies must be conducted to elucidate the mPR-mediated physiological actions of P_4 in the brain.

Physiological functions of mPRβ

Induction of oocyte maturation

A classic example of rapid non-genomic effects of steroids is the induction of oocyte maturation. Transcripts for mPRβ have been found in the membrane of the small Xenopus oocyte fraction that is unable to undergo germinal vesicle breakdown (GVBD) in response to P_4. The mPRα, which otherwise is involved in oocyte maturation in other species, was not present in Xenopus oocytes. Attempts to clone the Xenopus mPRα had failed, suggesting that in this species this physiological process is attributed exclusively to mPRβ [186] (Fig. 6). The expression of this receptor protein is increased by gonadotropins [212], although at this developmental stage (stage IV), the quantity of receptors is lower than in other oocytes upon P_4 treatment. Changes in receptor abundance are, however, consistent with the role for the receptor in P_4-induced oocyte maturation [186]. Interestingly, in other species, although hCG induced oocyte maturation, mPRβ was not affected by hCG and varied only slightly during the reproductive cycle [191]. Supporting evidence for the role of mPRβ in induced oocyte maturation has been demonstrated by studies in zebrafish and pigs [206, 244].

Functional effects of progesterone in the human myometrium and T-lymphocytes

In human myometrial cells, mPRα and mPRβ are expressed and differentially regulated by ovarian hormones. The expression of the mPRα gene was up-regulated by both E_2 and P_4, with a greater regulation by P_4 after 4 h. The PRβ gene was solely up-regulated by E_2 after 8 h of treatment. However, this differential regulation by P_4 suggests different functions for mPRα and β in the myometrium [201]. During pregnancy, the levels of P_4 gradually increase, causing relaxation of

the muscle layer, whereas at term, the levels of P_4 drop dramatically to initiate contractile activity. In rats, the expression of the mPRβ gene remained constant during gestation [188], whereas the onset of parturition at term coincided with a drop in mPRβ expression in humans [181]. Thus, a central role in the regulation of luteal functions has been attributed to mPRα. However, it has been shown that mPRβ is associated with $G_{\alpha i}$-protein and dissociates upon P_4 treatment. The signaling cascades activated by the $G_{\alpha i}$ protein appear to occur due to differential contributions of the mPRs present in myometrial tissue (Fig. 6). However, the exact role of mPRβ in mediating the effects of P_4 during pregnancy and at labor remains to be elucidated. Several studies must be conducted in order to explore this question in more depth.

An immunomodulatory role of P_4 has been previously demonstrated, but the mechanisms of action are still unclear. The presence of PGRs or alternative receptors to PGR has been suggested to be responsible for mediating such effects, but this has been a matter of dispute, since PGR is absent in lymphocytes [245]. However, mPRβ is present in T-lymphocytes at both the gene and the protein level. The high binding affinity (K_d 4.25 nM) of P_4 to the plasma membrane in Jurkat cells has been demonstrated and the activation of $G_{\alpha i}$-protein was not affected by RU486 or glucocorticoid receptor antagonist (Fig. 6). Of note, RU486 acted both as a strong antagonist to PGR and as a weak antagonist to GR. The receptor protein in the plasma membrane of Jurkat cells binds P_4 with high affinity [202]. The presence of mPRβ and mPRα in T-lymphocytes has been demonstrated, and the immunomodulatory role of P_4 has been mainly attributed to mPRα, due to its changes in expression during pregnancy and the menstrual cycle. However, the function of mPRβ in mediating the immunomodulatory functions of P_4 is still under investigation.

Effects of progesterone on GnRH release

Ovarian hormones are important regulators of gonadotropin release, acting on both the hypothalamic and pituitary axes. Considering the P_4-induced regulatory effects on LH secretion observed in experiments with PGRKO mice, in vitro and in vivo studies have demonstrated that mPRs are present in POAH in several species [195, 221]. Localization of mPRβ, along with that of mPRα, on the plasma membrane has been confirmed by Western blot and IHC. Furthermore, treatment with P_4, but not allopregnanolone (a P_4 metabolite acting on $GABA_A$ receptors) suppressed LH secretion in vivo independently of PGRs. The suppression of LH secretion was affected by the decreased GnRH secretion and not by reduced pituitary responsiveness to GnRH. The rapid inhibition of cAMP accumulation by the activation of G_i protein coupled to mPRs was specifically mediated by mPRα (Fig. 6). From experiments with mPRβ siRNA, it was suggested that mPRβ contributes to the total binding of P_4 in GT1-7 cells, but it does not mediate rapid effects on cAMP accumulation [195]. However, another signaling pathway might be considered.

Physiological functions of mPRγ

There are limited studies on the possible functions of mPRγ. Similar to the other two mPRs, specific P_4 binding characteristics of the human mPRγ have been demonstrated [176, 177]. Due to its high levels of expression in male and female kidneys, it was speculated that mPRγ might have effects on renal function. However, other studies showed the transcript for mPRγ also to be expressed in several reproductive tissues [181, 188, 201]. Saturation analysis of P_4 binding to the

recombinant human mPRγ in an *E. coli* expression system presented a single high affinity (K_d = 35 nM) and saturable P_4 binding (B_{max} = 0.5 nM). Furthermore, no homologies to the ligand binding domain of PGR were found in any region of the mPRs. Competition binding of steroids to hmPRγ demonstrated no or low affinity for binding other steroids such as E_2, androgens and corticosteroids, whereas P_4 was the specific ligand for hmPRγ [177]. The work of this thesis has led to new proposed roles for mPRγ and (mPRβ) (see results and discussion section).

Potential role of progesterone and mPRs in tubal transport

Both early and late effects of P_4 in regulating the CBF of ciliated cells from the fallopian tube have been reported in vitro/ex vivo [107, 234]. Rapid effects of P_4 had only been reported previously in one study (cow), and the rapid effect of P_4 could not be blocked by the nuclear receptor antagonist RU486, suggesting an involvement of mechanisms independent of PGR [107]. The recently discovered mPRs are present in spermatozoa and are implicated in regulating flagellar movement [207, 221]. In general, there is a well-known association between increased CBF of cilia in response to increased cAMP and Ca^{2+}. Such an example is the Ca^{2+}-stimulated CBF in airway cilia [246] and the progestin-mediated stimulation of sperm hypermotility [147]. The major classical sources of cAMP are the regulated isoforms of G-protein-sensitive transmembrane adenylyl cyclases (ACs). Indeed, increased AC activity has been reported upon G-protein activation of progestins [147]. However, a soluble distinct AC (sAC) reported many years ago in rat testes [247] was found in the axoneme itself and has been suggested to contribute to CBF regulation [248]. In cells transfected to overexpress mPR, intracellular Ca^{2+} increased upon stimulation of ER-localized mPR [198]. From the literature, the general effects of P_4 on the plasma membrane mPRs appear to involve activation of the G_i protein. Moreover, the $G_{\alpha i}$ protein-mediating immunoregulatory role of P_4 [202] was strengthened by studies in $G_{\alpha i}$ knockout mice [249]. $G_{\alpha i}$ was found in the motile cilia of rat, human and mouse fallopian tubes [233, 250]. Indeed, silencing the function of $G_{\alpha i}$ protein in the CNS of rats resulted in ciliostasis [233, 234]. In contrast to phospholipase A (PKA), which gives rise to increased CBF when phosphorylated, an effect consistent in all mammalian cilia, protein kinase C (PKC) has been implicated in slowing ciliary activity [235]. A ciliary membrane PKC phosphorylation target, p37, was found to be associated with the ciliary membrane in ovine cilia [251]. Even if the regulation of CBF has been studied in many systems, the greatest amount of information has come from studies on airway cilia. The structure and function of mammalian respiratory and fallopian tube cilia is similar. Whether equivalent signaling mechanisms are involved in the fallopian tube ciliary cells as in the lungs remains to be elucidated. Although the exact mechanism of P_4 signaling in tubal cells is not established yet, existing information may be sufficient to propose putative pathways that might control ciliary beat frequency.

AIMS OF THE THESIS

An overall aim of the thesis was to identify the distribution and regulation of mPRβ and mPRγ in different tissues, with a focus on the fallopian tube. Another main focus was to assess whether P_4 rapidly affects CBF in the mouse fallopian tube, thus implicating an involvement in the regulation of gamete transport.

Specific aims of papers I-III

1. To investigate the tissue and cellular distribution of mPRβ and γ in female and male mice (Papers I and II)
2. To characterize the hormonal regulation of mPRβ and γ in fallopian tubes of mice and humans (Paper II)
3. To examine whether progesterone rapidly regulates ciliary beat frequency in mouse fallopian tubes (Paper III)

METHODOLOGICAL CONSIDERATIONS

Detailed descriptions of the methods that have been used in this thesis are given in each paper and references therein. In this section, additional considerations of some methods are discussed.

MODELS

Mouse model

The animal experiments included in this thesis were performed in C57BL/6 mice (*Mus musculus*). Mice have most genes and gene products in common with humans, making them a very useful model for studying fundamental physiological functions as well as aspects of human disease. Among the general advantages of using mouse models is their small size, which makes them easy to house, the abundance of data in the literature and the availability of genetically modified strains. With regards to the reproductive system, similarities as well as differences exist between mice and humans. Despite the anatomical differences (Fig. 2) the hormonal regulation of transport and the duration of tubal egg transport are similar in mice and humans compared to other species [9].

The periovulatory interval in rodents is shorter compared to that in humans [252], but considering the non-pregnant mouse model, the estrous cycle in mice and the menstrual cycle in humans are comparable regarding the hormonal profile [253]. Mice are versatile models that allow precise hormonal manipulation. However, there are examples in which in vivo responses to hormonal stimuli appears to be shorter and in some cases even opposite in mice compared to humans [253].

Immature mouse model (Papers I, II, and III)

In Papers I, II and III, our choice of model for studying the fallopian tube was the immature mouse model (26 days old; 13-15 g). As endogenous steroid levels are low in these mice [80, 254], it is comparatively easy to interpret effects of added steroids. In studies of the effects of steroid hormones on steroid-responsive tissues, immature mice have, in some cases, been shown to be more sensitive than ovariectomized adult animals [253]. The immature model also allows study of the reproductive tissues without involvement of endogenous gonadotropins. By using immature rather than cycling mice, it is also easier to obtain animals that are at a similar developmental stage.

Gonadotropin-induced mouse model (Papers I and II)

To induce folliculogenesis, immature mice were injected with equine chorionic gonadotropins (eCG, 5 IU, i.p.), also known as Pregnant Mare Serum Gonadotropin or PMSG (Fig. 3). This horse protein, which corresponds to human chorionic gonadotropin, is a glycoprotein with both FSH and LH-like activity and has a long half-life in vivo [255, 256]. Treatment of immature mice with eCG results in follicular growth and preovulatory maturation within 48 h.

To induce synchronized ovulation and luteinization in mice, additional hormonal treatment with hCG (5 IU hCG, i.p.) is necessary (Fig. 3). The action of hCG in vivo is more extended compare to

that of LH, partially due to a higher affinity for LHR [257]. In our animal model, ovulation occurs approximately 12-14 h after hCG treatment [258]. A characterization of the eCG and/or hCG mouse models has been performed in our laboratory [80]. The ovarian weight of immature female mice increased upon eCG treatment over 48 hours, mimicking the preovulatory phase of a reproductive cycle. Subsequent treatment with hCG (6 to 48 hours) mimicked the period preceding ovulation. The serum levels of E_2 and P_4 in immature mice treated with gonadotropins is similar to that in adult cycling mice [81].

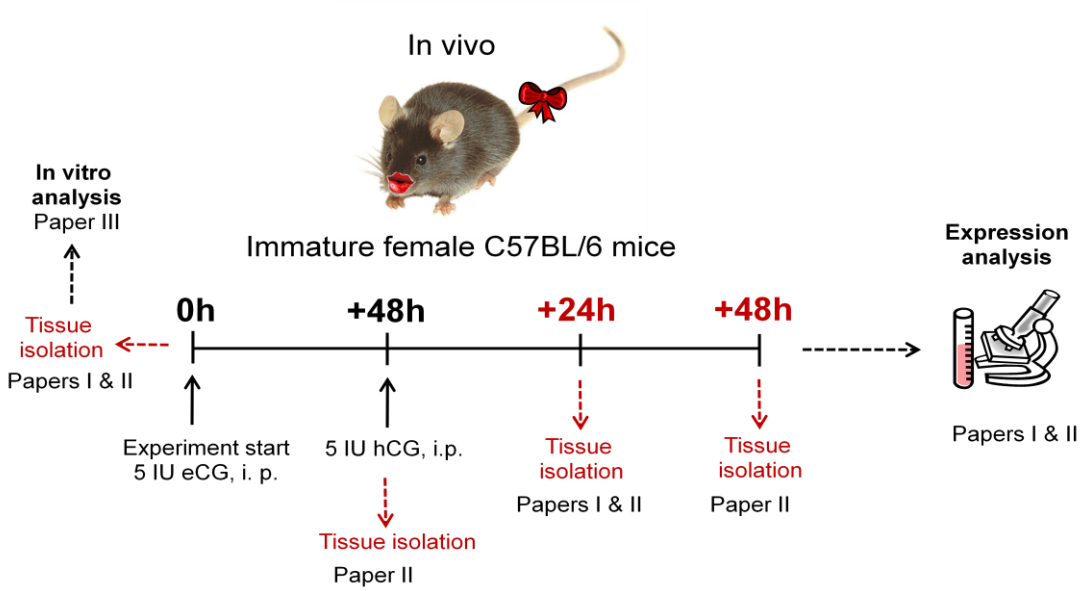

FIGURE 8: Schematic representation showing the animal model systems used throughout the thesis. eCG, equine chorionic gonadotropin; i.p., intraperitoneal injection; hCG, human chorionic gonadotropin

Human model (Papers I and II)

In Papers I and II, we used fallopian tube tissues from fertile women aged 28-42 years undergoing tubal ligation at Sahlgrenska University Hospital. Tubal ligation is used as a contraceptive method worldwide, and it is done with the intention of permanent female sterilization. Still, through microsurgery, the fallopian tubes can be repaired afterwards if required, and fertility is restored in 98% of women. Before the surgical procedure to collect samples for this study, all patients received a clinical examination and had a regular menstrual cycle. Accordingly, the tissue samples were classified in three groups based on the menstrual cycle: follicular, periovulatory and luteal phase. The patients had not used any hormonal drugs within three months before the surgery.

Isolation of tubal cells (Paper III)

In paper III, we aimed to study ciliary activity in vitro. The in vitro study of tubal activity has advantages as well as disadvantages. One advantage that the in vitro experiments have over the in vivo study is that they allow a very controlled exposure to hormones or other substances in the cells of interest without interference from other organs or systemic factors. In vitro studies also have disadvantages. For example, the tissue may not behave in the same way in vitro as it does in

vivo because of the stress exerted by taking out the tissue and because it is difficult if not impossible to perfectly mimic in vivo conditions.

In order to study ciliary activity in the fallopian tube epithelium in vitro, we first attempted to apply the method used by Wessel et al. (2004) on cow fallopian tubes [107]. The dissection principle adopted by Wessel was rather simple: the fallopian tube was opened longitudinally, and the pieces were attached in a gel-like material with the cell surface upward for further activity measurements. Even though the procedure appeared simple, it was one of the most challenging components in this thesis. One of the obstacles in sample preparation was the tiny size and the curled shape of the mouse fallopian tube. Although the connective tissues that keep the fallopian tube coiled were carefully removed, there is an inherent tendency of the tissue to return to its original shape. We tried to fasten the tissue, but it broke because of its fragility. Furthermore, the need to keep the tissue in a proper medium to prevent dehydration and to mimic the in vivo environment made the handling of the tissue sample even more difficult. In order to overcome the difficulties we came across during tissue preparation, we tested several methods.

We tried to straighten and fasten the tissue by inserting tubing with a small diameter into the fallopian tube. Next, we tried to cut open the tube-like shape with a scope to unfold it into a rectangular structure. As a result, the end of the pipe got trapped in the tube's curvatures and perforated its wall as the tube returned to its initial form. Since the straightened fallopian tube was difficult to handle in its full length, we cut the fallopian tube into smaller pieces and tried to apply the previous strategy to each individual segment. The insertion of tubing was, however, too difficult due to the small size of the tubal parts. We also pre-coated petri dishes with different substances (poly-L-lysine, collagen) to help pieces of tissue or cell clusters attach during incubation, but this did not help either. Next, we scratched out cells or small cell clusters from inside the tube; we observed, however, that this mechanical procedure damaged the cells. The approach finally selected was to chop the tissue to obtain clusters of ciliated cells. During dissection, the tissues were maintained and handled in a 37°C pre-warmed medium specially developed for the management and manipulation of oocytes and embryos in vitro. Lastly, keeping a clean medium that would not perturb the ciliary activity measurements proved to be a difficult task due to the loosening of tissue debris during cutting and handling. We tried to avoid incubation in dirty medium by transferring the tissue to new and clean medium after chopping.

IN VIVO AND IN VITRO TREATMENT

Hormonal treatment in vivo (Paper II)

To determine the specific effects of E_2 and P_4 on the regulation of mPRs, in experiments included in Paper II, we treated the immature mice with steroid hormones by intraperitoneal injection. All drugs were administrated in a volume of 100 µl in sesame oil. The i.p. injection is a efficient way to administer the hormone, and it was given in the lower part of the peritoneal-abdominal cavity without perforating the intestines. Sesame oil is a widely used solvent for the administration of fat-

soluble drugs. The treatment model of E_2 and P_4 was based on previous studies of the regulation of specific receptors in the mouse fallopian tube related to tubal function [80].

In paper II, both the ESR antagonist (ICI 182,780) and the PGR antagonist (CDB-2914) were injected in sesame oil suspension. The antagonists mainly prevent the action of any E_2 and P_4 present. Given in doses that range from 5 to 20 mg/kg, ICI 182,780 has been shown to ablate E_2 and ESR signaling in several mammalian species [259]. The dose of ICI 182,780 (8.3 mg/kg) that we injected appears to be relevant in this context. In rat, a single dose of 2 mg/animal CDB-2914 has been shown to interrupt pregnancy and P_4 reversed this effect [260]. The dose of CDB-2914 that we injected was of 1 mg/animal.

Treatment with ICI 182,780 (Faslodex ™) (Paper II)

The most widely used ESR antagonist, tamoxifen, is also a partial agonist. A new nonsteroidal antiestrogen named Faslodex (ICI 182,780; Fig. 9) has no agonistic activity and was therefore used.

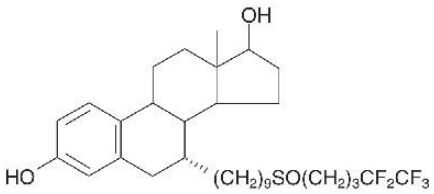

FIGURE 9: Chemical structure of the nuclear estrogen receptor antagonist ICI 182,780 (Faslodex™)

The addition of an akylamide side chain at position 7α of a normal E_2 molecule makes Faslodex bind with high affinity to ESRs while causing a rapid degradation of the receptor. As a result, the activation of transcription never occurs and this is what makes Faslodex a "pure" antiestrogen. [261]. Faslodex binds the ESR with approximately 100 times higher affinity than tamoxifen. Also, it does not cross the blood-brain barrier (and thus may not cause hot flashes) [262-264]. The current challenge is to create tissue- or even cell type-specific selective ESR modulators due to differences in tissue expression of ESR alpha and beta.

Treatment with CDB (VA)-2914 (Paper II)

CDB-2914 (CDB: Contraceptive Development Branch), also known as RTI 3021-012 and HRP-2000, is a synthetic steroid (produced by Southwest Foundation for Biomedical Research) with potent P_4 antagonist activity derived from 19-norP_4 (Fig. 10)

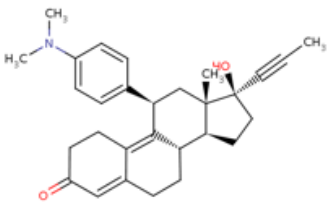

FIGURE 10: Chemical structure of the nuclear progesterone receptor antagonist CDB-2914

We preferred CDB-2914 to the much more widely used mifepristone (RU 486) due to the much reduced anti-glucocorticoid activity and increased anti-progestin activity in vivo [265]. It is not known if the CDB-2914 binds to the PGR. However, metabolites of CDB-2914 were showed to bind with high affinity to the PGR [266]. Administration of P_4 reversed the antagonistic activity of CDB-2914 on maintained pregnancy and on normal uterine development in immature rabbits [267]. As its metabolites have some activity, the pharmacological properties of CDB-2914 are still under evaluation. The half-life (½) for the elimination of mifepristone, a drug that has been used for many years, varies between 24 to 90 hours depending on the assay used [268].

Hormonal treatment in vitro (Paper III)

In Paper III, we aimed to study the effects of P_4 on ciliary activity in vitro. We were trying to overcome some of the troubles that in vitro handling causes by keeping the cells in a proper medium, at a physiologic in vivo temperature and by performing the sample preparation in a gentle way. To study the influence of P_4 on the CBF, we carried out two experimental series: one in which we measured the baseline CBF of the cells at regular time intervals, and one in which determination of the baseline CBF of each cell was followed by exposure to P_4 in ethanol or ethanol only, and subsequent recording of the frequency. We chose the concentration of P_4 (20 µM) based on the concentration used in a previous study on cows [107].

DETECTION OF THE RECEPTOR PROTEIN

Plasma protein purification (Paper I and II)

In Paper I and some of the experiments in Paper II, we used a procedure originally developed by Bordier [269] and later modified by Brusca and Radolf [270] to separate and concentrate integral plasma proteins from other proteins. This technique exploits the unique properties of the non-ionic detergent Triton X-114. Based on their hydrophobic structure, detergents are commonly used in the purification of integral plasma membranes. Non-ionic detergents, including Triton X-114, have a non-denaturing effect on antigens and are appropriate for the solubilization of integral membrane proteins. There are three values that describe the properties of a detergent: the HLB value, which stands for hydrophilic-lypophilic balance, the molecular weight of the micelle, and the CMC, which stands for critical micelle concentration. The HLB value describes the non-denaturing properties of the detergent based on the number of hydrophilic groups attached to the hydrophobic head group. For the more water-soluble Triton X-100, this value is 13.5, whereas for Triton X-114 it is 12.4. The critical micelle concentration, also called cloud point, was found to be 22°C for Triton X-114. At temperatures above the cloud point, non-ionic detergents form a cloudy suspension due to increased micellar size. The micellar aggregates can be pelleted by centrifugation to separate the detergent-rich phase from the detergent-poor phase. The content of Triton X-114 in each phase is about 12% and 0.04%, respectively. In Papers I and II, the protein concentrations in separated protein fractions were analyzed by the Bradford method and then further analyzed by Western blotting.

In Paper II, we used an alternative method to purify plasma membrane proteins based on subcellular protein fractionation by ultracentrifugation [271]. We briefly evaluated the purity of protein fractions and proper protein molecular weight by Western blotting and confirmed the results obtained using Triton X-114.

Antibody production

The peptides used to produce the polyclonal antibodies used in Papers I and II were synthesized for us by AgriSera, Vännäs, Sweden. The peptide sequences for mPRβ (GQQLRRLPKILEEGLPKMPC) and mPRγ (FGYRHPQSSATAC) were chosen from the non-membrane-spanning part near the N-terminus of the rat mPRβ and mPRγ cDNA sequences (GenBank accession numbers XM_236990 and XM_236314.3 respectively). In addition, the sequences were selected to match the mouse cDNA sequences with 100% homology and to avoid sequence similarities between the different mPRs or any other proteins. The length of the peptide sequence should preferably be between 4 and 14 amino acids, with a terminal cysteine to allow peptide orientation on the carrier molecule. The peptides were linked to keyhole limpet hemocyanin (KLH) and rabbits were the host animals for antibody production. The KLH is a relatively large protein aggregate containing copper, which readily dissociates with moderate pH changes. An advantage that we considered when choosing the rabbit for immunization was that they produce large amounts of serum. The antibodies were further affinity purified. A concentration of 0.52 mg/ml for mPRβ and 0.6 mg/ml for mPRγ and the specific activity of the antibodies were confirmed by ELISA. It should be kept in mind that antibody properties including affinity are pH-dependent.

Immunohistochemistry (Papers I and II)

Immunohistochemistry is a good qualitative method to detect which kinds of cells express or do not express a certain protein. One disadvantage of this method is that the absolute or even relative concentrations of an antigen cannot be determined easily. The basic principle for detection of antigen uses an enzyme-linked antibody. Specific antibodies possess a unique part called an epitope that recognizes the target antigens in the tissue. In Papers I and II, we used our own generated polyclonal antibodies and antibodies produced in the laboratory of Peter Thomas to detect the membrane P_4 proteins. Polyclonal antisera sometimes present lower specificity compared to monoclonal antibodies, since the antisera are composed of a mixture of heterologous antibodies. On the other hand, the use of monoclonal antibodies sometimes increases the risk of false negative results, since there is only one epitope that could be recognized. To eliminate false positive or negative results, we analyzed positive or negative control samples at the same time as the rest of the samples. In addition, we validated our primary antibodies using Western blotting in cell systems overexpressing the mPR or cell treated with short-hairpin RNA (shRNA) to reduce the mPR expression.

QUANTIFICATION OF THE RECEPTOR

Western blot assay (Papers I and II)

Western blot, or protein immunoblot, is an analytical method that allows detection of a protein in a sample. It does not give information on the localization of the protein within the cell or piece of tissue analyzed as immunohistochemistry does, but the molecular weight of the immunoreactive protein can be compared with the expected weight. Moreover, Western blot can evaluate whether the antibody binds more than one protein. In this way, it gives a higher certainty that the correct protein is being measured. Western blotting also allows a comparison between protein concentrations in different samples. To achieve a more quantitative assay, methods like enzume linked immuno sorbent assay (ELISA), radioimmuno assay (RIA) or enzyme immuno assay (EIA) may be used. However, in these methods, the molecular size of the immunoreactive protein in each sample cannot be controlled. The specificity and sensitivity of Western blotting depends largely on the antibodies used. The first step of the procedure is gel electrophoresis, which allows separation of proteins by size. The next step is the transfer of the separated proteins to a nitrocellulose membrane or some other type of protein-binding membrane (blotting), followed by detection (probing) with specific antibodies. The antibodies can sometimes be expensive or available only in limited amounts, although unbound antibody can often be reused between experiments.

RNA detection

In Paper II, total RNA was isolated from mouse fallopian tubes and ovaries using a commercial Micro column kit from Qiagen, Sweden. The mRNA expression was analyzed by two methods: reverse transcription polymerase chain reaction (RT-PCR) and quantitative polymerase chain reaction (Taqman).

Reverse transcription polymerase chain reaction (Paper II)

The PCR method was developed by Kary Mullis in 1984, who was awarded the Nobel Prize in Chemistry in 1994 for his work. This technique is a very sensitive method to detect expressed gene products though a specific amplification procedure. In Paper II, we used this method to confirm the expression of mPRs mRNA in mouse tissues. An advantage of the PCR method compared to, for example, Northern blotting is that it can detect very low levels of mRNA. The PCR reaction requires DNA polymerase (Taq polymerase), which enzymatically assembles the single-stranded DNA (cDNA) template, and DNA oligonucleotides (primers) to initiate new DNA synthesis. This process is repeated in a number of cycles, resulting in a doubling of the DNA amounts after each PCR cycle.

Quantitative real-time polymerase chain reaction (Paper II)

The qRT-PCR technique enables reliable detection and measurement of products generated during each cycle of the PCR process. This method requires a specific oligonucleotide probe or primers designed to hybridize within the target sequence. In our experiments in Paper II, we used specific primers designed to match the mPR genes. The probes/primers are fluorescently labeled with reporter fluorophores at their 5' ends and quencher fluorophores at their 3´ ends (Fig. 11). The quantitative real-time PCR technique has several advantages over the classical quantitative PCR

system. The use of fluorescent dye-labeled probes increases the sensitivity of the system by at least seven orders of magnitude and gives rise to a linear relationship between copy number and CT values. This method is used commonly nowadays; it can detect small differences in mRNA expression levels and quickly analyze several genes as well. The reporter fluorophore emits at a wavelength absorbed by the quencher fluorophore. The Taq polymerase starts extending primers moving toward the probes and, due to the 5´ nuclease activity, the probe is degraded and the reporter cleaved and separated from the quencher. The result is an increased emitted florescence that can be quantified. In our PCR, the generation of the product was determined by measuring the SYBR green fluorescence signal. Unbound SYBR Green exhibits little fluorescence, but during elongation, when SYBR Green binds to newly synthesized DNA, the emitted fluorescence signal increases proportionally with the amount of product formed. SYBR Green is not expensive to run, which makes it a good approach when wanting to test the expression of a number of genes. However, the design of the primers is important because SYBR Green can bind to all double-stranded DNA. Therefore, the primers should not be designed to span over long introns. The PCR cycle threshold (C_T) for a sample is used to define the real signal detected above the noise. Housekeeping genes are usually used to normalize gene expression and should not be influenced by the treatments. QPCR is optimized to have near 100% efficiency, facilitating the use of the $\Delta\Delta C_T$ method. This value is calculated from the difference between the C_T of the sample and the C_T of an endogenous control such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH). In paper II, we selected GAPDH, which is frequently used as a reference gene and worked best out of the reference genes we tested.

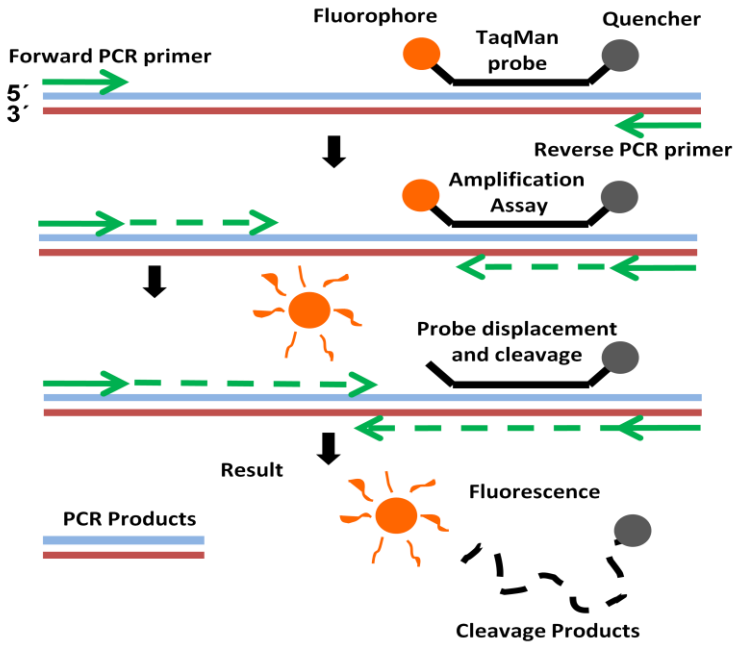

FIGURE 11 : Illustration of the chemical events taking place in the qRT-PCR reaction

MEASUREMENT OF CILIARY BEAT FREQUENCY (PAPER III)

Several studies have investigated changes in ciliary beat frequency in the tubal epithelium over the menstrual cycle. Different methods have been employed to study the in vitro ciliary motion in different tissues, including high speed cinematography combined with optical microscopy. In 1962, Dalhamn and Rylander described the first photometric method that could register CBF [272]. This method was based on imaging at relatively high magnifications of light microscopy and electronic detection of transmitted light fluctuations produced by the beating cilia. Normally this method demands a short working distance and is applicable only in vitro. Later, laser light scattering techniques were introduced for measurements of beating cilia [273], and they have provided highly precise measurement of CBF. Further technique development led to measurements of cilia movement in situ inside the trachea or the ampulla [274, 275]. However, the medical applicability of this method did not overcome low frequency signals caused by breathing, heart beating or animal/patient or environmental movements. A convenient method of studying ciliary activity in the fallopian tube is not easy to find.

In our study, we measured ciliary beat frequency using an inverted light microscope. The CBF readings (100 frames per second) were taken from an area of interest of approximately 50x50 pixels. This area allows the camera to take pictures of ciliary movement. As the cilia beat, the light intensity is changed and detected by the camera and computed into a frequency (in Hertz). The light change from the initial average is computed into a frequency by Fourier transformation. This transformation describes the frequencies that are present in the original signal. The cell or tissue fragment of interest was held still with the help of a micromanipulator. The typical application of micromanipulation in humans is intracytoplasmatic sperm injection (ICSI). This device controlled by a joystick is provided with a needle, which is driven by 3D step-motors. The movement reduction is obtained hydraulically using pistons connected to an oil-containing tube for persistent fast-holding. The baseline CBF was determined over a 30-minute period on the same cell at the same temperature and in the same medium prior to addition of the hormone. Therefore, each cell acted as its own control.

SUMMARY OF RESULTS AND DISCUSSION

MEMBRANE PROGESTERONE RECEPTORS (mPR) β AND γ ARE PRESENT AT THE TRANSCRIPTIONAL AND TRANSLATIONAL LEVELS IN BOTH HUMAN AND MOUSE FALLOPIAN TUBES (PAPER I AND II)

In Papers I and II, we aimed to investigate the general expression of mPRs in the mouse and human fallopian tubes. Analyzing mRNA expression is often easier and more straightforward than assessing protein expression directly. However, although the central dogma implies that a specific mRNA is translated into its corresponding protein, this is not always the case. Messenger RNA can be degraded before translation occurs. It is, however, agreed that both mRNA and protein expression measurements are useful to have for a better understanding of cell function. The expression of mPRα mRNA from spotted sea trout was initially identified by Northern blot analysis [176]. Most studies that have investigated the tissue-specific expression of mPR genes have applied RT-PCR techniques [181, 186-188, 195, 202, 206].

In Paper II, we used primers that target the specific cDNA sequences of the mPRβ and γ genes to demonstrate that the mPRs genes are expressed in the mouse fallopian tube and ovary (Fig. 3A,B and Fig. 4). Although several studies have demonstrated the expression of mPR mRNA in many tissues and species, to the best of our knowledge no other studies have investigated mPR gene expression in the fallopian tube. Although the expression of mPRα and β has been reported in an immortalized human fallopian tube cell line [234].

In order to study receptor protein expression, we developed specific antibodies designed to match a unique, presumably antigenic sequence from the extracellular N-terminal end of each mouse mPR protein. Antibodies were affinity purified on a column to achieve maximum purity, and the antibody-containing media was then evaluated by ELISA. Although we generated our own antibody against mouse mPRβ, we chose to use an antibody for mPRβ produced by another laboratory (the lab. of Peter Thomas) in our experiments, because it was able to detect the receptor at higher dilutions (lower concentration of antibodies) and appeared to have somewhat higher specificity (less cross-reactivity with proteins of different molecular masses). Most importantly, the specificity of this mPRβ antibody had already been validated and used in several species and reproductive tissues [177, 194]. In our studies, the specificity and cross-reactivity of both the mPRβ and γ antibodies were demonstrated by showing that specific blocking peptides completely abolished all binding in different assays techniques, and/or by using the antibodies in cell lines where either the mPRs genes were knocked down by RNA interference or the mPRs proteins were overexpressed.

Both the mPRβ and mPRγ antibodies detected a band of the expected size (approximately 40 kDa) in protein extract from mouse and human fallopian tubes (Paper I, Figs 3 and 4; Paper II, Figs. 2 and 6). Another study reported the expression of mPRα and β in the human fallopian tube using immunohistochemistry. However, the main purpose of that study was to investigate the expression of the mPRs in ovarian tumors. These tumors arise from epithelial cells, and fallopian tube tissues

were used as controls due to the higher content of epithelial cells in the fallopian tube compared to the ovary [204].

Taken together, the results of Paper I and II showed that mPRβ and γ are expressed at both the transcriptional and translational level (mRNA and protein level) in mice and humans.

MEMBRANE PROGESTERONE RECEPTORS (mPR) β AND γ ARE PRESENT IN BOTH REPRODUCTIVE AND NON-REPRODUCTIVE TISSUES IN MALE AND FEMALE MICE (PAPER I AND II)

In Papers I and II, we aimed to investigate the overall distribution of the mPRs in tissues from male and female mice, as well as in the fallopian tube. We found that both mPR β and γ are expressed in both male and female reproductive tissues. Both mPR β and γ were found to be expressed in the fallopian tube and ovary (Paper II, Fig. 3A). As reported in the literature, mPR mRNAs are broadly expressed in the reproductive tissues of a variety of species, including many fish species such as zebrafish [187, 191, 221], channel catfish [187] and goldfish [276], as well as in rat [188], sheep [198] and human [177, 181, 186, 199-202, 204]. In mice, mPRβ expression has been observed in the uterus [195], whereas mPRγ expression has been reported in P_4 responsive tissues outside the reproductive tract. We have previously investigated the expression of mPR genes in rats, where we found the strong expression of mPRα and β in reproductive tissues such as the ovary and uterus, and the expression of mPR γ in the uterus [205].

Using antibodies specific to mPRβ and γ, our results demonstrated the expression of receptor proteins with a molecular size of 40 kDa. The distribution of the mPRβ protein appears to be broader and more evenly expressed between tissues than that of mPRγ. In reproductive tissues, the expression of mPRβ was highest in testis, even though it was present also in prostate and epididymis (Paper II, Fig. 1). Interestingly, it appears that the expression levels of mPRβ and γ are similar in female reproductive tissues, while mPRβ predominates in the testis (Fig. 12). Western blot analysis detected protein moieties of 40 and 80 kDa, corresponding to monomers and dimers, in extracts from fish and human sperm [190, 199]; mouse and fish testis [199, 201, 202, 207], as well as from transfected cell lines [180, 201]. Interestingly, the ortholog of mPRβ in the Xenopus oocyte had an apparent molecular weight of 60 kDa [186], whereas cells transfected with the human mPRβ (HEK 293) yielded a protein of 30 kDa [182]. Our results showed that mPRβ and γ expressed in mouse tissues have the expected molecular weight of 40 kDa.

We have also evaluated the expression of mPRβ and γ in the mouse brain; our preliminary results suggest that the mPRs are involved in mediating the effect of P_4. However, in mouse brain, our antibody against mPRγ detected a strong band at 28 kDa in addition to weak expression of the 40 kDa protein (data not shown), which was only detected in protein preparations from the brain. This 28 kDa protein could be a truncated form of mPRγ that is present specifically in the brain;

alternatively, it could represent an unrelated protein that is highly expressed in the brain and is recognized non-specifically by the mPRγ antibody.

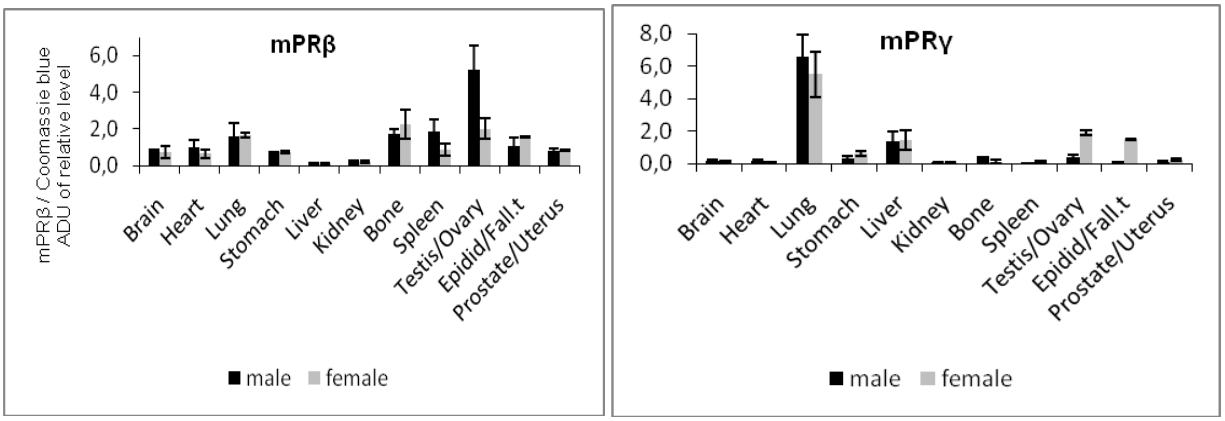

FIGURE 12: Tissue distribution of mPRβ (left; previously published in Paper II) and mPRγ (right; previously published in Paper I) in female and male mouse

In addition to the expression of mPRs in reproductive tissues, we observed the high and specific expression of mPRβ and γ in tissues outside of the reproductive tract (Fig. 12). The mPRs expression in various mouse tissues is essentially identical in both sexes. However, our studies on mice did not corroborate original reports of high mPRβ expression in brain tissues [176, 187]. This discrepancy could be attributed to the immature mouse model used in our studies. Our analysis of mPRγ revealed that its highest expression was in the lung and its lowest expression was in the kidney (Paper I, Fig. 2), in contrast to the first report which indicated very high expression in the kidney [177]. There is evidence that P_4 also affects pulmonary function and reduces muscle contractility in a rapid fashion [277]. We have found strong expression of mPRγ in mouse lung tissue and observed differential expression between female and male mice (Fig. 13).

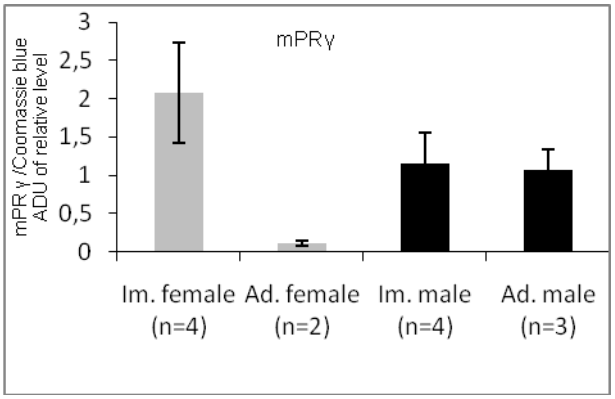

FIGURE 13: Relative intensity of endogenous mPRγ expression in immature and adult female and male mice (data not published)

We also found mPRγ to be highly expressed in the mouse liver, although the physiological function of P_4 in this tissue is less well known. Our findings in Paper I (Fig. 5) indicate a marked expression of the mPRγ protein in female mouse hepatocytes adjacent to the central veins. Rapid membrane depolarization in the liver (involving K+ channels) has been observed in response to P_4 [278]. In humans, mPRβ has been found in T-lymphocytes [202] and in ovarian tumors [204], implying an immuno-regulatory role and a possible involvement in tumor development.

MEMBRANE PROGESTERONE RECEPTORS (mPR) β AND γ ARE CO-EXPRESSED IN THE EPITHELIAL CELLS OF MOUSE AND HUMAN FALLOPIAN TUBES BUT HAVE A DISTINCT CELLULAR DISTRIBUTION (PAPERS I AND II)

Our current knowledge of the tissue/cellular distribution of mPRs has been primarily obtained via non-histological assays. However, one disadvantage of these methods is that they overlook the relationships between protein expression and histological structures, therefore limiting the understanding of the receptor's functions. A common strategy in elucidating the physiological function of a receptor is to investigate its cellular localization.

We used several immunohistochemistry techniques to investigate the localization of the mPRβ and γ receptors in the fallopian tube. The fallopian tube epithelium is mainly equipped with ciliary and secretory cells. We found that mPRβ and γ (Paper I, Fig. 3 and Paper II, Fig. 2) were specifically expressed in the ciliated cells of the fallopian tube epithelium in both mouse and human. Furthermore, the expression of mPRβ in human fallopian tube epithelial cells was also confirmed by another study that used the same antibody [204].

Interestingly, even though the expression of mPRβ and γ was consistently detected within the same cells in mouse and human tubal epithelia, the localization within these cells was distinct and specific for each mPR subtype (Paper I, Figs. 3 and 4; Paper II, Figs. 2 and 6A). In the fallopian tube, mPRβ was found in the motile cilia of epithelial cells, whereas mPRγ was found to associate with the apical membrane of the ciliated cells (Fig. 14). This co-occurrence within the same cell raises the possibility of a cooperative role in mediating effects of P_4.

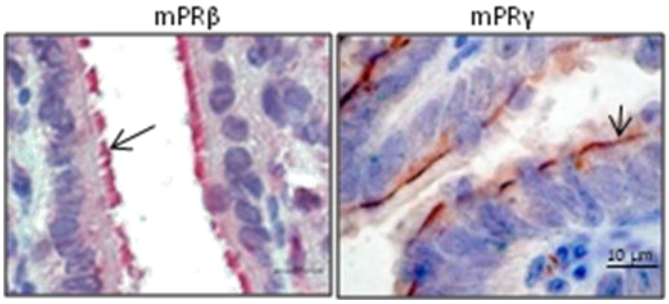

FIGURE 14: Distinct localization of mPRβ (left; published in Paper II, Fig. 2D) and mPRγ (right; published in Paper I, Fig. 3B) in ciliary cells of the immature mouse fallopian tube.

Even though the apical cell membrane and the cilia membrane contain different sets of proteins, the specialized ciliary membrane is continued from the apical membrane of the cell [279]. The co-localization of mPRβ and β-tubulin IV, which is a cellular marker for cilia, provides evidence of its ciliary localization (Paper I, Figs. 3 and 4; Paper II, Figs. 2 and 6A). In contrast to our findings regarding the localization of mPRβ, Romero-Sanchez et al. [204] reported unclear immunostaining of mPRs in all epithelial cells of the human fallopian tube, including secretory cells and ciliated cells, when using the same mPRβ antibody used in our study [204].

In the literature, the specific localization of mPRα and β to the plasma membrane was investigated using IHC assays, but was conducted with more of a focus on species differences. The IHC and/or

IF techniques revealed the plasma membrane localization of mPRs in human [199, 201], mouse [195] and fish [176, 190, 206-208]. However, mPRα and β were both found in plasma membrane preparations from testis [199], oocyte [206], gonadotrophes [195] and uterus [194, 201]. The mPRγ form is the least-studied of all mPRs subtypes. To the best of our knowledge, the subcellular localization of mPRγ had not been reported prior to our studies.

Although mPR localization to the plasma membrane has been reported for many tissues from many species since 2003, some studies do not support these findings [189]. In most of these studies, the plasma membrane localization was investigated using antibodies specific to the N-terminal portion of the mPR. However, Qui and colleagues showed that the staining method can be crucial, as the addition of Triton X-100 altered the observed staining pattern in porcine cumulus cells [244]. Triton X-100 is a non-ionic detergent which can damage the plasma membrane and allow the antibody to access the antigen. When we use 0.5% Triton X-100, the fluorescence appears as an intracellular staining of all epithelial cells (Fig. 15A) most likely because the plasma membrane is damaged, whereas specific staining to the plasma membrane, and only to the membrane of cililated cells (not secretory cells) is observed when Triton X-100 is excluded (Fig. 15B). We have also noted that different fixation and staining techniques can influence the general cellular localization using the same antibody.

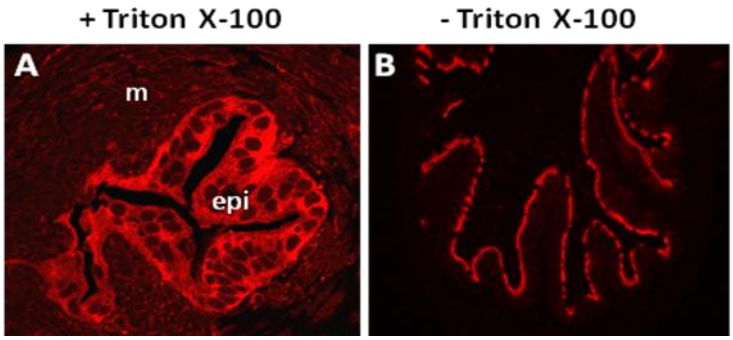

FIGURE 15: Immunohistochemical analysis of mPRβ localization in immature mouse fallopian tube cells treated with Triton X-100 (A) or untreated (B). epi: epithelial cell; m: muscle layer

On the other hand, it also appears that the mPR localization to the ER observed in mouse embryonic fibroblasts could not be explained by a detergent effect. The localization to the ER in some cells is also supported by other studies [182, 197, 204, 244] and is predicted by the presence of motifs for endocytosis internalization and ER export found in the mPRβ transcript [280]. Furthermore, steroid hormones, including P_4, easily pass through the plasma membrane and thus could reach the receptor inside the cell. Another matter of debate has been the orientation of the N- and C- termini of the mPRs in the plasma membrane, and their ability to sense and respond to P_4. The mPRs are Class II PAQR receptors and have some features that distinguish them from other PAQR receptors [144]. The mPRs begin a signaling cascade by activating G-proteins [180], while PAQR family members such as adiponectin receptors do not [179]. In contrast to PAQRs, both the hmPRα and smPRα possess an extracellular N-terminal domain and an intracellular C-terminal domain that acts with the third intracellular loop to activate G-proteins [180]. The originally reported orientation (extracellular N terminus and intracellular C terminus) was recently confirmed

by the permeabilization of transfected cells expressing the mPRs [180]. The association between the presence of recombinant and wild-type mPRs in cells and increased progestin binding has also been demonstrated [147].

Previous studies have proposed a cooperative role for mPRs in fish oocyte maturation induction and the transactivation of PGR in human myometrial cells [186, 201, 213, 214, 220, 221, 276]. Furthermore, the flagellar activity of human and fish sperm upon progestin stimulation is suggested to be mediated by mPRα [208]. The abundance of the receptor on the sperm membrane is related to sperm motility, as was demonstrated in both human and fish [199, 208]

In summary, Papers I and II demonstrated that mPRβ and γ are present in the ciliated cells of the fallopian tube of mice and humans. Both receptors are associated closely with the cilia, although they exhibit distinct cellular localizations. Taken together, these results suggest that these proteins might play a role in regulating some aspect of ciliary function in the fallopian tube.

PROGESTERONE REGULATES THE EXPRESSION OF mPRβ AND γ IN THE MOUSE FALLOPIAN TUBE (PAPER II).

In both paper I and paper II, the distinct localization of the mPRs to the fallopian tube epithelium was demonstrated. Part of paper II was designed to address whether ovarian steroid hormones regulate the expression of mPRs in the mouse fallopian tube.

The results presented in Paper II showed that P_4, a ligand of the mPRs, rapidly down-regulates the expression of both the mPRβ and γ genes in the fallopian tube of gonadotropin-primed mice (Paper II, Fig. 4). In line with our findings, treatment with the functional progestin 17α,20β-DHP causes the down-regulation of mPRβ mRNA expression in the ovary of the channel catfish [191]. Conversely, P_4 does not affect the expression of mPRβ in human myometrial cells [201]. In the rat corpus luteum (CL), mPRs appear to be independently regulated. The mPRα gene appears to be regulated in both the development of the CL and during pregnancy. The expression of mPRβ is decreased in late pregnancy, when a dramatic drop in P_4 level occurs, whereas the expression of mPRγ increases with advancing gestation. However, toward the end of the pregnancy the expression of mPRγ remains constant [188].

In the gonadotropin-stimulated mouse model, the regulation of mPRs could be directly mediated by P_4 in the target tissue, or by feedback acting on gonadotropin release from the pituitary gland. Ovarian hormones can regulate LH/FSH secretion in the brain via feedback mechanisms and thus influence their own production in the ovary, in addition to other pathways. Although the expression and function of FSH receptors in the ovary are well-established, their presence in the fallopian tube has been reported but their physiological function is only poorly understood [281]. In E_2-primed, castrated rats, P_4 induce the release of both FSH and LH [282]. This model is in many aspects similar to that presented by immature mice injected with eCG, which leads to an

endogenous E_2 peak. We demonstrated the regulation of both mPRβ and mPRγ by P_4 in mice primed with eCG, but not in immature mice. This lack of effect in the immature mouse is consistent with a model of P_4-induced regulation of mPRs via gonadotropin release from the hypothalamus/pituitary gland, although this hypothesis needs to be tested further.

Consistent with the expected feedback on gonadotropin release, we observed a similar trend of reduced mPRβ and mPRγ expression in E_2-treated eCG-primed mice, but the effect was not statistically significant (Paper II, Fig. 4). In immature mice, however, E_2 rapidly and significantly down-regulated the expression of mPRβ in the fallopian tube (Paper II, Figs. 3B and C), clearly suggesting a gonadotropin-independent regulation. The regulation of the mPRα gene has also been investigated in an immortalized fallopian tube cell line, where it was reported to be down-regulated by E_2 and up-regulated by P_4 [234]. This result suggests that E_2 and P_4 may directly regulate at least one mPR in the fallopian tube. In both human myocytes and teleost ovaries, a direct regulation of mPRα and β by P_4 and other progestin hormones has also been demonstrated in vitro [176, 201]. On the transcriptional level, P_4 causes a greater up-regulation of the mPRα gene, whereas the mPRβ gene was regulated solely by E_2. The observed regulation on the transcriptional level was associated with an increased abundance of mPRα and β proteins after 18 h of treatment. Differential regulation of mPRα and β by P_4 in human myometrial cells suggests that the two receptors have divergent functions [201]. After ovulation, the continuously P_4-producing cumulus cells surrounding the oocyte [90, 91, 283, 284] probably expose the epithelium of the fallopian tube to much higher P_4 levels than are experienced by any other tissue in the body, at least for a brief period of time. Thus, it is possible that circulating steroids might be of less importance than local levels of P_4 for the regulation of receptor levels. Indeed, the activation of sperm swimming is thought to be caused by a gradient of locally produced P_4 as the spermatozoa approach the ovum inside the fallopian tube [285]. Notably, there is no intra-tubal steroid production in either the immature or the eCG mouse model.

Direct regulation of the mPRs by E_2 and P_4 in the fallopian tube could conceivably be mediated either by stimulation of the classical nuclear receptors ESR and PGR, or by non-genomic signaling pathways, possibly involving the mPRs themselves. Tentative E_2 and P_4 response elements (EREs and PREs) are present upstream of both the mPRβ and mPRγ genes [187], suggesting a possible regulation via ESR and PGR. Indeed, PGR and ESR are both expressed in the epithelial, stromal and muscle cells of the fallopian tube [80, 137, 286]. The expression of these receptors positively correlates to plasma E_2 levels, whereas a negative correlation to plasma P_4 was found [287-289]. A few studies have proposed that the classical PGR is localized outside of the nucleus, possibly in the plasma membrane [212, 217, 290]. The classical PGR is reported to be expressed in the ciliated, stromal and muscle cells of the mouse [80, 137] and human fallopian tube [291]. Teilmann and colleagues (2006) showed intense staining of the classical PGR in the cilia proper [139], i.e., outside the nucleus, quite similar to the staining for mPRβ reported in Paper II. This localization could indicate a role for the PGR in non-genomic signaling (see also discussion p. 59-63).

The ESRs have been reported to be expressed in the ciliated, secretory and smooth muscle cells in the fallopian tube of the immature rat [137, 292, 293]. In contrast, one study demonstrated a strong immunostaining for the ESRβ specifically along the cilia proper of mice, whereas the secretory cells remained unstained [286]. It remains to be determined how the PGR and the ESRβ are localized to the cilia, whether they act as functional receptors there and if so, what signaling pathways are involved. It has been suggested that up to 5% of ESRs can be associated with plasma membrane caveolae. For example, ESRs were found to associate with caveolae in endothelial [294] and human breast cancer cells [295]. It has been shown that ESR association with caveolae could be coupled to $G_{\alpha i}$ and stimulate endothelial nitric oxide synthase (NOS) [296], or epidermal growth factor (EGF) activity to ultimately activate MAPK signaling pathways [297]. In human endothelial cells, it has been shown that E_2 activates ESR-associated non-nuclear $PI(_3)K$, similar to the mechanisms of nuclear glucocorticoid (GR) and thyroid receptors activation upon treatment by dexamethasone and thyroid hormone [298]. Different structural models of the LBD with alternative ligand pockets (for genomic and non-genomic responses) have been proposed for ESRα and the vitamin D receptor (VDR) [299].

In conclusion, the results of Paper II show that P_4 and E_2 regulate the expression of mPRs in the mouse fallopian tube. Steroid receptors are commonly regulated by their own ligands, thus our findings add support for a role of mPRβ and γ as functional progesterone receptors in the fallopian tube. The ciliated cells of the fallopian tubes are equipped with both nuclear and membrane-associated steroid receptors, which all could be involved in mediating the steroid-dependent regulation of the mPRs.

THE MOUSE FALLOPIAN TUBE PROVIDES A GOOD MODEL SYSTEM FOR STUDYING CILIARY ACTIVITY (PAPER III)

In Paper III, we aimed to establish a method for recording the CBF of epithelial cells using a mouse model.

A number of studies have looked for a change in CBF in the fallopian tube mucosa during the menstrual/estrous cycle, though none of these studies have been conducted in mice. Several techniques for measuring CBF have been developed, most of which are adapted for larger pieces of tissue than can be obtained from mice. The wide range of the reported baseline CBF (between 5-20 Hz) obtained from human studies also suggests that experimental conditions will often influence the CBF.

As ethics concerns dictate that we must work with animal models to study the mechanisms that regulate gamete transport, we should consider that there may be significant differences across species. For example, the interval between ovulation and implantation varies markedly among species. Mice are the most common animal model for human physiology because they are

genetically relatively close to humans, they are easy to breed and their physiology is very well understood. Furthermore, mice are amenable to hormonal manipulations, and genetically modified models are available or can be produced. Knockouts of the PGR gene (PGRKO mice) are available and will be useful for further evaluating the roles of different P_4 receptors on fallopian tube function. In addition, the estrous cycle in mice and the menstrual cycle in humans are comparable. Despite the anatomical differences between humans and mice, the duration of tubal transport is similar: 80 h in human and 72h in mice [9]. Nevertheless, the human egg spends almost 90% of this period in the ampulla region in the fallopian tube, whereas in mouse the egg remains in the ampulla for only 25% of this time.

Other laboratories have used immortalized human oviductal cell lines to study the hormonal regulation of the fallopian tube mucosa [233, 234]. However, one limitation of this approach is that the morphological characteristics of the ciliary epithelial cells may be lost over time. In our study, we performed measurements of CBF in ciliary cells from mouse fallopian tube immediately after tissue removal. Ex-vivo recordings of CBF over at least two hours demonstrated that the mouse model is compatible with our methodology. The establishment of a baseline CBF, the addition of hormone, and a subsequent recording of ciliary activity were possible. Establishing a baseline CBF is important and also required in order to examine factors that control the mechanism of ciliary activity. In Paper III, we found a mean CBF of approximately 22.5 ± 3.8 Hz in the mouse fallopian tube. In humans, however, the baseline CBF from in vitro studies ranges between 5 and 20 Hz [61, 100], whereas in cow a mean CBF of 23.1 Hz with a standard deviation of 4.7 Hz was reported in vitro [107]. Furthermore, deviations in CBF might be caused by pathological conditions, especially in the observations in humans, and/or by the methodology used. In some ex vivo studies, the CBF increased after several hours/days in culture [100, 101]. Several factors, such as P_4, E_2, interleukin 6, prostaglandins and Ca^{2+} have been reported to affect CBF in different species [100, 107, 300-302].

Another important factor to consider is the temperature at which the measurements are performed. To overcome muscular contractility ex vivo, several studies have been performed at temperatures lower than 37°C. In order to make our studies consistent with those of Wessel et al., and to reduce the effect of muscular contractility, we performed the CBF measurements at 35°C [107]. The effect of temperature on female reproductive tissues may have been previously underestimated. In the male reproductive system, the temperature gradient between the testis and the deep body temperature is well-characterized and is known to be important for maintaining fully functional sperm production. The temperature of human, rabbit, pig and cow follicles are on average 1.3 - 1.7°C cooler than the ovarian stroma [303]. Temperature differences are thought to occur due to a reaction within the Graafian follicles. This gradient might be important for gamete development in females. Interestingly, in mated pigs the isthmus appears to be about 0.7°C cooler than the other segments of the fallopian tube [303]. The arrest of spermatozoa in the caudal portion of the isthmus has been observed in several species including cow [304], and sperm motility is reduced in this segment of the fallopian tube. Furthermore, rabbit and human spermatozoa have the ability to sense temperature differences smaller than 0.5°C [305]. Temperature differences of at least this degree have been observed in pig and rabbit at ovulation, and lasts for at least 10 hours after

ovulation [306]. Similar observations in humans revealed that the temperature decreases from the isthmus to the uterus at ovulation [307]. Human spermatozoa have been reported to move toward warmer temperatures [305], and thermotaxis could be complementary to chemotaxis as a mechanism for sperm guidance in the fallopian tube. Taken together, it appears that gametes are exposed to decreased temperatures in the hours before sperm-egg interaction and fertilization occur. Temperature deviations of as little as 1°C may negatively influence cell organelles, protein chemistry and folding, especially in the oocyte maturation process [308]. However, further studies are required to establish the importance of a temperature gradient in the female reproductive tract, as well as to elucidate any differences between mouse and humans.

Ciliary activity is also affected by fluctuations in the intracellular pH. The pH of tubal fluid changes during the menstrual cycle, ranging from 7.1-7.3 during the follicular phase to 7.5-8.0 after ovulation. The pH is regulated by electrolytes and ions that pass through the lumen. The influence of pH on the CBF has been extensively studied in airway epithelia, where alkalization of the intracellular pH has shown to increase the CBF while acidification reduces it [309].

Our results show that the mouse is an appropriate model for the study of ciliary activity in the fallopian tube. Many factors affect the CBF and should be controlled for in any experimental setup.

PROGESTERONE TREATMENT CAUSES A RAPID DECREASE IN CBF IN THE MOUSE FALLOPIAN TUBE (PAPER III)

Progesterone has been proposed to regulate the CBF in the fallopian tube of several mammalian species [2, 4, 60, 105]. To investigate the effects of P_4 on the CBF, we treated ciliary epithelial cells from immature mouse fallopian tubes with P_4. Our results, presented in Paper III, show a significant drop in CBF (about 2.5 Hz, or 11%, compared to control cells) 30 minutes after the addition of P_4. This result is very similar to the results found in the cow, where P_4 reduced the CBF by 11% within 15 minutes after the addition of P_4 [107]. The CBF of control cells was reduced by an average of 0.7 Hz by the end of the recording period. This apparent difference was not statistically significant. Our observations appear to be similar to those of Wessel and colleagues despite the relatively great evolutionary distance between the cow and mouse within the mammalian taxon. These data suggest that rapid effects of P_4 on the CBF of the fallopian tube most likely exist in other mammalian species as well. Recently, a poster at the 6th International meeting on "Rapid Responses to Steroid Hormones" 2009 in Spain, presented results supporting the rapid P_4-induced effect on CBF in mouse fallopian tube epithelial cells [310]. In this study, a rapid increase in CBF was reported to occur in mouse ciliary oviduct cells in primary culture upon P_4 treatment (10 µM), an effect that appears to involve the activation of the TRPV4 channel. This is not surprising, since activation of the TRPV channels results in increased Ca^{2+} levels, which are known to increase CBF.

In paper III we showed that treatment with 20µM of P_4 decreased the CBF in mouse in a rapid fashion. The same concentration caused a similar effect in cow [107]. However, this concentration of P_4 is considerably higher than physiological levels found in the general circulation. In a study in the cow [107], the use of a µM concentration of P_4 was based on the estimated P_4 concentration in the human placenta [311]. In mice, the serum levels of P_4 normally range between 25 and 50 nM [81], whereas in humans they are often between 1 and 25nM [312, 313]. However, ovarian steroid levels are often significantly higher in the arterial and venous uterine blood than in systemic circulation [314]. Furthermore, the concentration of P_4 measured close to the corpus luteum in the peritoneal cavity (136 nM) was 4-fold higher than in other peritoneal measurements and 5-fold higher than in the general circulation [312]. Casslén and colleagues (1986) reported up to 342nM of P_4 in the peritoneal fluid of a post-ovulatory woman [315]. These findings suggest that the high concentration of P_4 released from the corpus luteum enters the peritoneal cavity and influences the function of nearby tissues, such as the fallopian tube, although the authors also reported low nM levels of P_4 in human fallopian tube fluid. In rodents, but not humans, the ovary and the fallopian tube are surrounded by a common bursa. Thus, as the bursa of mice prevents the fluid surrounding the P_4-producing ovary to spread freely into the peritoneal cavity, the mouse fallopian tube is probably exposed to even higher concentrations of P_4. In addition, the cumulus cells surrounding the oocyte after ovulation continue to produce P_4 in both mice and humans [90, 283] and are thus likely to contribute to an increased local concentration of P_4 in the fallopian tube. Therefore, the P_4 concentration can be expected to be much higher in the environment immediately surrounding the cumulus complex. Thus, association between circulating levels of P_4 and the CBF of the fallopian tube may be of limited value [2, 51, 60, 61, 104, 316, 317]. To date, we cannot state conclusively whether our use of 20µM P_4 represents a physiologically relevant concentration similar to the levels of P_4 encountered by cilia close to the cumulus complex.

An important question is what receptor or receptors mediate the rapid effects of P_4 on the CBF in the fallopian tube. The rapid nature of the response suggests that a non-genomic signaling pathway is involved.

Immunoreactivity to the classical PGR has been demonstrated in the cilia proper (i.e., plasma-membrane associated, outside the nucleus) of the epithelial cells in mouse and human fallopian tubes [139]. One possibility, therefore, is that the non-genomic action of P_4 could be mediated via the nuclear receptor as well. Indeed, several studies have presented evidence of PGR localization outside of the nucleus [318-320]. There, the PGR has been shown to mediate ligand-independent gene transcription. It has also been shown that this interaction can occur directly through the poly-proline motif of PGR, which acts as a ligand for the SH3 domain of the c-Src protein, or indirectly through the association of PGR and ESR, which further activates the SH2 domain of c-Src. Active c-Src will then trigger the activation of the MAPK signaling pathway [121, 321]. Another report proposed that cross-talk mediated via the plasma membrane-situated mPRs is important for signal transduction. In cultured myometrial cells that simultaneously express both mPRs and PGR, PGR activation was achieved through signaling mechanisms started at the plasma mPRs after P_4 treatment [201]. Interestingly, Wessel et al. (2004) found that pretreatment with the PGR antagonist RU486 could not block the effects of P_4 on CBF in the cow. This could be interpreted

as an indication that the PGR is not involved in controlling CBF. However, as the mechanisms for non-genomic signaling mediated by PGR are not as clear as those for its role as a transcription factor, it is not certain that RU486 would be able to block PGR's ability to signal non-genomically.

The expression of mPRβ and mPRγ in ciliary cells and their highly specific localization to the cilia or base of cilia suggest that they play a role in the P_4 regulation of CBF. Ligand association and dissociation from mPRs is rapid (2-4 minutes) [180] and much shorter than that of the PGR (80-100 minutes) [318], but typical for membrane receptors. Also, mPRs are known to transduce changes P_4 into physiological changes within minutes in other tissues or cell types [180, 195, 201, 206, 221]. The mPRα is also present in the spermatozoa of both fish [190, 207, 221] and mammals [199], another cell type with motile cilia in which progestin rapidly affects the CBF [199, 207, 221]. Furthermore, no homology to the ligand binding domain of the PGR has been found in any region of the mPRs. Consequently, neither RU486 nor Org31710 interact with human mPRs [177]. This lack of interaction is consistent with the lack of effect of RU486 on P_4-mediated changes in CBF in the cow fallopian tube [107]. Taken together, these results suggest that the mPRs are the receptors that regulate the effects of P_4 on the CBF.

If secreted P_4 from the cumulus cells indeed acts as a beacon to reveal the exact position of the ovum to the fallopian tube, cells of the fallopian tube should be able to differentiate between the levels of P_4 found very close to the ovum and the rest of the fallopian tube. For example, the recombinant mouse mPRβ and human mPRγ proteins produced in an E. coli expression system both had high affinity (K_d = 30-40 nM) and saturable (B_{max} = 0.3–0.5 nM) P_4 binding sites [177]. The nuclear PGR in sea trout displayed a 4-fold higher binding affinity (K_d = 1.9 nM) [318] than did s-mPRα (K_d = 8 nM), and the affinity of P_4 binding to the human PGR-B was 5-fold higher (K_d = 0.8 nM) [322] than human mPRα (K_d = 4.2 nM). Given the affinities of the PGR and the mPRs to progesterone, all would be expected to be fully activated at 20μM. It is quite possible that the level of P_4 represents a supra-physiological concentration. Determining whether lower concentrations of P_4 have a similar effect on the CBF is therefore an important next step.

The intracellular signaling pathway in the fallopian tube that connects P_4 concentration to changes in the CBF is not yet known. Information on both mPR activities in other cell systems as well as knowledge of various intracellular events that influence the CBF of different organs, particularly airway cilia, can be used to suggest possible pathways (Fig. 16). The structure and function of the mammalian respiratory and fallopian tube cilia are similar in many aspects.

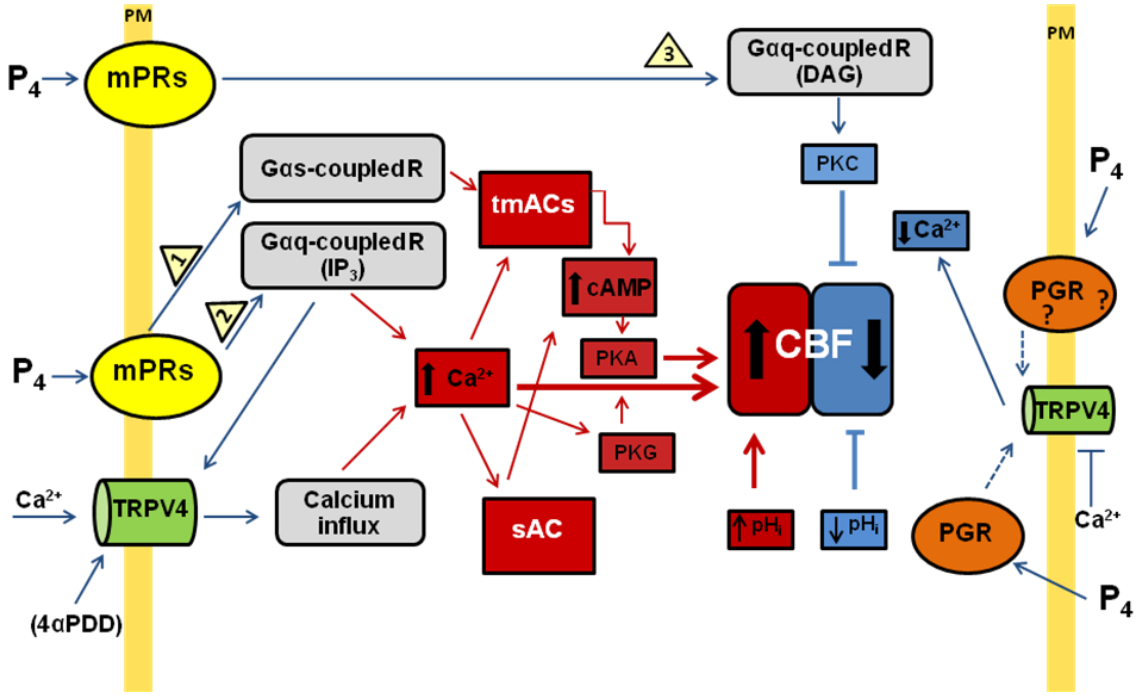

FIGURE 16: Summary of proposed pathways regulating mammalian CBF in the fallopian tube. Red and blue arrows represent factors known to stimulate the CBF; blunt blue lines represent known inhibitory interactions. Pathway 1 - Activation of membrane AC with increased cAMP, demonstrated in the Atlantic croaker [208]; Pathways 2 and 3 – Activation of a cAMP-independent signaling pathway (IP_3) has been proposed to be involved in oocyte maturation in Atlantic croaker [209]. The remaining signaling pathways involving cAMP activation of PKA, Ca^{2+} activation of PKG and the effects of pH and Ca^{2+} on CBF have been established in mammalian airway ciliary cells and spermatozoa [235]. The plasma membrane TRPV4 channel contributes to increase the CBF via Ca^{2+} influx [323] and/or through IP_3R [324]; the involvement of TRPV4 in decreasing CBF is regulated by PGR [325]. Abbreviations: tmAC: transmembrane adenylyl cyclase; sAC: soluble adenylyl cyclase; PKA, PKC, PKG: protein kinase A,C,G; TRPV: transient receptor potential vanilloid; 4αPDD 4α-phorbol 12,3-didecanoate; P_4: progesterone; PM: plasma membrane.

There is a well-known association between increased CBF and elevated Ca^{2+}. The calcium that mediates increased CBF is derived from both intracellular stores and external Ca^{2+} influx [326, 327]. Increased cAMP and PKA phosphorylation can also lead to an increased CBF [328, 329]. Classically, the major sources of cAMP are the G-protein-sensitive transmembrane or soluble adenylyl cyclases (ACs) [147, 247, 248]. It is possible that upon P_4 binding, mPRs could activate intraciliary and/or membrane localized ACs to modulate the CBF. The physiological roles of $G_{\alpha i}$ in mediating responses to P_4 [147] has been strengthened by studies in $G_{\alpha i}$ knockout mice [249]. Interestingly, $G_{\alpha i}$ is specifically localized to motile cilia in rats and humans, suggesting that these proteins might play a cilia-specific physiological role [233, 250]. In fact, the $G_{\alpha i}$-protein has been clearly demonstrated to participate in the regulation of CBF in the central nervous system of the rat, and further evidence for this involvement was provided by $G_{\alpha i}$-knockdown mice [330]. The injection of specific antisense oligonucleotides resulted in damage and loss of cilia, thus causing ciliary stasis [330]. Furthermore, 1-100 nM P_4 treatment affects the release of GnRH by the hypothalamus in gonadotrophic mice in vivo and in GnRH-producing cells in vitro through a

mechanism involving the mPRs and G proteins (G_i), but not PGR [195]. In contrast to PKA, which is consistently activated in all mammalian cilia, protein kinase C (PKC) has been implicated in slowing ciliary activity [235]. A ciliary membrane PKC phosphorylation target, p37, was found to be associated with the ciliary membrane in ovine tracheal cilia [251]. Interestingly, acute effects on the phosphorylation of p38 and MLC were observed after exposure to free P_4 in myometrial cells, which also express mPRs [201]. Recently, the involvement of the transient receptor potential vanilloid (TRPV) channel (TRPV4) in P_4-mediated modulation of CBF in the mouse fallopian tube has been suggested [310, 325]. TRPV4 is a thermosensor that is activated at temperatures below 33°C, as well as by other stimuli including heat, acidic pH and endogenous agonists, such as P_4. It has been proposed that TRPV channels are permeable to ions such as Ca^{2+} and can be controlled by P_4 in the mouse fallopian tube, thus allowing the regulation of the CBF. However, an indirect effect of P_4 mediated via PGR is to decrease the expression of TRPV4 channels, thus resulting in decreased Ca^{2+} influx and reduced CBF in human tracheal epithelial cells [325]. Indeed, PGR expression has been reported in both ciliated cells from rat and mouse [80, 137] and in the cilia proper of the mouse and human fallopian tube [139]. Furthermore, the PGR has been found in airway epithelial cells of other species [331, 332]. Together, these observations suggest that multiple intracellular pathways may be involved in the regulation of CBF by P_4, but our understanding of this process is far from complete.

In summary, the results of Paper III show that ciliary cells of the mouse fallopian tube beat at a frequency of approximately 22 Hz ex vivo. Treatment with P_4 causes a rapid decrease in CBF, an effect that is likely mediated via non-genomic signaling. The mPRβ and γ are the primary receptor candidates proposed to mediate the effects of P_4 in the fallopian tube, thus influencing tubal transport. Multiple intracellular pathways may be involved in translating the receptor binding into a modulation of the CBF.

CONCLUDING REMARKS

The studies in this thesis have resulted in the following main conclusions:

1. Membrane progesterone receptors (mPR) β and γ are present at transcriptional and translational level in both human and mouse fallopian tube

2. The distribution of the receptor proteins is present in both the reproductive and non-reproductive tissues in male and female mice

3. The mPRβ and γ specifically and distinctly co-localize in the epithelial cells of female mouse and human fallopian tube

4. Progesterone rapidly regulate the expression of mPRs in the mouse fallopian tube

5. The mouse model provide a good model system for measurement of CBF

6. Progesterone treatment caused a rapid decrease in CBF in the ciliary cells from the fallopian tube of mouse

The role of progesterone and the recently discovered membrane progesterone receptors (mPR) in mediating physiological functions in reproduction is certainly comprehensive. In this thesis, we have hypothesized that the mPRs might play a role in gamete transport in the fallopian tube. Understanding the dynamic interaction between tubal epithelium, tubal fluid contents including the hormones, gametes and embryo is crucial for a successful pregnancy. The knowledge of endocrine regulation of tubal transport to ensure fertilization, embryo development and transport to the implantation place is however far from complete.

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