GRANULOSA CELL APOPTOSIS

TRANSCRIPTIONAL REGULATION BY THE NUCLEAR PROGESTERONE RECEPTOR

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Happy is he who gets to know the reasons for things

Publius Vergilius Maro
70-19 BCE

ABSTRACT

Ovarian follicle atresia caused by granulosa cell apoptosis is a central process in normal female physiology. Progesterone has been reported to be a survival factor in granulosa cells at several developmental stages. This thesis focuses on the local functions of progesterone relating to the control of granulosa cell apoptosis during the periovulatory interval. The well-characterized gonadotropin-primed immature rat model was used to generate periovulatory granulosa cells, which were subsequently subjected to serum-free cell culture. The effects mediated by the nuclear progesterone receptor were investigated using two progesterone receptor antagonists, RU 486 (mifepristone) and Org 31710. The transcriptional regulation mediated by the nuclear progesterone receptor was investigated using the Affymetrix microarray technique. Decreased de novo synthesis of cholesterol was found to be one of the major effects of high concentrations of Org 31710. Recent studies have demonstrated that inhibition of cholesterol synthesis results in substrate limitation for post-translational isoprenylation, which has interesting implications for the cellular control of apoptosis. We found that cholesterol synthesis and protein isoprenylation are important factors maintaining granulosa cell survival; however, decreased protein isoprenylation cannot explain the induction of apoptosis by progesterone receptor antagonists. In addition to transcriptional regulation, progesterone also initiates rapid cellular responses that have been suggested to regulate granulosa cell apoptosis. We have demonstrated that Org 31710, which acted on the nuclear progesterone receptor, specifically and reversibly induced apoptosis of periovulatory granulosa cells in vitro. We found no support for any contributing non-genomic signaling of progesterone. Furthermore, we could not corroborate previous reports suggesting rapid effects of progesterone in immature rat follicles. Expanded microarray studies focused on early and late transcriptional effects of low doses of Org 31710. Gene ontology analysis was used to select biologically relevant functional groups for further analyses, including genes involved in apoptosis, reproductive processes, cell adhesion, cell cycle regulation, transcriptional control and angiogenesis. In conclusion, we found that progesterone is a central, survival-promoting regulatory factor that acts via the nuclear progesterone receptor during the periovulatory interval. The identification of novel gene targets of progesterone expands our knowledge of the events that occur in granulosa cells during ovulation and luteinization.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Äggstockarna hos en kvinna har två uppgifter; de innehåller könsceller, ägg, som ungefär en gång per månad under den fertila perioden av kvinnans liv genomgår ägglossning för att kunna befruktas. Dessutom producerar äggstockarna de kvinnliga könshormonerna östrogen och progesteron. Alla ägg som finns i äggstockarna bildas under fostertiden och vid puberteten finns ca 400 000 ägg. Ett snabbt överslag ger att ungefär 400 ägglossningar sker fram till klimakteriet, då äggen är i det närmaste slut. Vad händer med alla andra ägg? Äggen ligger i vilande äggblåsor som kontinuerligt rekryteras att börja växa. Den äggblåsa som genomgår ägglossning har vuxit och utvecklats under flera månader till en stor vätskefylld struktur som producerar östrogen som frisätts till blodet. De flesta äggblåsorna når aldrig ägglossning utan tillbakabildas i en process som kallas atresi och som beror på att stödjeceller i follikeln, de s.k. granulosacellerna genomgår programmerad celldöd, apoptos. Programmerad celldöd är kroppens sätt att göra sig av med oönskade celler utan att skada andra celler runt omkring och är bland annat ett viktigt skydd mot uppkomsten av tumörer. Vilka folliklar som växer och vilka som tillbakabildas styrs av en balans av många hormoner och andra molekyler. En kort tid före ägglossning slutar äggblåsorna producera östrogen och börjar istället bilda progesteron. Detta sker i samband med att äggblåsan förbereds för att släppa ut ägget och omvandlas till en gulkropp i en process som kallas luteinisering (lutein är det färgämne som ger gulkroppen dess färg). Progesteron är nödvändigt för att äggblåsan ska spricka under ägglossningen och för att förbereda livmodern för att kunna ta emot ett befruktat ägg. Tidigare studier har visat att progesteron också minskar förekomsten av programmerad celldöd under den här perioden och skulle därför kunna vara en viktig överlevnadsfaktor för äggblåsan under tiden för ägglossning.

I den här avhandlingen har målsättningen varit att i mer detalj undersöka hur progesteron påverkar granulosacellerna före ägglossning och hur hormonet kan förhindra programmerad celldöd. Progesteron är ett steroidhormon som likt andra steroider påverkar vilka gener en cell ska aktivera och därigenom vilka proteiner cellerna ska bilda. Vi har använt en djurmodell där unga råttor behandlas med hormoner för att deras äggstockar ska utvecklas till precis det stadium som ska studeras. Vi har sedan isolerat granulosaceller och studerat dem ”in vitro”, det vill säga i provrör. För att se vilka gener som påverkas av progesteron har vi blockerat den receptor (mottagarstruktur) som progesteron binder till och som sedan påverkar cellens gener i cellkärnan. Vi har använt en teknik, microarray, som

möjliggör att man kan mäta aktiviteten hos alla cellens tiotusentals gener på samma gång. I två olika studier har vi närmare studerat gener som påverkas av låga eller höga koncentrationer av receptorblockerare samt efter kort respektive lång behandlingstid. Lång behandlingstid med hög koncentration medförde förändringar i cellernas förmåga att bilda kolesterol. Kolesterol används som grundsten i produktionen av steroidhormoner, men substanser som bildas under processens gång är också viktiga för speciella proteinmodifieringar som kan ha betydelse för programmerad celldöd. Vi har visat att både kolesterol och resulterande proteinmodifieringar har betydelse för programmerad celldöd i granulosaceller. Progesteron påverkar emellertid processen i alltför låg grad för att detta ska kunna vara förklaringen till hur progesteron styr cellernas överlevnad.

I en mer omfattande undersökning av de förändringar av genaktivitet som en lägre koncentration receptorblockerare ger upphov till, hittade vi flera grupper av gener som kan ha betydelse för olika viktiga processer under ägglossningen. Vi identifierade en stor samling gener som skulle kunna påverka programmerad celldöd och som man tidigare inte känt till att de kontrolleras av progesteron.

Progesteron kan, förutom att påverka cellens gener i en relativt långsam process, även ha snabba effekter på andra signalsystem i cellerna. Sådana effekter har i andra studier föreslagits bidra till progesterons kontroll av celldöd. Det har inte varit helt klarlagt huruvida progesterons förändringar av genaktivitet bidrar till regleringen av celldöd. I en delstudie undersökte vi därför de olika signalvägarna närmare för att se om de är inblandade i progesterons effekter. Vi visade att progesteron med säkerhet verkar via långsamma förändringar av cellernas genaktivitet. Däremot kunde vi inte hitta några bevis för att andra signalsystem skulle vara involverade.

Sammanfattningsvis har vi visat att progesteron är en överlevnadsfaktor för granulosaceller vid tiden för ägglossning, samt att progesteron verkar genom en förändring av cellernas genaktivitet. Progesteron tycks också påverka flera olika viktiga processer som är nödvändiga för att ägglossning ska ske. Många av de gener som vi har identifierat har tidigare varit okända i de här sammanhangen. Fördjupade studier av deras funktion och betydelse behövs för att helt förstå hur ägglossning och luteinisering går till.

LIST OF PUBLICATIONS

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

I **Progesterone-receptor antagonists and statins decrease de novo cholesterol synthesis and increase apoptosis in rat and human periovulatory granulosa cells in vitro.**
Rung E, Friberg PA, Shao R, Larsson DGJ, Nielsen E, Svensson PA, Carlsson B, Carlsson LM, Billig H.
Biol Reprod 2005; 72: 538-545.

II **Apoptotic effects of a progesterone receptor antagonist on rat granulosa cells are not mediated via reduced protein isoprenylation.**
Friberg PA, Larsson DGJ, Rung E, Billig H.
Mol Reprod Dev 2007; 74: 1317-1326.

III **Dominant role of nuclear progesterone receptor in the control of rat periovulatory granulosa cell apoptosis.**
Friberg PA, Larsson DGJ, Billig H.
Biol Reprod 2009; In Press as DOI:10.1095/biolreprod.108.073932.

IV **Nuclear progesterone receptor in rat periovulatory granulosa cells: genome-wide transcriptional regulation by Org 31710 in vitro.**
Friberg PA, Larsson DGJ, Billig H.
Submitted.

CONTENTS

ABBREVIATIONS

ADAMTS	a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin motif
ADCYAP1	adenylate cyclase activating polypeptide 1, commonly known as PACAP
AF	activation function
AKT	thymoma viral proto-oncogene
APAF1	apoptotic peptidase activating factor 1
BAD	BCL2-associated agonist of cell death
BAK	BCL2-antagonist/killer 1
BAX	BCL2-associated X protein
BCL2	B-cell leukemia/lymphoma 2
BCL2L1	BCL2-like 1, commonly known as BCL-XL
BH	BCL2 homology domain
BID	BH3-interacting domain death agonist
BOK	BCL2-related ovarian killer protein
cAMP	cyclic adenosine monophosphate
CASBAH	CAspase Substrate dataBAse Homepage
CRYAB	crystallin alpha B
CYP11A1	cytochrome P450, family 11, subfamily a, polypeptide 1, commonly known as P540scc
DAVID	Database for Annotation, Visualization and Integrated Discovery
DBD	DNA-binding domain
DIABLO	diablo homolog (Drosophila)
DISC	death-inducing signaling complex
eCG	equine chorionic gonadotropin
EDN2	endothelin 2
EGF	epidermal growth factor
FAS	tumor necrosis factor receptor superfamily member 6
FASL	Fas ligand (TNF superfamily, member 6)
FDR	false discovery rate
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
GABA	gamma-aminobutyric acid
GO	gene ontology
hCG	human chorionic gonadotropin
HDL	high density lipoprotein
HMGCR	3-hydroxy-3-methylglutaryl-Coenzyme A reductase
HMGCS	3-hydroxy-3-methylglutaryl-Coenzyme A synthase
HRE	hormone response element
HSD3B	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase cluster, commonly known as 3β-HSD
HSD17B	hydroxysteroid (17-beta) dehydrogenase, commonly known as 17β-HSD
i.p.	intraperitoneal injection
IAP	inhibitor of apoptosis
IGF1	insulin-like growth factor 1

LBD	ligand-binding domain
LDL	low density lipoprotein
LH	luteinizing hormone
LHR	luteinizing hormone receptor
MAPK	mitogen activated protein kinase
MOMP	mitochondrial outer membrane permeabilization
MVK	mevalonate kinase
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NR5A1	nuclear receptor subfamily 5, group A, member 1, commonly known as SF-1
NR5A2	nuclear receptor subfamily 5, group A, member 2, commonly known as LRH-1
PAQR	progestin and adipoQ receptor family
PARP	poly (ADP-ribose) polymerase
PCR	polymerase chain reaction
PGR	progesterone receptor
PGRMC1	progesterone receptor membrane component 1
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PPARG	peroxisome proliferator activated receptor gamma
QPCR	quantitative polymerase chain reaction
RAB11A	RAB11a, member RAS oncogene family
RAS	rat sarcoma viral oncogenes
RQ	relative quantity
s.c.	subcutaneous injection
SAM	Significance Analysis of Microarrays
SCARB1	scavenger receptor class B, member 1
SERBP1	serpine1 mRNA binding protein 1
SGK1	serum/glucocorticoid regulated kinase 1
SH3	SRC homology 3
SIGC	Spontaneously Immortalized Granulosa Cell
SMAD	MAD homolog (Drosophila)
SRC	Rous sarcoma oncogene
SRE	sterol regulatory element
SREBP	SRE binding protein
STAR	steroidogenic acute regulatory protein
TGFA	transforming growth factor alpha
TGFB	transforming growth factor beta
TLC	thin layer chromatography
TLDA	TaqMan low density array
TNF	tumor necrosis factor
TRP53	transformation related protein 53, commonly known as P53
VEGFA	vascular endothelial growth factor A
ZBTB16	zinc finger and BTB domain containing 16, commonly known as PLZF

INTRODUCTION

Human ovaries are oval intra-abdominal organs that are approximately the size of walnuts in adult women. They have two main functions. First, they are reproductive organs, harboring the female gametes and releasing a fertilizable oocyte approximately once a month during the fertile life of a woman. In addition, they are endocrine structures, producing the major female sex hormones necessary for reproductive functions. Externally, the ovary is covered by a serous epithelium resting on a basement membrane. Underneath this layer is a dense connective tissue known as the tunica albuginea. Two main regions make up the ovary itself. The inner medulla contains nerves and branching blood vessels, while the oocyte-containing follicles are situated in the outer cortex. Resting follicles are found in the relatively avascular region close to the tunica albuginea, while growing follicles are closer to the vascularized medulla.

Preovulatory follicle development

Folliculogenesis is the process of follicle growth and development, starting with recruitment of resting follicles and ending with the release of a dominant follicle(s) (Fig. 1). There are approximately 400,000 resting follicles in the ovaries at the

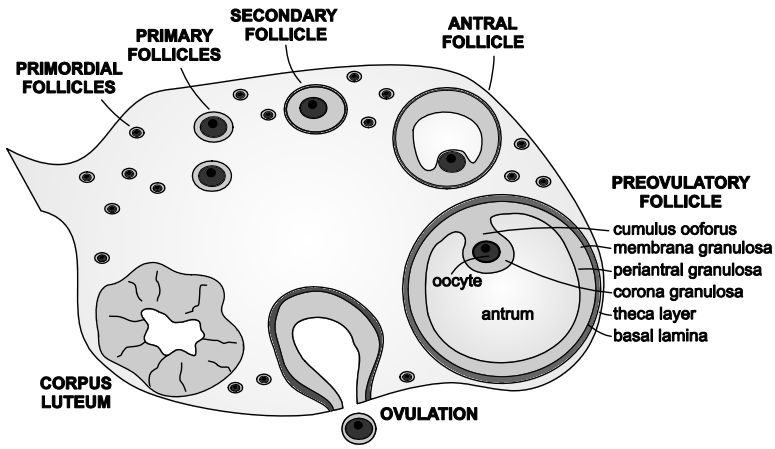

FIGURE 1

Schematic illustration showing the process of folliculogenesis and the structural components of the growing follicles. In humans, the entire process takes at least several months to complete. Although it appears as if the growing follicles migrate along the edge of the ovary, this is not the case.

onset of puberty [1]. Each primordial follicle consists of a single layer of squamous pregranulosa cells, a small oocyte arrested in meiosis I and a basal lamina enclosing the entire unit. When primordial follicles are continually directed to initiate growth by factors that are thought to be intrinsic to the ovary [2], they transform into primary follicles, which are characterized by growing oocytes and a single functional layer of cuboidal granulosa cells that nurture the oocyte. A recent review has described the process of follicle growth and development [3]. Extensive gap junctions connect the granulosa cells both to their neighboring cells and to the oocyte, thus enabling communication in the form of small molecules, such as cyclic adenosine monophosphate (cAMP) and Ca^{2+}, and formation of a metabolic syncytium [4]. Follicles with two to eight layers of granulosa cells, termed secondary follicles, begin recruiting fibroblast-like cells, external to the granulosa layer. Vascularization of this so called theca layer leads to the first direct exposure of the follicle to the surrounding endocrine milieu. The initial proliferation of the granulosa cells is extremely slow, making it difficult to distinguish resting from early growing follicles and to estimate the extended time passing from recruitment to the later stages of folliculogenesis [3, 5]. In tertiary or pre-antral follicles, cavitation, i.e., the formation of a fluid-filled cavity, marks the transition to the antral stages of folliculogenesis.

In antral (also known as Graafian) follicles, the theca layer is divided into the fibroblast-dominated collagenous connective tissue called theca externa and the steroidogenesis-competent theca interna. The granulosa cells are differentiated according to their intrafollicular localization and are divided into corona, cumulus, periantral and membrana granulosa cells. Follicle development up until antrum formation is considered to be independent of the cycling levels of the pituitary gonadotropins follicle stimulating hormone (FSH) and luteinizing hormone (LH), whereas antral follicles depend on these tropic hormones for their continued growth. In addition to the gonadotropins, local factors are important in follicular development. The expression of these local factors is often regulated by the gonadotropins. In most cases, the same factors also regulate follicle atresia, which is described below. Interestingly, the oocyte has recently been shown to orchestrate the rate of early folliculogenesis, indicating a reciprocal dependency between the oocyte and the somatic cells [6]. The increase in follicle size from 400 μm to 2 cm (in humans) is largely due to an increase in the volume of follicular fluid in antral follicles. In humans, one dominant follicle is selected for ovulation from a cohort of large antral follicles, influenced by the secondary rise in FSH during the late luteal phase of the menstrual cycle. Dominance is likely established based on the

threshold levels of FSH that are required by individual follicles for their continued growth and development [7].

A continued differentiation process induced by FSH results in the induction of aromatase and eventually luteinizing hormone receptor (LHR) expression in the granulosa cells of the largest growing follicle, enabling it to produce increasing

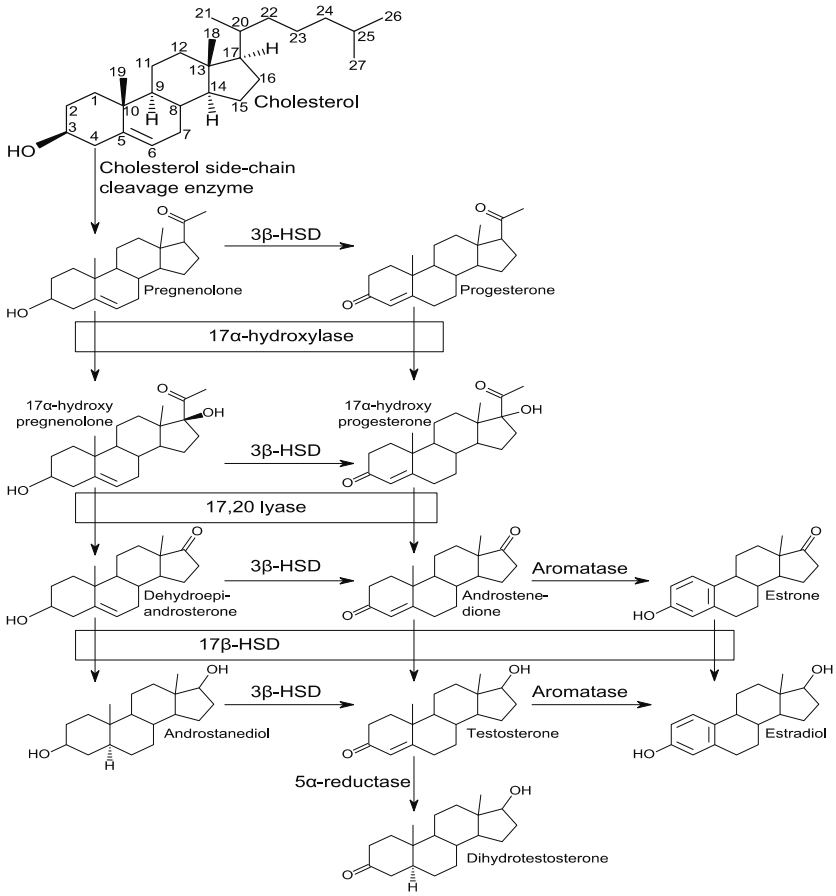

FIGURE 2

Schematic overview demonstrating the enzymatic steps of steroidogenesis. All steroids are produced from cholesterol, which serves as the basic building block, in the following order: progestagens, androgens and estrogens. Additional steps not included here include, for example, the synthesis of corticosteroids and mineralocorticoids. The carbon atoms in the cholesterol skeleton are numbered as a reference for the enzymatic transitions. All enzymes are indicated by their common names. 3β-HSD, hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase cluster (HSD3B); 17β-HSD, hydroxysteroid (17-beta) dehydrogenase (HSD17B).

amounts of estrogens and to respond to the impending LH surge. According to the established two-cell, two-gonadotropin theory [8], estradiol is produced by granulosa cells from androstenedione, which diffuses across the basement membrane from the LH-stimulated theca interna. FSH and LH, acting via the cAMP / protein kinase A (PKA) intracellular signaling pathway, stimulate the steroidogenic capabilities of granulosa and theca cells, respectively, even though the enzymes expressed in the two cell types differ. Among the enzymes induced is steroidogenic acute regulatory protein (STAR), which is considered the rate-limiting step in the production of steroids, as it performs a critical step in the transport of cholesterol from the outer to the inner membrane of the mitochondrion [9]. An overview of the steroidogenic pathways is presented in Figure 2.

Atresia: most follicles never ovulate

The oocytes stored in the ovaries are formed as primordial germ cells at the beginning of the fourth week of embryogenesis. Their number is greatest around the 20th week of embryonic development and has been estimated to be approximately 7 million [1]. This number is rapidly diminished, however. The number of oocytes remaining at birth has been estimated to be 1–2 million, and out of this number, less than 400,000 remain at the onset of puberty. Considering the approximately 400 ovulations that take place during the fertile life of a woman, one can appreciate that more than 99.9% of all oocytes available at puberty fail to ever reach ovulation. The normal final destination of most oocytes is a process termed atresia, which is Greek for “closure of hollow space”. In preantral follicles, the oocyte is the first follicular component to disappear, whereas the granulosa cells are the first to be affected in antral follicles [10]. In primates, atresia is estimated to occur in relatively few follicles, approximately 30%, during the preantral stages and 15% in small antral follicles [5, 10]. The incidence is higher in large antral follicles, where 50–75% of the follicles degenerate. The atretic process can be divided into four steps, as described in [10, 11] and summarized below:

1) Pyknotic nuclei in 10–20% of the granulosa cells, especially periantral cells; floating granulosa cells in the follicular fluid, and decreased mitotic activity;
2) 10–50% of the granulosa cells of the follicular wall are missing; the basement membrane loses its integrity; and leukocytes invade the granulosa layer;
3) The entire follicle appears shrunken, and the shapes of the follicle and oocyte are irregular; the theca layer is hypertrophied in some species;
4) In the final stage of degeneration, granulosa and theca cells are entirely missing, and the antrum is entirely filled with invading fibroblasts.

Metabolic effects are associated with these morphological changes, including increased progesterone synthesis and decreased estrogen synthesis [10, 11].

Apoptosis

The atretic process is caused at the cellular level by apoptosis or programmed cell death of the granulosa cells of the degenerating follicles [12, 13]. The term ‘apoptosis’ was introduced by Kerr et al. in 1972 to describe a specific morphological pattern of dying cells that is distinct from necrosis [14]. Apoptosis is Greek for “falling off”, as leaves fall from a tree. An individual cell faces three possible choices: proliferation (mitosis), specialization (differentiation) or death (apoptosis). Thus, apoptosis allows tissues to maintain a balance between too many cells and too few cells. During embryonic development, apoptosis is used to sculpt the developing organs, e.g., ductal morphogenesis of mammary glands, and to remove undesired cells or tissues, as exemplified by removal of the Wolffian duct during female embryonic development [15]. The critical role of apoptosis in development can be seen in the nematode *Caenorhabditis elegans* (*C. elegans*), where 131 out of the total 1,090 cells undergo precise and ordered programmed cell death [16]. For this reason, *C. elegans* has been extensively used as a model organism for the study of apoptosis. Sydney Brenner, H. Robert Horvitz and John E. Sulston were awarded the Nobel Prize in Medicine in 2002 "for their discoveries concerning 'genetic regulation of organ development and programmed cell death.'"

Apoptosis can be defined as caspase-mediated (see below) cell death with specific morphological features [17]. It is an active process that is controlled at the level of transcription as well as translation. An affected cell shows chromatin condensation, cell shrinkage, membrane blebbing, nuclear condensation, nuclear fragmentation and formation of apoptotic bodies that are targeted by neighboring cells or macrophages for phagocytosis. Importantly, the cellular membranes remain intact, and ATP levels remain normal throughout the process. Apoptosis often occurs quickly, removing affected cells without a trace in a few hours time. These characteristics are in distinct contrast to what is seen in necrotic cell death, which describes the sum of changes that occur in cells dying by, for example, oncosis [18]. In necrotic cell death, the cells swell instead of shrink, and the cellular membranes rupture, which results in release of the cellular contents and subsequent inflammation of the surrounding tissue. In apoptosis, poly (ADP-ribose) polymerase (PARP) is inactivated, whereas in necrosis, PARP struggles to repair DNA damage, rendering ATP-dependent ionic pumps at the cellular membrane

ineffective and eventually causing osmotic imbalance, cellular swelling and membrane rupture [19].

Apoptosis is executed by caspases

The morphological changes that occur in apoptotic cells are caused by the caspase family of proteases (Cysteine-dependent ASPartate-specific proteASEs) [20]. Caspases can be functionally divided into two groups, one of which is involved in the apoptotic machinery (caspases 2, 3, 6, 7, 8, 9 and 10), and the other, in the processing of inflammatory cytokines (caspases 1, 4, 5, 13 and 14 (as well as the murine caspases 11 and 12)). This functional categorization, however, is not entirely strict. In addition, the caspase groupings can be based on structure. Caspases with long prodomains (caspases 1, 2, 4, 5, 8, 9, 10, 11 and 12) are functionally initiator caspases, mediating signals from upstream adapter molecules. Caspases with short prodomains (caspases 3, 6 and 7) are effector caspases, mechanistically carrying out apoptosis by cleaving specific cellular components. The effector caspases are typically activated by the initiator caspases. All caspases are produced as inactive latent zymogens, enabling fast initiation of the apoptotic response. The CASBAH (CAspase Substrate dataBAse Homepage) database currently lists 405 known caspase substrates [21]. Several targets are well defined, including PARP, which was mentioned above. Proteolytic cleavage of fodrin and gelsolin contributes to effects on cell shape and membrane blebbing [22]. Inactivation of nuclear lamins mediates the corresponding nuclear effects [23]. One of the hallmarks of apoptosis, internucleosomal DNA fragmentation [24], is caused by DNA fragmentation factor, beta subunit (previously known as caspase-activated DNase, CAD), which is activated after caspase-mediated cleavage and separation from its inactivating partner, DNA fragmentation factor, alpha subunit (previously known as inhibitor of CAD, ICAD) [25, 26]. A characteristic DNA ladder, consisting of multiple integers of 180 base pair fragments, can be visualized when the cleaved DNA is separated by electrophoresis. A precise functional significance has not been identified for all caspase substrates, however, suggesting that many caspase targets could be “innocent bystanders”.

The intrinsic mitochondrial pathway of apoptosis: the apoptosome

Caspases can be activated in several ways. The intrinsic or mitochondrial pathway is activated by diverse cell stressors, such as genotoxic damage or growth factor withdrawal (Fig. 3). Pro-apoptotic signals induce the release of cytochrome c from the mitochondrion in a process called MOMP (mitochondrial outer membrane permeabilization). Once cytosolic, cytochrome c induces the formation of the apoptosome, a multimeric complex containing several molecules of cytochrome c,

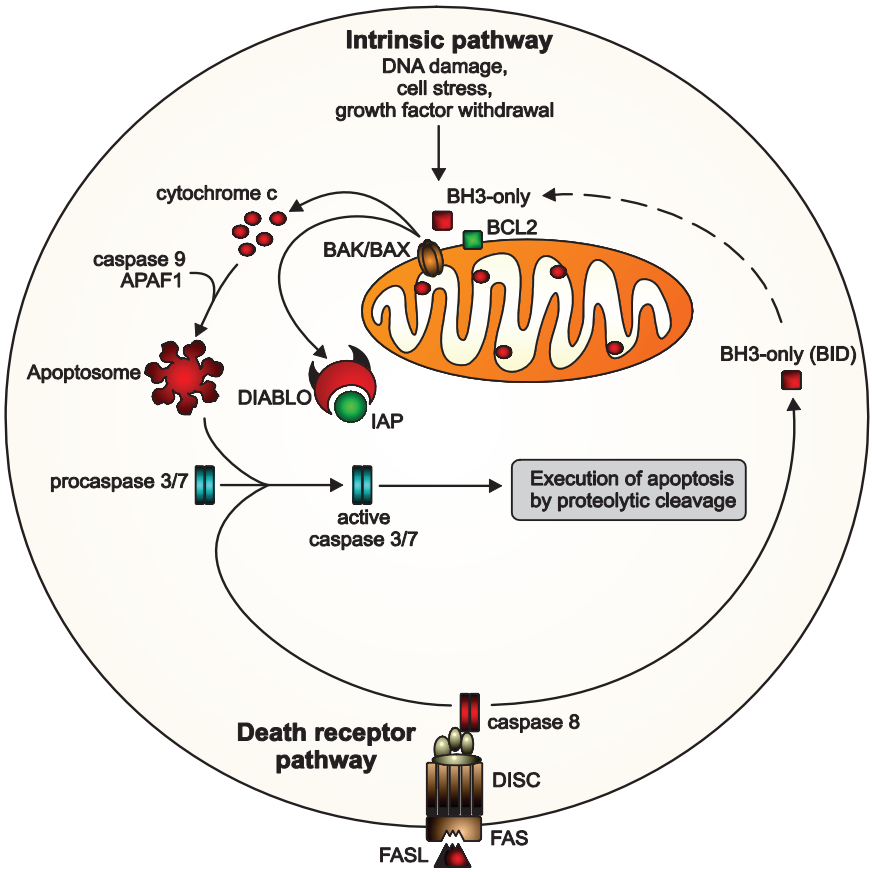

FIGURE 3

Schematic overview of the intracellular apoptotic pathways. The convergence of the intrinsic (mitochondrial) and death receptor pathways on the caspase cascade system is indicated. APAF1, apoptotic peptidase activating factor 1; BAK, BCL2-antagonist/killer 1; BAX, BCL2-associated X protein; BCL2, B-cell leukemia/lymphoma 2; BID, BH3-interacting domain death agonist; BH, BCL2 homology domain; DIABLO, diablo homolog (Drosophila); DISC, death-inducing signaling complex; FAS, tumor necrosis factor receptor superfamily member 6; FASL, Fas ligand (TNF superfamily, member 6); IAP, inhibitor of apoptosis.

apoptotic peptidase activating factor 1 (APAF1) and caspase 9 [27]. The formation of the apoptosome activates the proteolytic activity of caspase 9, setting in motion the downstream effector caspase cascade. Additional pro-apoptotic components are simultaneously released from the mitochondrion, such as diablo homolog (Drosophila) (DIABLO) [28, 29]. The inhibitor of apoptosis proteins (IAPs) are post-mitochondrial apoptosis modulators [30] that are inactivated by interaction with DIABLO [28].

Considering the critical implications of setting this system in motion for the individual cell, one can appreciate the need for tight control of cytochrome c release. This task is performed by the B-cell leukemia/lymphoma 2 (BCL2) family of proteins. These proteins are divided into three groups based on the presence of 1–4 BCL2 homology domains (BH1–4). The proteins that contain all four domains (e.g. BCL2 itself) are anti-apoptotic and typically reside in the outer mitochondrial membrane, where they dimerize with other BCL2 proteins. The two remaining groups are pro-apoptotic and comprise an effector group and a BH3-only group. Two competing hypotheses exist for how these proteins interact to regulate the release of cytochrome c [31]. In the anti-apoptotic protein neutralization model, the effectors BCL2-antagonist/killer 1 (BAK) and BCL2-associated X protein (BAX) must be continually inhibited by binding to anti-apoptotic BCL2 members, or MOMP ensues upon homo-oligomerization of BAK and BAX to form proteolipid pores in the membrane. In this model, the BH3-only proteins sequester the anti-apoptotic proteins, thereby preventing their binding to the effectors. The direct activation model states that the effectors must be activated by BH3-only proteins to cause MOMP. In this scenario, activator-BH3-only proteins can be sequestered by anti-apoptotic members, and other BH3-only proteins act as de-repressors or sensitizers, thereby shifting the balance of apoptosis.

The death receptors

Examples of death receptor family members are tumor necrosis factor receptor superfamily member 1 and tumor necrosis factor receptor superfamily member 6 (FAS). The death receptors thus mediate apoptotic signaling by cytokines such as tumor necrosis factor (TNF) and Fas ligand (TNF superfamily, member 6) (FASL). Once activated by ligand binding, the receptors multimerize and thereby form a death-inducing signaling complex (DISC), which mediates downstream signals via adapter molecules to caspase 8 [20]. Depending on the cell type, activation of caspase 8 suffices to activate downstream effector caspases and apoptosis, or

interaction with the mitochondrial pathway by means of activation of the BH3-only BH3-interacting domain death agonist (BID) is necessary [32, 33].

Regulation of apoptosis in the ovary

The life or death of an individual follicle is regulated differently depending on the stage of development of the follicle [34]. In primordial and primary follicles, survival factors derived from the oocyte determine the fate of the follicle, while the somatic (granulosa) cells are critical for survival once the antral stage is reached [35]. Ultimately, the viability of granulosa cells during development is controlled by a number of factors, the most important of which are the gonadotropins, but locally produced growth and differentiation factors are also important. In this section, the focus will be on factors that are important in the regulation of growth and atresia of antral and luteinizing follicles.

Regulatory factors in antral and luteinizing follicles

Antral follicles are critically dependent on FSH stimulation for survival. In immature or hypophysectomized animals, where serum levels of gonadotropins are low, follicles undergo increased atresia [36, 37], and addition of gonadotropins can rescue these atretic follicles. FSH or human chorionic gonadotropin (hCG) / LH can also prevent the spontaneous onset of apoptosis in cultured early antral and preovulatory follicles [38, 39]. Insulin-like growth factor 1 (IGF1) is a locally produced factor that can suppress apoptosis in antral follicles [38]. The local levels of IGF1-binding proteins regulate the action of IGF1. Fibroblast growth factor 7 (previously known as KGF) suppresses the spontaneous onset of apoptosis in cultured preantral, as well as preovulatory follicles [40]. Additional growth factors of importance are fibroblast growth factor 2 (previously known as bFGF), transforming growth factor alpha (TGFA) and epidermal growth factor (EGF), all of which inhibit the apoptosis of cultured preovulatory granulosa cells [41]. In preovulatory follicle culture, the cytokine interleukin 1 beta induces nitric oxide production, which stimulates production of cyclic GMP and inhibits apoptosis [42]. Estrogens are of central importance for the growth of antral follicles. Consequently, estrogen withdrawal has been shown to induce apoptosis of antral follicles after a two-day treatment of immature hyophysectomized rats with estrogens [43]. In contrast to estrogens, androgens were shown to induce rather than inhibit apoptosis in the same study. Progesterone has also been described as a

regulator of ovarian apoptosis and will be discussed in detail in subsequent sections.

The transforming growth factor beta (TGFB) superfamily of cytokines has attracted a great deal of attention recently as possible regulators of follicle growth and atresia [44]. Members of this family include activin, inhibin, TGFA, TGFB, bone morphogenetic proteins, growth differentiation factor 9 and nodal. Many of these proteins are involved in oocyte-granulosa cell communication directing early follicle growth and in modulation of hormone production by antral follicles [44]. Nodal acts in a pro-apoptotic manner on granulosa cells and appears to be produced in the theca layer [45]. Treatment with TGFB1 has been demonstrated to reduce apoptosis in human luteinized granulosa cells, but this effect appears to depend on the species studied [46].

Adenylate cyclase activating polypeptide 1 (ADCYAP1, previously known as PACAP) belongs to the secretin-glucagon-vasoactive intestinal peptide family and is produced locally in the ovary. The LH surge induces transient expression of ADCYAP1, which has also been demonstrated to suppress apoptosis of periovulatory follicles [47].

Important atretogenic factors in the ovary include the death receptor ligands, such as TNF and FASL. TNF dose-dependently induces apoptosis in early antral follicles cultured in presence of FSH [48]. FAS is expressed in the granulosa cells of atretic antral rat follicles [49, 50]. Treatment of isolated human granulosa/luteal cells with a monoclonal anti-FAS antibody induces apoptosis if the cells are pre-treated with interferon gamma [51]. The FASL/FAS interaction has also been suggested to cause apoptosis in bovine granulosa cells after serum withdrawal [52]. Studies in isolated granulosa cells from rat antral follicles suggest that the orphan nuclear receptor peroxisome proliferator activated receptor gamma (PPARG) may induce apoptosis [53]. A synthetic PPARG ligand, troglitazone, induced expression of transformation related protein 53 (TRP53, commonly known as P53) and decreased the expression of BCL2 [53]. Additional pro-apoptotic factors include interleukin 6 [54] and gonadotropin releasing hormone 1 [55].

Intracellular pathways

The endocrine, paracrine and autocrine factors controlling the survival or death of growing follicles often act via common and redundant intracellular signaling pathways. One of the most central pathways is the cAMP/PKA system, mediating not only the effects of FSH and LH, but also of locally produced ADCYAP1.

Apoptosis of cultured early antral rat follicles can be prevented by treatment with cAMP [39]; however, the PKA pathway is not the only system that mediates FSH- and LH-regulated granulosa cell apoptosis. Other survival systems that are activated in parallel include the thymoma viral proto-oncogene (AKT), mitogen-activated protein kinase (MAPK) and serum/glucocorticoid regulated kinase 1 (SGK1) signaling pathways [56, 57].

The AKT family of kinases is activated by phosphorylation as a result of receptor binding of various growth factors, including IGF1 and TGFA, and activation of the phosphatidylinositol 3-kinase (PI3K) pathway [58, 59]. In support of an anti-apoptotic role for the AKT family, studies have shown that the AKT inhibitor LY294002 induces apoptosis in cultured rat granulosa cells [60]. SGK1 is a protein kinase similar to AKT, which is also activated by in the PI3K pathway. SGK1 is up-regulated by both FSH and LH and has been implicated in the growth and differentiation of granulosa cells [56, 61, 62]. EGF- and TGFA-induced phosphorylation of MAPK1 (commonly known as ERK) has been suggested to promote the survival of granulosa cells, whereas growth factor withdrawal has been associated with loss of MAPK activity and induction of apoptosis [58, 63, 64].

Binding of TNF to death receptors can have both pro- and anti-apoptotic effects in granulosa cells. The pro-apoptotic actions include caspase 8 activation, as described above, among others. The survival-promoting effects are mediated by activation of the transcription factor protein complex nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) [65, 66].

TRP53 is one of the most studied tumor suppressor genes. TRP53 halts cell cycle progression in case of DNA damage in order to allow for repairs and, if the damage is extensive, it induces apoptosis. In the rat ovary, TRP53 is down-regulated after FSHR stimulation and up-regulated as a result of culture under serum-free conditions, indicating a role in granulosa cell apoptosis [67]. Similarly, the human ortholog, TP53, is up-regulated in atretic follicles of the human ovary in vivo, as well as during serum-free culture of luteinizing granulosa cells [68]. The addition of hCG to serum-free cultures inhibits the induction of TP53 [68].

The TGFB family members signal via the MAD homolog (Drosophila) (SMAD) pathway, resulting in transcriptional regulation [69]. Studies of *Smad3*-knock-out mice suggest an important function for this pathway in follicle growth and atresia, since *Smad3*−/− mice are infertile and show aberrant follicle growth as well as anomalies in the expression of BCL2 family members [70]. There are reports of

crosstalk between the PI3K and SMAD pathways, demonstrating that activation of AKT can inhibit the transcriptional activity of SMAD3 [71, 72].

Ovulation and luteinization

The current understanding of the process of ovulation is that the ovulatory LH surge induces follicle changes similar to those seen in an acute inflammatory reaction [73]. This reaction involves the induction of protease activity, which degrades the extracellular matrices of the connective tissues of the follicle, leading to eventual rupture of the follicle and release of the cumulus-oocyte complex. Genetic analyses have identified a large number of genes that are likely involved in the ovulation process. Most of these genes are involved directly or indirectly in inflammation, either as immediate-early transcription factors or as pro- or anti-inflammatory agents. In addition, genes are induced that help protect the cells from the increased oxidative stress that is associated with inflammation [74]. Three major groups of biochemicals have received substantial attention as regulators of ovulation, namely progesterone, prostaglandins and proteolytic enzymes. The role of progesterone will be further discussed in a subsequent section.

Prostaglandins are formed from membrane phospholipid-derived arachidonic acid and are associated with inflammation. Their role in ovulation was proposed after it was reported that the nonsteroidal anti-inflammatory agent indomethacin (which inhibits prostaglandin synthesis) blocks ovulation [75-79]. The prostaglandin level is known to transiently increase after hCG administration in the immature rat model. Accordingly, prostaglandin-endoperoxide synthase 2 (commonly known as COX2), the enzyme responsible for prostaglandin synthesis, is also up-regulated after hCG treatment [80]. The varying duration of the periovulatory interval in different species might be a function of the time between the LH surge and the induction of prostaglandin synthesis [73]. The exact functional role of the prostaglandins has not been clearly elucidated, but stimulation of angiogenesis [81] and proteolytic enzyme activity [82] have been suggested.

Involvement of protease activity in ovulation was proposed as early as 1916, but support for the theory did not emerge until the mid-1960s. Plasminogen activators and related factors have been implicated in ovulatory mechanisms, but reported data are contradictory, and the function of the plasminogen activators has not been firmly established [73]. Additional proteolytic enzymes that have been investigated are the matrix metalloproteinase family and the ADAMTS enzymes (a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin motif) [73].

When the ovulatory follicle ruptures and collapses, the corpus luteum forms from the remnants. The transition is known as luteinization and involves tissue remodeling, morphological changes, massive angiogenesis, cell cycle regulation and changes in the steroidogenic machinery [83]. These changes take place in parallel with the inflammatory ovulation process; thus, the luteinization of follicular cells can be considered to commence at the time of the LH surge. While the LH surge is clearly the main inducer of the luteinization process, an influential role for the oocyte has been suggested [83].

During luteinization, the follicular cells enter their terminal differentiation stage and exit from the cell cycle. In the rat, the cell cycle is arrested by 4 h after gonadotropin stimulation [84, 85], and in primates granulosa cell proliferation is decreased 12 h after the LH surge [86]. Studies on bovine granulosa cells indicate that exit from the cell cycle renders the cells insensitive to FASL-induced apoptosis [87].

An important shift in steroidogenic synthesis occurs during luteinization. Whereas the preovulatory follicle mainly produces estrogens, the main product of the corpus luteum is progesterone (in primates, parallel synthesis of estrogens remains in the corpus luteum). This shift and the general steroidogenic increase are mediated by the transcriptional regulation of several genes, including expression of STAR, which is increased in a biphasic manner in the rat with a transient peak shortly after the LH surge, followed by second rise after a few days [88]. Expression of cytochrome P450, family 11, subfamily a, polypeptide 1 (CYP11A1, commonly known as P540scc) [89] and HSD3B [90] is induced in the granulosa cells with the net effect that the granulosa cells become highly competent for progesterone production.

Many of the factors that have been described to be involved in ovulation and inflammation appear to affect angiogenesis as well. Angiogenesis is critical for the establishment of a functional corpus luteum, in which a large capillary network supplies progesterone to the circulation. The process includes modulation of the existing extracellular matrices by proteolysis and involves proteolytic enzymes [91]. The inflammatory mediators of ovulation induce increased expression of vascular endothelial growth factor A (VEGFA), which is an important angiogenic factor that acts as a mitogen and survival factor for endothelial cells [92]. While in general, angiogenic factors like VEGFA are produced during tissue hypoxia, the expression of VEGFA in the ovary is under hormonal control [93]. In addition, VEGFA has been reported to promote survival in an auto/paracrine manner in granulosa cells of bovine follicles [94].

Extensive tissue remodeling takes place during corpus luteum formation. This remodeling likely plays an important functional role. Relating to the previous paragraph, VEGFA has been reported to mediate the effects of hCG on cell-matrix interactions [95]. Both VEGF and hCG induce expression of fibronectin and its integrin receptors in human luteinized granulosa cells, thereby stimulating adhesion, migration and survival. Furthermore, during ovulation, a specialized hyaluronan-rich extracellular matrix forms around the oocyte and between the cumulus cells; a process known as cumulus expansion. This process is considered critical for oocyte release and is influenced by prostaglandins and oocyte-derived factors [73].

Progesterone

Progesterone is a member of the steroid hormone family and functions as a central hormonal regulator of normal female reproduction and pregnancy. The major site of progesterone production in the non-pregnant female is the corpus luteum. The physiological functions of progesterone include effects on the ovary in conjunction with ovulation and follicle rupture, and effects on the uterus that are necessary for implantation and the maintenance of pregnancy. In the mammary glands, progesterone is required for lobular-alveolar development and inhibition of milk production during pregnancy. Progesterone is also a neuroactive steroid that affects, for example, sexual response behavior. Furthermore, progesterone, or rather a progesterone metabolite, allopregnanolone, mediates actions in the brain by binding to the gamma-aminobutyric acid (GABA-A) receptor [96].

Nuclear progesterone receptors

Classically, the effects of progesterone are mediated by the nuclear progesterone receptor (PGR), a ligand-activated transcription factor. The structure of PGR has been well studied since it was first cloned and characterized in 1986 in chicken [97]. The basic structure of PGR is shared by all nuclear receptors and includes five functional domains. The ligand-binding domain (LBD) is located C-terminally, and the DNA-binding domain (DBD) is located centrally, separated from the LBD by a hinge region. There are transactivation domains, known as AF (for Activation Function), that are located N-terminally to the DBD (AF1) and in the LBD (AF2) [98] (Fig. 4). Two major receptor isoforms are transcribed from the *Pgr* gene. PGR-A is a 164 amino acid N-terminally truncated isoform, relative to PGR-B. The two PGR isoforms are functionally different: PGR-B is a stronger

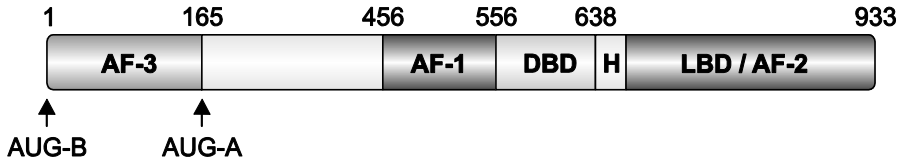

FIGURE 4

Schematic overview depicting the structure of the progesterone receptor. The start codons (AUG) for PGR-A and PGR-B are indicated. AF, activation function; DBD, DNA-binding domain; H, hinge region; LBD, ligand-binding domain. The numbering indicates the amino acids of the protein.

transactivator than the A isoform due to a third transactivation domain called AF3, which is located in the PGR-B-unique sequence [99]. As a result, the PGR isoforms recruit specific subsets of co-regulators and regulate different subsets of genes in a cell- and promoter-specific manner [100, 101]. In cell types in which the PGR-A form is not transcriptionally active, PGR-A instead functions as a dominant repressor of PGR-B activity, as well as of the activity of other receptors, including the estrogen receptor [102, 103]. The balance between the PGR isoforms in a target tissue could determine the response to progesterone. This balance is regulated in a hormone- and differentiation-dependent manner [104]. The individual genes that are under the control of the respective PGR isoforms have been investigated in the T47D breast cancer cell line [101].

The free inactive PGR resides in the cytoplasm or nucleus bound to heat shock proteins. This interaction seems to be of importance for activation of PGR upon ligand binding [105]. When progesterone binds to PGR, the heat shock proteins dissociate from the receptor, and two receptor molecules dimerize and are translocated to the nucleus. The active PGR dimer binds to hormone response elements (HRE) in the promoter regions of target genes. There, the AFs recruit a multitude of co-regulators that in turn mediate interactions with the transcriptional activation machinery and chromatin-remodeling proteins. The HREs are common for PGR and the receptors for glucocorticoids, androgens and mineralocorticoids. The consensus sequence consists of two half-site imperfect palindromic sequences separated by a short spacer, 5'-AGTACA-XXX-TGTTCT-3' [106]. Despite the fact that these receptors share a common HRE, they induce different responses in gene expression in cells were they are co-expressed. It is thought that small differences in the HREs and surrounding bases confer conformational changes to the entire bound receptor, thereby affecting the recruitment of co-regulators and ultimately the receptor-specific response [107]. The AF1 sites of different nuclear

receptors have a low degree of sequence homology, which could contribute to differential co-regulator recruitment [98].

In addition to the regulation of classical progesterone target genes, there are several examples of genes that are regulated by progesterone, but lack HREs in their promoter regions. Such regulation is caused by ligand-dependent PGR cross-talk with other transcription factors [108]. Interestingly, this type of gene regulation can take place even if the ligand binding to the PGR is an antagonist. In addition to the various transcriptional effects of progesterone, rapid, non-genomic responses have been demonstrated to be mediated via the PGR. Such effects can be mediated via ligand-dependent direct interaction of the PGR with the SRC homology 3 (SH3)-domains of the Rous sarcoma oncogene (SRC), which leads to activation of the MAPK signaling pathway [109]. Many aspects of PGR signaling have been shown to be influenced by phosphorylation of the receptor as well as of the involved co-regulators [110]. One reported effect of phosphorylation is ultrasensitization of the receptor, with sensitivity reaching down to picomolar levels of progesterone [111]. The cell cycle kinase cyclin A / cyclin-dependent kinase 2 has been reported to increase the activity of PGR by phosphorylation of several co-regulators, inferring cell cycle-dependent regulation of PGR activity, which accordingly peaks during the S-phase of the cell cycle [112].

Membrane progesterone receptors

As described in a recent review by Gellersen et al [113], it has been known for decades that progesterone can initiate rapid effects independent of transcriptional regulation. This occurs even in the absence of PGR expression, suggesting that other mediators may be responsible for these effects. Typically, rapid effects occur in seconds or minutes; they cannot be abolished by inhibitors of transcription or translation, and they are inducible by cell-impermeable progesterone. Importantly, the studies of rapid progesterone effects and characterization of the receptors have been considered controversial due to several technical difficulties. The most promising candidates for membrane progesterone binding are progesterone receptor membrane component 1 (PGRMC1) and membrane progestin receptor family members.

The membrane progestin receptors are part of the progestin and adipoQ receptor family (PAQR). They were initially cloned as three isoforms: mPRα, β and γ, now termed PAQR7, 8 and 5, respectively [114, 115]. The current knowledge regarding the functional properties of the PAQR family of membrane progestin receptors has

been described and discussed in two recent reviews [113, 116]. These receptors have been suggested to be involved in oocyte maturation in fish and amphibians and in the onset of parturition in humans via several lines of evidence [115, 117]. The initial characterization of these proteins suggested a 7-transmembrane domain structure, typical for classical G-protein-coupled receptors. A study by Krietsch et al., however, failed to corroborate these findings [118]. Recently, a study by Smith et al. using a yeast PAQR expression system provided support for the progesterone-binding properties of the membrane progestin receptors, but at the same time, questioned the notion that the receptors signal via G-proteins [119].

PGRMC1 was first purified from porcine liver microsomal fractions in the search for membrane-associated non-genomic receptors for progesterone [120]. It was recognized to be part of an approximately 200 kDa large oligomeric protein complex that binds progesterone. Its complex functions have been described in several recent reviews [113, 121-124]. The intracellular localization of PGRMC1 are not clearly defined, and it has been reported to be present in the plasma membrane, endoplasmic reticulum, nucleus and cytoplasm [122]. Progesterone has long been thought of as a ligand for PGRMC1, but clear evidence of this hypothesis is lacking, and other proteins in the 200 kDa microsomal complex have been suggested as the progesterone-binding component [124]. Furthermore, steroid specificity was low in the original study, in which binding to the PGRMC1 complex was also demonstrated for testosterone, cortisol and corticosterone, as well as various drugs [120]. Regulation of expression of PGRMC1 by progesterone has been reported in several studies, but appears to be tissue-specific [124].

It appears that PGRMC1 interacts with different binding partners depending on the cell type, suggesting multiple possible functions. In the ovary, PGRMC1 has been proposed to regulate granulosa cell apoptosis by interaction with serpine1 mRNA binding protein 1 (SERBP1) [125, 126]. The PGRMC1/SERBP1 complex has been suggested to regulate granulosa cell apoptosis in developmental stages during which the nuclear receptor is not expressed, including the antral follicles from immature rats and the luteinized granulosa cells of the corpus luteum. The intracellular signaling mechanisms involved have not been completely established, but involvement of regulated Ca^{2+} levels [127] and activation of protein kinase G [128] has been suggested. A direct interaction between PGRMC1 and cytochrome P450 proteins has been demonstrated recently, implicating PGRMC1 in the metabolism of drugs, cholesterol and hormones [129]. In addition, PGRMC1 has been proposed to play a role in the regulation of cholesterol synthesis in COS-7 cells [130].

Progesterone in ovulation and luteinization

Expression of PGR in granulosa cells is transiently induced by LH in rodents [131, 132], in contrast to humans, where PGR is also expressed in the corpus luteum [133, 134]. In general, PGR is not thought to be expressed before the LH surge; however, a recent study reported expression of the PGR-B isoform in granulosa cells at virtually all developmental stages, as well as in the corpus luteum in the rat [135]. The expression of PGR in other ovarian cell types appears to be species-specific.

An intraovarian role for progesterone during ovulation as a mediator of the effects of gonadotropins was postulated by Rondell in 1974 [136]. In 1981, Rothchild proposed that progesterone could promote the development of the corpus luteum [137]. Today, it is well established that progesterone is necessary for ovulation, as was demonstrated by Snyder et al., who showed that ovulation in rats could be blocked by inhibition of steroidogenesis in a manner that was reversible by add-back of progesterone [138]. Furthermore, preovulatory treatment with RU 486 inhibits ovulation in gonadotropin-primed immature mice [139], and PGR-knock-out mice fail to ovulate [140], demonstrating a critical role for PGR as a mediator of these effects of progesterone. Studies of mice in which the PGR isoforms have been selectively ablated have revealed the physiologically different functions that they control [141]. It appears that ablation of PGR-A severely reduces superovulation, but without completely abolishing it, whereas ablation of PGR-B has no apparent effect on ovulation. Both receptor isoforms, therefore, seem to be involved in the ovulatory response, but only the PGR-A isoform is critical. Furthermore, studies on selective PGR isoform knock-outs demonstrated that PGR-A is necessary for uterine development, whereas PGR-B is necessary for mammary gland development during pregnancy.

The specific functions of progesterone in ovulatory mechanisms are not clearly established, but inhibition of proteolytic activity and follicle rupture has been demonstrated. Progesterone and PGR are necessary for the increased periovulatory expression of ADAMTS1 [140, 142], ADCYAP1 [143] and cathepsin L [140], all of which have been implicated in the ovulatory process. Treatment with PGR antagonists has also been reported to reduce the levels of prostaglandins in the rat, suggesting a connection between progesterone and prostaglandin signaling [144]. Recently, transient periovulatory expression of endothelin 2 (EDN2), which acts in an auto-/paracrine manner to control follicle rupture was shown to be regulated by PGR [145]. Subsequent studies demonstrated PPARG to be a mediator of the PGR-regulated expression of EDN2 [146].

Whether progesterone plays a role in luteinization is not clearly established. It is possible that the importance of progesterone is species-specific. In rats, which have a short periovulatory interval, the importance of progesterone is less clear than in primates and other species with long periovulatory intervals and functional corpora lutea with prolonged PGR expression. Initial studies on PGR-knock-out mice reported that luteinization was absent [147], but subsequent studies showed that, despite the lack of follicle rupture, the granulosa cells expressed markers of luteinization [140]. In contrast, in the macaque, the essential role of progesterone for luteinization has been established [148, 149].

Several studies have identified progesterone as a survival factor in the periovulatory interval and in luteal cells. The PGR antagonists Org 31710 and RU 486 have been reported to induce apoptosis in rat [150], human [151, 152] and mouse [153] luteinizing granulosa cells. Progesterone and RU 486 have also been reported to affect survival in the bovine corpora lutea of early pregnancy [154]. There have been suggestions that progesterone might stimulate its own synthesis. ADCYAP1, which is regulated by PGR, have been shown to stimulate progesterone secretion in gonadotropin-primed immature rats [155]. Furthermore, progesterone-mediated regulation of HSD3B has been suggested, but not clearly established [156-158]. The suggested local effects of progesterone during the periovulatory interval include effects on proliferation and the cell cycle. In bovine luteal cells, increased resistance to FASL-induced apoptosis seen after the LH surge has been reported to be dependent on PGR-mediated cell cycle exit [87]. During the late periovulatory interval of the macaque, progesterone was shown to affect the expression of cyclins, supporting a possible role in cell cycle exit [86].

The cholesterol synthesis pathway and its derivatives

The common basic substrate in the synthesis of all steroids is cholesterol (Fig. 2). Cholesterol exists in the cell either as free cholesterol, mainly bound to the plasma membrane, or stored in the form of cholesteryl esters in cytoplasmic lipid droplets. There are two available sources to replenish the cellular supply of cholesterol: 1) de novo synthesis from acetate; and 2) receptor-mediated extracellular uptake of cholesteryl esters from circulating lipoproteins, low density lipoprotein (LDL) in humans and high density lipoprotein (HDL) in rodents [159].

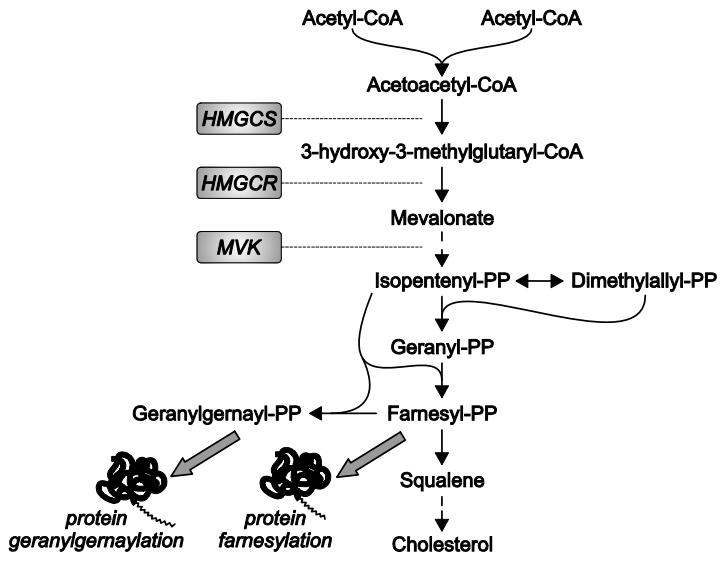

FIGURE 5

Schematic overview showing the cholesterol synthesis pathway, including the isoprenylation branch-point reactions. The enzymes catalyzing the major initial steps of the pathway are shown as grey boxes. HMGCS, 3-hydroxy-3-methylglutaryl-Coenzyme A synthase; HMGCR, 3-hydroxy-3-methylglutaryl-Coenzyme A reductase; MVK, mevalonate kinase; -PP, -pyrophosphate.

The de novo biosynthesis of cholesterol is a well-characterized process that is highly regulated to balance cellular needs and avoid toxic sterol accumulation (Fig. 5). This balance is maintained by feedback inhibition of several of the enzymatic steps in the biosynthetic pathway [160]. The rate-limiting enzymatic step is the conversion of 3-hydroxy-3-methylglutaryl-Coenzyme A into mevalonate, which is catalyzed by 3-hydroxy-3-methylglutaryl-Coenzyme A reductase (HMGCR), residing in the smooth endoplasmic reticulum. The activity of HMGCR can be inhibited by a family of drugs known as statins, which are used clinically to reduce the cholesterol levels in hypercholesterolemic patients [161]. Physiologically, the activity of HMGCR is regulated at the transcriptional, translational and post-translational levels [162]. Transcriptional regulation is sterol-sensitive and is mediated through sterol regulatory elements (SRE) located in the promoter regions of the target genes. The SRE is bound by mature SRE binding proteins (SREBP), thus initiating gene transcription [163].

The cellular demand for cholesterol increases after the LH surge, when the luteinizing granulosa cells begin producing progesterone. As described in the section covering luteinization, transcriptional regulation of several genes mediate

the increase in steroidogenesis, and ensures adequate cellular access to cholesterol. In highly steroidogenic cells, such as luteinized granulosa cells, the major source of cholesterol used for steroid production is of extracellular origin, i.e., from circulating lipoproteins. This has been demonstrated in human luteinized granulosa cells, which produce normal amounts of progesterone even if the de novo biosynthesis of cholesterol is inhibited, as long as the cells have access to serum lipoproteins [164]. Accordingly, the gene encoding the receptor that mediates uptake of cholesteryl esters from HDL in rodents, scavenger receptor class B, member 1 (*Scarb1*) [165], is one of the genes that are induced by the LH surge [166].

Protein isoprenylation

Cholesterol is but one of the end products of the cholesterol synthesis pathway. Farnesyl pyrophosphate is the common substrate for the so-called branch-point reactions responsible for the synthesis of not only cholesterol but also ubiquinone- and heme-A side chains, dolichol, isopentenyladenine, and protein isoprenylation substrates [167, 168]. When the supply of extracellular sources of cholesterol is high, the resulting low flux in the pathway is preferentially shunted into these higher affinity critical non-sterol pathways. This is known as the flux diversion hypothesis and serves as an additional regulatory system in the mevalonate pathway [160, 169].

Protein isoprenylation is the post-translational attachment of a lipophilic isoprenoid moiety, farnesyl or geranylgeranyl, to a C-terminal cysteine residue of a target protein (Fig. 5). Target proteins are defined by a C-terminal recognition sequence, CAAX, CAC or CC, where A is an aliphatic amino acid and X is any amino acid. The acquired lipophilic moiety enables membrane anchoring of the protein, but isoprenylation has also been suggested to play a role in protein-protein interactions [170]. The enzymes catalyzing the isoprenylation process, polyisoprenyltransferases, have been well characterized and include protein farnesyltransferase [171], protein geranylgeranyltransferase type I [172], and RAB geranylgeranyltransferase [173]. Subsequent to completed isoprenylation, the CAAX target proteins are further modified by a prenylation-dependent endopeptidase, which cleaves between C and A_1 [174]. The prenylated cysteine is then methylated, adding further to the protein's lipophilicity, at least concerning farnesylated proteins [175].

Inhibition of the isoprenylation process is generally considered to cause loss of function of the target protein. Isoprenylated proteins are often involved in important cellular functions, such as signal transduction pathways. Examples include the small GTPases, notably the rat sarcoma viral oncogene (RAS) protein family, and the heterotrimeric G-proteins. The RAS proteins have been subjected to extensive studies, since mutations of RAS proteins are found in 30% of all human cancers [176]. One of the RAS subfamilies, the RAB proteins, facilitate vesicular trafficking between compartments of exo- and endocytotic pathways [177]. The double geranylgeranylation of RAB proteins is necessary both for membrane association of the active form of the proteins and also for the interaction between cytosolic RAB proteins and GDP-dissociation inhibitors [178].

Studies of primary cells derived from several different tissues, and established cell lines, have shown that depletion of isoprenylation substrates can lead to the induction of apoptosis [179-183], as well as inhibition of proliferation [184], cell migration [184] and angiogenesis [185]. The effects on proliferation and apoptosis do not seem to be related to the other pathways originating from farnesyl pyrophosphate [186, 187]. A study by Gadbut et al. is one of few supporting regulation of isoprenylation in a physiological context [188].

AIMS OF THE THESIS

The overall aim of this thesis was to characterize the role of progesterone during the periovulatory interval in the rat ovary. The main focus was on the specific role of progesterone in the regulation of granulosa cell apoptosis and the transcriptional changes mediated by the nuclear progesterone receptor.

Specific aims of papers I–IV

Paper I

1. To characterize the transcriptional effects of specific PGR antagonists inducing apoptosis in isolated rat periovulatory granulosa cells.
2. To investigate the effect of PGR antagonists on de novo cholesterol synthesis.
3. To study the influence of de novo cholesterol synthesis on granulosa cell apoptosis.

Paper II

1. To determine the involvement of protein isoprenylation as a mediator of PGR antagonist-induced granulosa cell apoptosis.

Paper III

1. To investigate the specific role of PGR as a regulator of granulosa cell apoptosis.
2. To study the involvement of alternative receptors for progesterone signaling in granulosa cell apoptosis.

Paper IV

1. To characterize early and late transcriptional changes caused by low doses of specific PGR antagonists in periovulatory granulosa cells.
2. To identify novel genes potentially mediating the effects of progesterone on apoptosis regulation.

METHODOLOGICAL CONSIDERATIONS

The materials and methods used in this thesis are described in detail in the individual papers. Here, a general description is presented, covering the most central methods.

The gonadotropin-primed immature rat model

The basic animal model system used in this thesis was the common laboratory rat (*Rattus norvegicus*) of the Sprague-Dawley stock. Some differences in the rat and human reproductive systems deserve discussion. In contrast to humans, the rat has a shorter periovulatory interval and transient expression of PGR during this period [131, 132]. The corpus luteum is less developed in the rat, and the luteal phase is short unless pregnancy ensues.

Immature (26 days old) female rats were injected with equine chorionic gonadotropins (eCG, 10 IU, s.c.) to induce folliculogenesis (Fig. 6). Equine CG is the horse equivalent of human CG and has some useful properties, including its high carbohydrate content, which extends its half-life in vivo relative to FSH [189]. In contrast to hCG, eCG has both FSH- and LH-like properties [190]. Approximately 48 h after eCG injection, a cohort of follicles will have reached the preovulatory stage of development. A subsequent injection of hCG (50 IU, i.p.) serves to mimic the endogenous LH surge and, thus, induces ovulation of mature follicles. While hCG, like LH, binds to LHR, it has the advantage of a longer half-

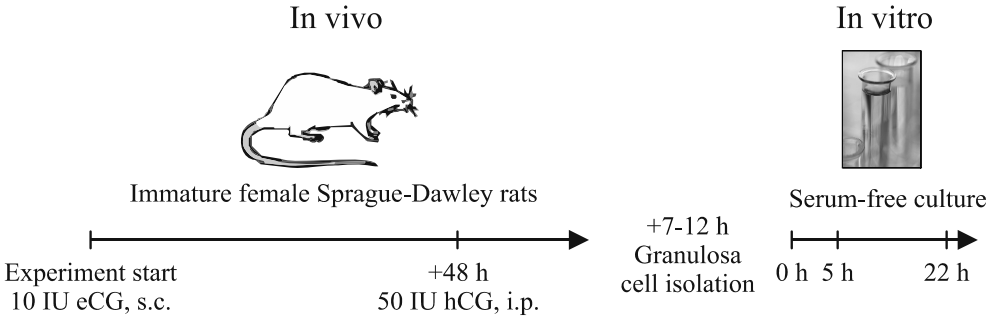

FIGURE 6

Schematic depiction showing the animal model system used throughout this thesis. eCG, equine chorionic gonadotropin; s.c., subcutaneous injection; hCG, human chorionic gonadotropin; i.p., intraperitoneal injection.

life and higher receptor affinity than LH, making it more useful in an experimental setting [191]. The technique of inducing follicle growth, development and ovulation by injections of gonadotropins bypasses the inherent problems of studying ovaries in adult animals. While adult ovaries contain a heterogeneous collection of follicles of various sizes, as well as corpora lutea, gonadotropin-primed immature ovaries enable the study of follicles at a precise and uniform developmental stage [41]. In addition, the absence of endogenous gonadotropins in immature animals bypasses pituitary-related issues that are encountered when animals are treated in vivo.

In vitro model of the periovulatory interval

The periovulatory interval is defined as the time between the endogenous LH surge (or hCG injection) and follicle rupture [192]. In the rat, this interval is approximately 14 h. Periovulatory granulosa cells were isolated from gonadotropin-primed immature rat ovaries by follicle puncture. In Paper I, the time of isolation was selected to be 12 h after the hCG injection to closely resemble the human model that was used in parallel studies. In Papers II–IV, granulosa cells were isolated at 7 h post-hCG to better enable study of the effects mediated by transient PGR expression, which peaks at this time [131, 153]. The follicles that responded to hCG were identified by their increased size and increased vascularization of the follicle wall. This isolation technique yields a relatively pure population of granulosa cells if care is taken to avoid inclusion of sheets of the theca layer. A small amount of contaminating cells, including oocytes, erythrocytes, adipocytes and immune cells, is expected.

The use of isolated granulosa cells to study physiological periovulatory events has advantages as well as disadvantages. Of major importance in these investigations was that studies on primary isolated granulosa cells facilitate the study of cellular events specific to particular differentiation stages, which is not possible to mimic in cell lines. Some of the factors known to affect the survival of whole follicles in vitro or in vivo, however, are ineffective in isolated granulosa cells, indicating the necessity of paracrine interactions for factors such as IGF1 [41]. These interactions cannot be studied in isolated cells, but their study in cultured follicles is hindered by the fact that isolation of whole follicles is a cumbersome and slow process.

Progesterone receptor antagonists

Considering the diverse functions of progesterone, there are obvious pharmacological applications for anti-progestins in areas such as contraception, uterine bleeding and cancer. Numerous compounds have been synthesized since the discovery of mifepristone at the beginning of the 1980s, ranging from pure antagonists to partial agonists/antagonists. Unfortunately, due to the politically sensitive issue of abortion and the first clinical application of mifepristone in pregnancy termination, the development of these compounds has been slow [193].

Mifepristone (Roussel-Uclaf 38486, commonly known as RU 486) was the first clinical PGR antagonist, and is consequently the most studied. The RU 486/PGR complex behaves like progesterone/PGR in most respects, including DNA binding, but the interaction with co-regulators is different. RU 486 has a slightly higher affinity for PGR than progesterone, but it is a more effective competitive antagonist than predicted by its affinity for the receptor. There appear to be three explanations for this: 1) the RU 486/PGR complex binds more strongly to DNA than R5020/PGR (R5020 is synthetic progestin) [194]; 2) the RU 486/PGR complex can heterodimerize with R5020/PGR, resulting in decreased activity of the entire complex [195]; and 3) the RU 486/PGR complex recruits additional co-repressors, contributing to its antagonist action [196]. In vitro, some PGR antagonists, including RU 486, can be converted into agonists in a cell- and context-dependent manner [197]. Only the PGR-B isoform mediates such partial agonist actions. Treatment with 8-bromo-cAMP triggered an antagonist/agonist transition in T47D breast cancer cells [198]. In addition, RU 486 was only partially effective in antagonizing the effects of R5020 in the presence of 8-bromo-cAMP [199]. In addition to its affinity for PGR, RU 486 is also a glucocorticoid receptor antagonist.

In contrast to RU 486, Org 31710 is more specific for PGR; its affinity for the glucocorticoid receptor is 30-fold lower than that of RU 486 [200]. Both Org 31710 and RU 486 bind weakly to the androgen receptor (2.3 and 4.7%, respectively, compared to that of dihydrotestosterone). The binding of Org 31710 to PGR has been reported to be similar to that of RU 486.

Detection of apoptosis

To detect and quantify apoptosis in the model system used in this thesis, a method was required that was sensitive enough to detect subtle changes in affected cells.

The method should also work with relatively few cells in suspension. In paper I, we used a technique to quantify the degree of apoptosis based on the amount of fragmented DNA present in each sample. As described above, the fragmentation of DNA is a hallmark of apoptosis, but the detection of fragmented DNA is not a qualitative proof of apoptosis, given that necrosis also leads to nonspecific DNA fragmentation. In the subsequent papers, this method was replaced with a faster and more efficient technology based on quantification of activated caspases 3 and 7. The assay is based on a luminogenic caspase 3/7 substrate containing the tetrapeptide target amino acid sequence for caspase 3/7, DEVD. Following caspase cleavage, amino-luciferin is released leading to production of light after reaction with luciferase. The luminescent signal is proportional to the amount of active caspases in the sample. When interpreting the measured caspase activity, the following limitations should be kept in mind: 1) some or all cells could have been immediately affected by the investigated treatment, such that the peak caspase activity has passed; 2) all of the cells could have been affected, but only a subset entered the apoptotic process; or 3) only a subset of cells could have been affected by the treatment, e.g., due to lack of receptor expression. Considering these limitations, time course studies should be performed when investigating caspase activity in a cell population. Throughout this thesis, ‘caspase 3/7 activity’ and ‘apoptosis’ are used interchangeably.

Cholesterol synthesis assay

In Paper I, the observed transcriptional regulation of cholesterol synthesis enzymes, suggested regulated de novo cholesterol synthesis. This was functionally validated by quantification of cholesterol synthesis products. Granulosa cells were incubated in the presence of ^{14}C-labeled acetic acid during the final 5 h of cell culture. Sterols and steroids were extracted from each sample by chloroform/methanol-extraction and separated by thin layer chromatography (TLC). The incorporation of ^{14}C into cholesterol, cholesteryl esters and progesterone was subsequently quantified.

In TLC, samples are loaded as bands onto a thin 20×20 cm silica solid phase plate. The plate is placed in a closed chamber containing the mobile phase (solvent), which travels by capillary action through the plate. The samples on the plates are dissolved in the migrating solvent and move along up through the plate. The components of the sample are differentially retained in the solid phase based on their affinities for the solvent and the solid phase. Thus, the choice of solvent or

solvent mixture will affect which components are separated. The strength of the solvent will determine how fast the components of interest migrate relative to the solvent front. The selectivity of the solvent will affect the resolution of the separation, i.e., the ability to separate two bands. The correct choice of solvent will, therefore, affect the ability to draw conclusions from a TLC experiment.

The bands separated by TLC were visualized by iodine staining and identified by comparison to included known references. Quantification was based on scraping of bands followed by scintillation counting in Paper I and by densitometry in Paper II. The advantage of the second approach is that all bands are visualized, minimizing factors such as unknown interference of nearby bands in the quantification.

Quantification of protein isoprenylation

In Paper II, a lipophilicity-based cellular separation technique was used to quantify changes in protein isoprenylation. A non-ionic detergent known as Triton X-114 was used as described in a protocol developed by Bordier in 1981 [201] and modified by Brusca and Radolf in 1994 [202]. Non-ionic detergents are non-denaturing and suitable for solubilization of membrane proteins. During the process, the detergent replaces most of the lipids in contact with the hydrophobic domains of integral membrane proteins, and soluble protein-detergent mixed micelles are formed. Furthermore, non-ionic detergents have a special property in that they tend to form aggregates if the temperature is raised a few degrees over the critical micelle temperature. This is thought to be the result of decreased hydration of the polar head-groups (polyoxyethylene-moieties) as the temperature increases. After this so-called cloud point is reached, a detergent-rich phase (containing approximately 12% Triton X-114) and an aqueous phase (containing approximately 0.04% Triton X-114) are formed. Conveniently, the cloud point for Triton X-114 is only 22°C. This property is used to separate solubilized membrane proteins from hydrophilic proteins, which are not affected by the presence of the detergent. In Paper II, the separate protein fractions were subsequently analyzed by Western blotting to determine the relative contents of specific proteins.

Alternative means of studying the partitioning of proteins include cell fractionation and microscopy techniques. We briefly evaluated subcellular fractionation by ultracentrifugation, confirming the results obtained using Triton X-114. Microscopic evaluation of the intracellular localization of proteins is commonly used to study the regulation of protein isoprenylation. Unfortunately, the cell culture system used in this thesis, with cells in suspension, was poorly adaptable to

this method. The commonly used method of labeling cells with [^{3}H]mevalonolactone to quantify the incorporation of ^{3}H into isoprenylated proteins cannot be used when an expected effect on substrate availability is to be studied. Several alternative approaches to quantify relative isoprenylation have been evaluated and rejected in the laboratory, including size shift of modified proteins in Western blotting and development of antibodies directed against the farnesyl moiety.

Microarray

In Papers I and IV, Affymetrix microarrays were used to study the transcriptional changes induced by the PGR antagonist Org 31710. The microarray technology enables simultaneous detection of the mRNA levels of thousands of expressed genes across the genome in a sample. The microarray system developed by Affymetrix is oligonucleotide-based. Each transcribed sequence present on a specific array is represented by a probe set containing 11–20 probe pairs. Each probe pair consists of a perfect match probe that is exactly complementary to the target sequence and a mismatch probe in which the middle nucleotide has been replaced. A 25-mer synthetic oligonucleotide makes up each probe. The perfect match / mismatch system allows for control of nonspecific hybridization. Isolated RNA from samples of interest is reverse transcribed into complementary DNA, and then transcribed into biotin-labeled complementary RNA. The hybridization of fragmented complementary RNA allows for signal detection from each of the hundreds of thousands of probes in each array and relative quantification of each transcript. In Paper I, an early rat genome array (Rat Genome U34A) was used, containing approximately 8,800 probe sets. In Paper IV, the Rat Genome 230 2.0 array was used, containing more than 31,000 probe sets.

In recent years, the statistical approaches for handling the huge data sets obtained by microarrays have been refined. Importantly, the challenge of multiple inferences must be managed. In Paper I, the relative expression levels in the four arrays were compared using the Affymetrix empirical algorithm MAS4.0, followed by functional validation of the conclusions drawn. In Paper IV, Significance Analysis of Microarrays (SAM) [203] was used to compare relative expression levels. By using false discovery rate (FDR) control, this analysis software is specifically adapted to handle problems of multiple inferences that have been inherent in microarray analyses. The software computes a statistic d_i for each gene i by measuring the strength of the relationship between gene expression and

explanatory variable (e.g. treated or untreated). Significant changes in the data are found using repeated permutations with a user-defined tuning parameter *delta*. The selection of *delta* affects the number of genes reported and the FDR associated with the list of genes. A *q*-value is also reported for each gene, representing the lowest FDR at which the gene is called significant.

While statistical analysis can manage reliable identification of the probe sets affected in a microarray experiment, functional inferences depend on correct annotation of the probe sets. Gene annotations are constantly being updated, and many probe sets still represent unknown genes, thus preventing further studies. Functional conclusions drawn from microarray studies are usually based on gene ontology (GO) analysis. The Gene Ontology project is a collaborative effort to address the need for consistent descriptions of gene products in different databases [204]. The three organizing principles of GO are "cellular component", "biological process" and "molecular function." Statistical methods can be used to provide estimates of whether associations between the regulated genes in a microarray study are greater than what one would expect by chance. This type of analysis is useful for providing biological hypotheses, but should not be thought of as proof of causal relationships. There are several reasons for this: 1) regulation of mRNA levels is not necessarily reflected at the level of protein activity; 2) regulation of a specific set of genes might be irrelevant if another gene product is rate-limiting in the process at hand; 3) the indicated process could be parallel to a different process that is affected by the experimental conditions; and 4) the annotations for the probe sets included in a microarray are continually updated, often involving the inclusion of multiple possible genes within a single probe set, and therefore, it is possible to obtain results of falsely overrated functional groups. In Paper IV, The Database for Annotation, Visualization and Integrated Discovery (DAVID) 2008 was used for GO-based functional annotations of genes [205].

Quantitative polymerase chain reaction

Although the quality of the data obtained from microarray experiments has greatly improved with continued development of the technology in recent years, it is valuable to validate array data using alternative approaches. For positive validation, the observed changes should be confirmed to be reproducible in a larger number of independent samples. In Paper IV, quantitative polymerase chain reaction (QPCR) was used to confirm a subset of the microarray results.

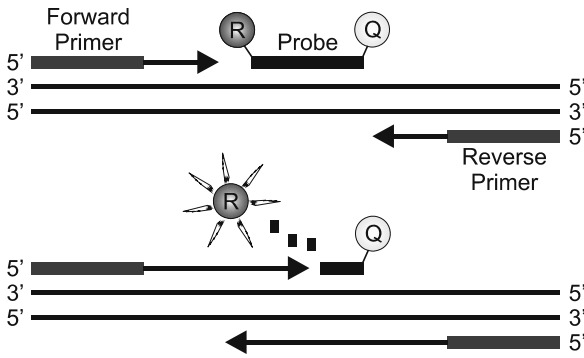

FIGURE 7

The mechanism behind the QPCR reaction. 5' nuclease-mediated cleavage of the probe during strand elongation releases the fluorescent reporter dye (R) from the vicinity of the quencher (Q). As a result, the amount of PCR product in each sequential cycle can be monitored.

The polymerase chain reaction (PCR) is a very sensitive and fast technique that is used to amplify small amounts of DNA. In QPCR, which is a real-time reverse transcription PCR method, the conditions are adapted to quantify a specific mRNA in an unknown sample. The TaqMan QPCR technology is based on the relationship between the starting amount of complementary DNA and the amount of PCR product at any given PCR cycle number. The amount of PCR product present is continually detected using a probe with a fluorescent reporter dye at the 5' end and a quencher at the 3' end (Fig. 7). The result is increased fluorescence as the probe reporter dye is cleaved and separated from the quencher by the exonuclease activity of the DNA polymerase. The PCR cycle (C_T) in which a preset threshold fluorescence is reached is used to compare the relative starting amounts of complementary DNA in unknown samples.

The TaqMan low density array (TLDA) has been developed to allow high throughput QPCR-based microarray validation. In TLDA, customized 384-well plates are used to enable 384 simultaneous QPCR reactions using factory-loaded inventoried primers and probes. The inventoried assays are optimized to have PCR efficiency close to 100%, enabling the use of the $\Delta\Delta C_T$ method of relative quantification. The relative quantity (RQ) of an unknown sample to a calibrator sample is given by:

$$RQ = 2^{-\Delta\Delta C_T}$$

where $\Delta\Delta C_T$ is given by:

$$\Delta\Delta C_T = \Delta C_{T_{target}} - \Delta C_{T_{calibrator}}$$

where target and calibrator could be treated and untreated samples, respectively, and ΔC_T is given by:

$$\Delta C_T = C_{T_{sample}} - C_{T_{endogenous\ control}}$$

where the endogenous control is an included reference gene whose expression should be unaffected by the conditions under investigation.

In Paper IV, ten TLDA plates were used to validate the expression of 85 target genes in four different samples. The endogenous control was selected from a group of eleven candidate genes included in the assays based on their traditional use and/or high and stable expression in the microarray experiments.

SUMMARY OF RESULTS AND DISCUSSION

PGR antagonists inhibit cholesterol synthesis (Paper I)

This paper aimed to investigate the transcriptional regulation mediated by PGR during the periovulatory interval. For this purpose, we used isolated rat granulosa cells, cultured for 24 h with or without treatment with the PGR antagonist Org 31710. Transcriptome analysis was performed using four Affymetrix Rat Genome U34A arrays that contained approximately 8,800 probe sets. The concentration of Org 31710 was selected based on previous studies in which 10 μM was the lowest effective concentration inducing apoptosis, measured as an increase in DNA fragmentation [150]. In that study, the effect was specific to the periovulatory interval during which PGR is expressed [131, 132] whereas no increase in apoptosis was seen in immature granulosa cells. Furthermore, in our experience, the magnitude of the response correlates well with PGR levels during the periovulatory interval. The selection criteria used in the microarray analysis resulted in 51 up-regulated and 40 down-regulated transcripts. Manual function-based categorization of these genes identified several groups, prominent among which was a group of genes encoding several enzymes in the de novo cholesterol synthesis pathway.

Further investigations focused on cholesterol synthesis and the functional implications of its regulation by Org 31710. The four regulated genes were phenylalkylamine Ca^{2+} antagonist (emopamil) binding protein (previously known as 3-beta-hydroxysteroid-delta-8,delta-7-isomerase), *Mvk* and *Hmgcs1* and *-2* (Paper I, Fig.1). In addition to the cholesterol synthesis genes detected by the Affymetrix MAS4.0 algorithm, a complementary analysis of other genes in this pathway was performed. Interestingly, the expression of the majority of the genes in the pathway was decreased in the Org 31710-treated cells. The other genes that were apparently regulated included the rate-limiting *Hmgcr*. This broad regulation of the enzymes of cholesterol synthesis indicates that these enzymes are not individually regulated by progesterone; instead, these effects are likely mediated at the level of SREBP and its associated proteins. SREBP itself, however, is mainly regulated post-translationally, suggesting that the actual targets of PGR are even further upstream. To validate the apparent regulation of cholesterol synthesis, the ability of granulosa cells treated with Org 31710 or RU 486 to produce cholesterol was investigated. The incorporation of ^{14}C-acetate into cholesterol, cholesteryl esters and progesterone, during the final 5 h of cell culture was assessed after

separation of these compounds by TLC. Both PGR antagonists decreased compound-labeling by approximately 50%, functionally confirming a decrease in cholesterol synthesis (Paper I. Fig. 2).

Since progesterone is a reported survival factor in periovulatory granulosa cells [150-153], and PGR antagonists accordingly induce apoptosis, the possibility of a connection between the decreased cholesterol synthesis and induction of apoptosis warranted further investigation. These two events (i.e., increased apoptosis and decreased cholesterol synthesis) could be either isolated or causally related effects of PGR antagonist treatment (Fig. 8). To distinguish between these possibilities, granulosa cells were treated with three different drugs of the statin family. Statins are used clinically to treat hypercholesterolemia and they effectively inhibit cholesterol synthesis by inhibiting the rate-limiting enzymatic step, HMGCR. As expected, treatment of granulosa cells with statins efficiently decreased the incorporation of ^{14}C into cholesterol (Paper I, Fig. 3A). If the PGR antagonists induced apoptosis by inhibiting cholesterol synthesis, statin treatment should have a similar effect. Indeed, in agreement with their relative efficiency in inhibiting cholesterol synthesis, all statins investigated induced apoptosis (Paper I, Fig. 3B and C). The increased apoptosis seen with statins was partially reversed by adding mevalonic acid, restoring cholesterol production (Paper I, Fig. 4). Mevalonic acid

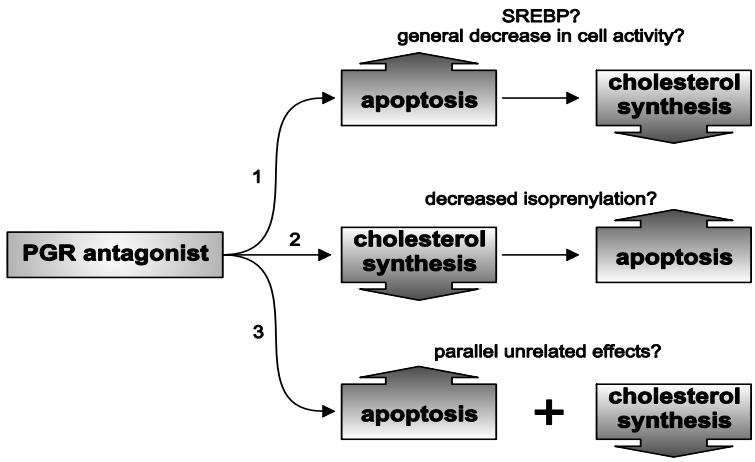

FIGURE 8

The three possible relationships between the events found to occur after treatment of periovulatory granulosa cells with PGR antagonists in vitro. 1) PGR antagonists induce apoptosis which in turn affects the activity of the cholesterol synthesis pathway; or 2) PGR antagonists directly inhibits cholesterol synthesis which results in increased apoptosis, possibly due to the insufficient availability of isoprenylation substrates; or 3) PGR antagonists induce apoptosis and inhibit the cholesterol synthesis as two parallel and independent events.

did not reverse the apoptosis induced by PGR antagonists as expected, however, since MVK, converting mevalonate to 5-phospho-mevalonate, was one of the enzymes down-regulated at the transcriptional level.

Considering that cholesterol is the basic substrate for all steroid synthesis, the effects of statins on apoptosis could simply be a result of decreased progesterone synthesis and could thereby be regarded as an alternative way to decrease PGR signaling. To investigate this possibility, granulosa cells were treated with cyanoketone, an irreversible inhibitor of the activity of HSD3B. Whereas cyanoketone dose-dependently inhibited progesterone accumulation in the culture medium and induced apoptosis as expected (Paper I, Fig. 5), the much higher apoptogenic potency of the statins could not be explained by a similar effect on progesterone production alone.

The apoptosis-inducing effect of the statins suggests a common mechanism for statins and PGR antagonists, connecting cholesterol synthesis and apoptosis. Such a relationship could involve the well-known ability of statins to induce apoptosis by decreasing protein isoprenylation [179-183]. The mechanism by which statins induce apoptosis is generally held to be related to their effects on cell cycle progression [206]. An alternative possibility is that the decrease in cholesterol synthesis occurs as a direct result of the increase in apoptosis. In fact, apoptosis has been reported to affect cholesterol synthesis, given that SREBP is a known caspase target [207, 208]. Notably, this would imply increased rather that decreased cholesterol synthesis as a result of increased apoptosis. In addition, unpublished observations from our group indicate that alternative means of inducing apoptosis do not appear to decrease cholesterol synthesis at a functional level. This also speaks against the possibility of decreased cholesterol production due to a reduction in viable, metabolically active cells.

In summary, the results of Paper I suggest that progesterone maintains a high level of de novo cholesterol synthesis in luteinizing granulosa cells, in spite of the mainly extracellular sources of cholesterol for increased steroid synthesis. One possible explanation for this could be increased levels of isoprenylated proteins involved in pro-survival pathways that are activated during the periovulatory interval.

Decreased isoprenylation does not mediate apoptosis induced by PGR antagonists (Paper II)

As indicated by the results of Paper I, decreased protein isoprenylation provides an appealing link between the progesterone receptor and apoptosis. The study presented in Paper II was designed to test the hypothesis that statins and PGR antagonists share a common mechanism in their induction of apoptosis. If the mechanism of action causing apoptosis were the same for both treatments, the principles of causality dictate that there should be a coherently timed relationship between the cause and effect [209]. In other words, if apoptosis was caused by decreased cholesterol synthesis, the decrease should occur before the onset of apoptosis. While statin treatment potently decreased cholesterol synthesis early in the culture and induced apoptosis gradually over time, Org 31710 had only a slight effect on the cholesterol synthesis early in the culture but still clearly induced apoptosis at that time (Paper II, Fig. 1 and 2).

To further investigate the putative involvement of isoprenylation in granulosa cell apoptosis, studies were conducted on the reversibility of the effects of statins and PGR antagonists by adding isoprenylation substrates back to the media. Previous data from our group (Rung et al.) suggest that isoprenylation could be implicated in human periovulatory granulosa cell apoptosis [210]. In the study by Rung et al., inhibitors of isoprenylation, farnesyl- and geranylgeranyl transferase inhibitors, induced apoptosis. Similar results were seen in unpublished studies on rat granulosa cells in this laboratory. In addition, the effects of both statins and PGR antagonists were partially reversed by the addition of isoprenylation substrates to human luteinizing granulosa cells, indicating that protein isoprenylation is important for granulosa cell survival.

Similar experiments performed in Paper II, however, did not corroborate these reported effects. Addition of the isoprenylation substrates farnesol, farnesyl-pyrophosphate, geranylgeraniol or geranylgeranyl-pyrophosphate did not reverse the effect of Org 31710 on apoptosis (Paper II, Fig. 3). In contrast, addition of these substrates partially reversed the effects of statins (Paper II, Fig. 4), indicating that statins indeed caused apoptosis by decreasing protein isoprenylation. The geranylgeranyl substrates were more efficient than the farnesylation substrates in reversing the effects of statins, suggesting a more prominent role for geranylgeranylated proteins in the control of apoptosis in granulosa cells. Some studies support a dominant role for geranylgeranylation while others demonstrate a major role for farnesylated proteins [183, 185, 211-214]. This is likely related to

the specific cell type and function under investigation, plausibly involving different subsets of proteins.

Finally, to assess the effects of Org 31710 and statins, a direct measurement of the isoprenylation status of a selected protein, RAB11a, member RAS oncogene family (RAB11A), was performed. A lipophilicity-based separation technique was employed using the non-ionic detergent Triton-X114. Whereas simvastatin caused a clear shift from the detergent-rich phase (containing isoprenylated proteins) to the aqueous phase (containing unmodified proteins), the corresponding protein ratios were unaffected by treatment with Org 31710 (Paper II, Fig. 5). In 2001, Kim et al. reported that both farnesyl transferase and geranylgeranyl transferase type I were cleaved and inactivated by caspase 3 during ongoing apoptosis in a mouse lymphoma cell line [215]. The authors suggested that the inactivation of these enzymes could contribute to the apoptotic process by inactivating pro-survival signaling pathways in the cells. Consequently, any induction of apoptosis should induce a degree of decreased protein isoprenylation, which is in conflict with our studies on RAB11A isoprenylation. RAB11A, however, is geranylgeranylated by a separate prenyltransferase, RAB geranylgeranyltransferase [173], which has not been reported to be affected by caspase 3, possibly explaining the discrepancy.

Taken together, the results of Paper II shows that disrupted isoprenylation results in apoptosis of granulosa cells, as in other investigated cell types, and also suggest that treatment with statins at least partially induces apoptosis by reducing isoprenylation substrate availability. It can also be concluded, however, that treatment of granulosa cells with PGR antagonists does not sufficiently [168] inhibit cholesterol synthesis to affect protein isoprenylation. Consequently, PGR antagonists induce apoptosis by a different mode of action. Moreover, the critical data provided by time-effect analysis indicate that the effect of PGR antagonists on cholesterol synthesis does not exclusively cause the increase in apoptosis, although a contributing delayed effect cannot be ruled out.

Regulation of periovulatory granulosa cell apoptosis by PGR (Paper III)

A common theme of the research in this laboratory has been elucidation of the mechanisms behind the anti-apoptotic effects of progesterone in granulosa cells during the periovulatory interval. Although several papers have demonstrated the anti-apoptotic function of progesterone at various stages of granulosa cell

development, controversies remain regarding the mode of action of progesterone. The involvement of PGR has been under some debate since many studies using PGR antagonists, including studies in our group, have used relatively high concentrations to demonstrate effects on apoptosis [123, 216]. Alternative progesterone receptors, mainly the PGRMC1/SERBP1 complex, have been reported to mediate the effects of progesterone on apoptosis [123]. In addition, it has been suggested that the effect of high concentrations of PGR antagonists could result in decreased synthesis of progesterone, indirectly affecting the activity of PGRMC1/SERBP1 [216]. Paper III aimed to take on the controversies surrounding the involvement of PGR in the regulation of periovulatory granulosa cell apoptosis.

To carefully investigate the apoptogenic effect and specificity of PGR antagonists, periovulatory granulosa cells were treated with increasing concentrations of Org 31710 and RU 486. Increased caspase 3/7 activity was seen at a lowest concentration of 10 nM (Fig. 9 and Paper III, Fig. 1), which corresponds well to the reported affinity of the antagonists for PGR [200]. These low effective concentrations indicate specific interaction with PGR, a finding which is further supported by complete reversal of the effect by adding back R5020, a synthetic progestin. Approximately a ten-fold higher concentration of R5020 was required for full reversal, in agreement with previous publications [198] and the known properties of PGR antagonists, discussed in the methodology section [194-196]. While PGR antagonist concentrations ranging from 30–1,000 nM induced an increase in apoptosis by approximately 20–30%, a concentration of 10 μM induced a sharp second increase in caspase activity by 200–300%. As expected and in

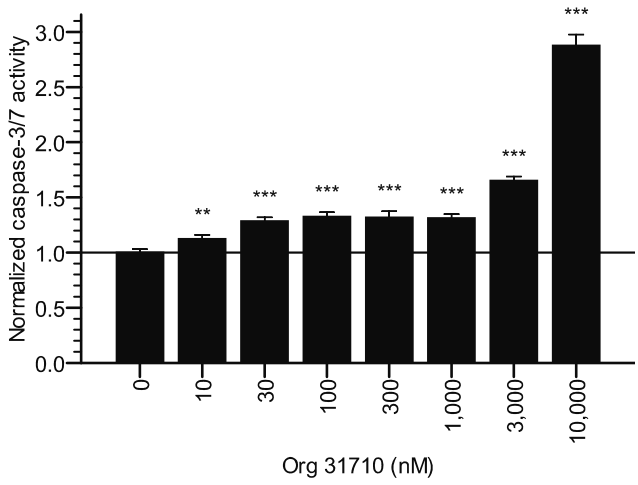

FIGURE 9

Effect of PGR antagonist Org 31710 on apoptosis measured as caspase 3/7 activity in rat periovulatory granulosa cells cultured for 22 h. Bars represent mean ± SEM. The statistical analysis used was general linear model followed by a single-sided Dunnet's post hoc test. **, $P < 0.01$, ***, $P < 0.001$. Taken from Paper III, Fig. 1.

agreement with previous results, the effect of 10 μM Org 31710 could not be reversed by addition of R5020 since the approximate 100 μM concentration required is in itself toxic and induces apoptosis (unpublished observations). In contrast to previous findings in Paper I, the 10 μM concentration of Org 31710 also induced apoptosis by approximately 30% in immature granulosa cells, which likely do not express PGR, suggesting a toxic component of the response (Paper III, Fig. 5).

It has been suggested that the effects of PGR antagonists could be mediated via a decrease in progesterone synthesis, and therefore, other progesterone receptors and signaling systems than PGR [216]. The effect of Org 31710 on progesterone accumulation was investigated, and treatment with 100 nM was found to cause an approximate 24% decrease (51 nM to 39 nM) in progesterone levels in the culture medium. To evaluate the impact of this decrease, we used the HSD3B inhibitor cyanoketone to examine the effect of pharmacological removal of progesterone. In agreement with the results of Paper I, increasing doses of cyanoketone induced apoptosis concurrently with a decrease in progesterone accumulation in the spent medium (Paper III, Fig. 3). Two major conclusions can be drawn from these experiments: 1) decreasing progesterone to undetectable levels in spent medium induces apoptosis by 45–50%; and 2) to induce a degree of apoptosis corresponding to the levels induced by PGR antagonists, the progesterone concentration must be reduced to at least below 5 nM. The effect of cyanoketone was fully reversed by the addition of 5 nM R5020, indicating that the effect was specific for progesterone, and complete progesterone-mediated apoptosis protection by concentrations of R5020 below 5 nM. Treatment of granulosa cells with reversible concentrations of cyanoketone induced a higher degree of apoptosis than PGR antagonists did, suggesting involvement of other progesterone receptors or signaling systems in apoptosis regulation. Co-treatment of granulosa cells with cyanoketone and PGR antagonists, however, limited the degree of apoptosis induced to the effect of PGR antagonists alone. This effect could be due to partial agonist activity of the PGR antagonists [197]. If this holds true, cyanoketone treatment would represent a complete removal of progesterone signaling, while PGR antagonists would represent a partial removal of progesterone signaling mediated via PGR. Since the full anti-progesterone effect is limited by PGR antagonists, perhaps the full effect is completely mediated via PGR as well. Indeed, this is a plausible conclusion.

Investigations of the possible implications of the effects of PGR antagonists on apoptosis mediated by other receptors have been carried out previously, excluding

the gamma-aminobutyric acid (GABA-A) receptor, the androgen receptor and the glucocorticoid receptor as possible candidates [150-152]. In addition, the effect of the androgen receptor antagonist flutamide did not to affect apoptosis in our model system (unpublished observations).

In 2000 and 2001, studies with high concentrations of PGR antagonists on rat and human periovulatory granulosa cells were published by Svensson et al. [150, 152]. In 2000, Makrigiannakis et al. also published results suggesting that progesterone is a survival factor in human luteinizing granulosa cells [151]. Makrigiannakis used a similar system with human granulosa cells retrieved in conjunction with in vitro fertilization. In contrast to previous results from our group, Makrigiannakis reported that the addition of low levels of exogenous progesterone rescued luteinizing granulosa cells from serum-free culture-induced apoptosis. This was somewhat surprising, since these cells should produce enough progesterone by themselves to saturate all available PGRs. In 2004, Quirk et al. reported on the role of progesterone in bovine luteinized granulosa cells [87]. In their study, granulosa cells were isolated at different times after an ovulatory stimulus and cultured first for 24 h in the presence of serum, followed by 24 h without serum but with the addition of FASL. The authors reported that the cells became insensitive to FASL when they were isolated at 14 h after the ovulatory stimulus, and that this effect was reversed by the addition of 500 nM RU 486. Furthermore, they reported that cell cycle exit correlated with the increased resistance to FASL, and that this cell cycle exit was mediated via PGR. Engmann et al. performed a study in 2006 in which human luteinized granulosa cells were cultured for 3 days in the presence of serum [216]. When the serum was subsequently removed, induction of apoptosis occurred within one hour and was reversed by the addition of progesterone to the medium. This effect of progesterone could not be reversed by 5 μM RU 486, and the authors proposed that rapid progesterone effects mediated via PGRMC1 were implicated, rather than PGR signaling.

In summary, the results of Paper III show that progesterone acts specifically via PGR to regulate apoptosis of rat periovulatory granulosa cells.

Regulation of granulosa cell apoptosis by PGRMC1 (Paper III)

In addition to PGR, PGRMC1 and its partner SERBP1 have been implicated in the control of granulosa cell apoptosis [123]. Therefore, the study presented in Paper III briefly investigated the possible involvement of PGRMC1 in the regulation of

rat periovulatory granulosa cell apoptosis. Circumstantial evidence from several papers suggest a role for PGRMC1 in granulosa cell apoptosis; however, only a few papers have functionally tested this hypothesis [126, 216, 217]. One of the functional tools used has been a rabbit polyclonal antibody serum directed against the N-terminal end of porcine PGRMC1 [218]. This N-terminal sequence is identical in the human and rat orthologs. Treatment of rat granulosa cells and human luteinized granulosa cells with this antibody has been reported to block the activity of PGRMC1 and induce apoptosis [126, 216].

Culture of periovulatory granulosa cells in the presence of this same anti-PGRMC1 antibody did not, however, result in an increased degree of apoptosis (Paper III, Fig. 5). In contrast, caspase activity was decreased by increasing concentrations of anti-PGRMC1. The lack of effect was not due to an absence of PGRMC1 in the cells or a non-functional antibody since both protein expression and antibody integrity were determined by Western blotting. A possible interpretation could be an apoptogenic rather than an anti-apoptotic role of PGRMC1 in the periovulatory interval. Alternatively and more likely, the decreased apoptosis seen was a result of the increasing concentrations of unspecified rabbit serum proteins. Indeed, a similar effect was seen when incubating granulosa cells with pre-immunized rabbit serum. In an attempt to establish a positive functional role for anti-PGRMC1, we used immature rat granulosa cells (i.e., obtained from small antral follicles of 26-days old rats). Immature rat granulosa cells [219-222] as well as Spontaneously Immortalized Granulosa Cells (SIGC) [126, 223] have been used to demonstrate the effects of progesterone at developmental stages lacking PGR expression, but the blocking activity of anti-PGRMC1 has only been reported in SIGC and human luteal cells [126, 216]. In our hands, progesterone did not reduce spontaneous apoptosis in immature granulosa cells, and the addition of cyanoketone did not induce apoptosis. Furthermore, inclusion of the anti-PGRMC1 antibody in the granulosa cell cultures had a similar anti-apoptotic effect as seen in periovulatory granulosa cells.

In summary, these results did not corroborate previous reports of an anti-apoptotic role for progesterone in immature granulosa cells. We also did not find any support for a functional role of PGRMC1 in the regulation of granulosa cell apoptosis. In addition to the data obtained from the experiments using the anti-PGRMC1 antibody, the results of the cyanoketone experiments in Paper III support a dominant role for PGR in the regulation of apoptosis. A third piece of supporting evidence is provided by a review of the reported affinity of progesterone for the various possible progesterone receptors that have been discussed here. Importantly,

5 nM R5020 was sufficient to induce a full progesterone-mediated anti-apoptotic response in the cyanoketone experiments (Paper III, Fig. 3C). This is reasonable, considering that the K_D of R5020 for PGR has been reported to be in the 1–4 nM range [224, 225]. In fact, phosphorylation of PGR can render the receptor even more sensitive to progestins [111]. In comparison, the reported progesterone binding sites of PGRMC1 have K_D values of 11 nM and 286 nM for progesterone [120], while the affinity for R5020 has not been reported. On a side note, PAQR family members have demonstrated K_D values of 5 nM for progesterone, but they have much lower affinity for R5020 and RU 486 with values in the micromolar range, at least regarding PAQR7 [116, 226]. Therefore, it follows that apoptosis induced by cyanoketone-mediated removal of progesterone is not likely mediated via PGRMC1 or the PAQR family.

Transcriptional regulation by progesterone (Papers I and IV)

The periovulatory granulosa cell transcriptional changes caused by PGR antagonists, studied in Paper I, were further investigated in Paper IV. The Rat Genome U34A arrays used in Paper I were replaced by the more comprehensive Rat Genome 230 2.0 arrays in Paper IV. Moreover, the array design was

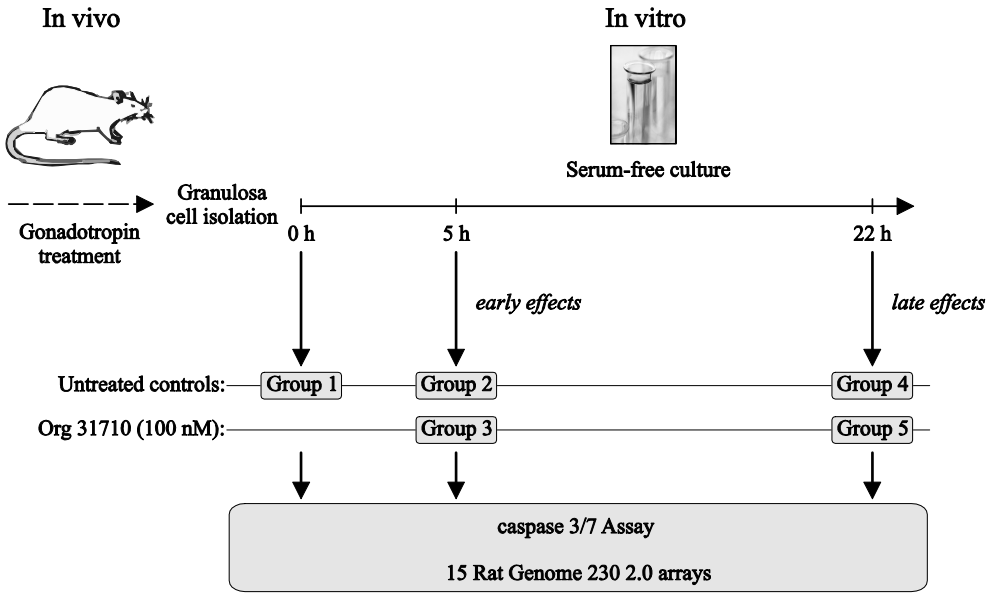

FIGURE 10

Schematic overview showing the experimental setup for the microarray analysis in Paper IV.

supplemented to include both early and late transcriptional changes mediated by PGR (Fig. 10). The concentration of the PGR antagonist Org 31710 was selected to be 100 nM in order to more specifically characterize regulated genes while minimizing possible toxic side effects, in agreement with the results of Paper III. In addition, in Paper IV, granulosa cells were isolated at peak PGR expression (i.e., at 7 h post-hCG).

The major transcriptional changes identified occurred over time during the serum-free cell culture (Paper IV, Fig. 1). Considering the cellular adaptations required for the cell culture conditions together with the physiological changes occurring during the periovulatory interval, these transcriptional changes were not unexpected. The main focus of the study, however, was to investigate the specific changes induced by treatment with Org 31710 after 5 and 22 h of cell culture, relative to the controls. Pairwise comparisons were made between the five treatment groups using SAM, and the 1,000 highest ranked regulated genes in each comparison were analyzed further. Functional categorization and enrichment analysis were performed using the DAVID bioinformatics tools. A subset of functional groups was selected based on a subjective evaluation of biologically relevant functions, and on overrepresentation of GO terms in DAVID. We focused primarily on genes that were GO annotated to be related to apoptosis and reproductive processes. In addition, genes involved in angiogenesis, cell adhesion, cell cycle control and negative transcriptional regulation were investigated. As a complement to the DAVID analysis, we also used the novel bioinformatics resource CoPub, which is a text mining tool that detects co-occurring biomedical concepts in abstracts from the MedLine literature database. The expression of 85 selected genes was further examined using TLDA to confirm the results of the arrays.

Transcriptional regulation of apoptosis

An increase in caspase 3/7 activity by Org 31710 could not be seen until between 10–15 h of culture (unpublished observations). This is important for the interpretation of the transcriptional data in the microarrays. The changes seen after 22 h could be not only late transcriptional effects reflecting the pathways leading to apoptosis, but many of these genes could quite likely be regulated as a consequence of increased apoptosis. In contrast, the early transcriptional effects are in relative terms more likely causative, affecting cellular pro- or anti-survival pathways. Accordingly, based on the CoPub analysis, apoptosis was an overrepresented term in the Org 31710 group at 5 h, suggesting that the genes

affected at that time point were the most important candidates to investigate further.

A total of 72 genes noted as GO "apoptosis" were found in the DAVID analysis (Paper IV, Fig. 2 and 3). There were 35 genes out of the 72 that were regulated in the Org 31710 group at 5 h. *Sgk1* was one of the most clearly regulated genes that was down-regulated by Org 31710 after 5 h. SGK1 has been suggested to be involved in the regulation of granulosa cell proliferation, differentiation and apoptosis [56, 61, 62] but its regulation by progesterone has not previously been demonstrated in the ovary. No direct evidence has been presented demonstrating SGK1-mediated regulation of ovarian apoptosis. However, as described in a recent review of the actions of SGK1 [227], the diverse reported functions of SGK1 include a survival-promoting function associated with regulation of forkhead transcription factors, NF-κB and BCL2-associated agonist of cell death (BAD). Transcriptional regulation of *Sgk1* has been reported for TGFB, endothelin and PPARG; thus, it is likely that progesterone partially regulates the expression of *Sgk1* via PPARG and/or endothelin [145, 146].

Zinc finger and BTB domain containing 16 (ZBTB16) is a transcriptional repressor and a likely mediator of the effects of PGR. Its regulation by progesterone, and also by glucocorticoids, has recently been investigated in human uterine tissues [228]. Furthermore, ZBTB16 has been implicated in the regulation of proliferation, differentiation and apoptosis. Brosens and Gellersen speculated that progesterone acts as a regulator of the balance of pro-apoptotic TRP53 signals and anti-apoptotic ZBTB16 in the endometrial stroma [229]. In our study, the *Zbtb16* gene was not strongly expressed in the granulosa cells but its transient profile and sharp suppression by Org 31710 at 5 h were clear. This is in agreement with the reported expression profile of *Zbtb16* as an early progesterone-induced gene in the endometrium.

An interesting subset of regulated genes was found suggesting that progesterone regulates the activity of the TGFB family and SMAD signaling. The Org 31710-mediated suppression of follistatin, which binds and inactivates activin [230], could result in increased activin-mediated SMAD signaling. This result is consistent with previous studies in the uterus [231]. In addition, the expression of latent transforming growth factor beta binding proteins 1 and 3 was found to be suppressed by Org 31710. These proteins bind TGFB in a latent complex in the extracellular matrix, thereby decreasing TGFB activity [69]. The increased expression of Kruppel-like factor 10 by Org 31710-treatment further supports an increased activity in SMAD signaling. Kruppel-like factor 10 acts as an important

enhancer and regulator of SMADs and is a known pro-apoptotic factor in many cell types [232]. Furthermore, the expression of *Smad3*, one of the receptor-regulated SMADs and a central mediator of TGFB action, was increased by Org 31710 treatment. Three additional genes that were indicated as regulated by Org 31710 have been reported to function as co-repressors of SMAD transcriptional activity. These genes include histone deacetylase 5 [233], TGFB-induced factor homeobox 1 [234] and homeodomain interacting protein kinase 2 [235]. The exact roles in ovarian physiology of the many members of the TGFB family have not been clearly established, but available studies predominantly report pro-survival functions [44]. Together, these data suggest the possible involvement of modulated action of the TGFB family in Org 31710-mediated apoptosis and/or other processes influenced by progesterone. Indeed, a recent characterization of the transcriptional effects of progesterone in the endometrium concluded that a substantial number of genes were regulated indirectly via modulation of for example the SMAD signaling system [236].

Direct regulation of mitochondrial apoptosis could be mediated via regulated expression of two members of the BCL2 family, the anti-apoptotic BCL2-like 1 (*Bcl2l1*, previously known as *Bcl-XL*) and the pro-apoptotic BCL2-related ovarian killer protein (*Bok*). PGR-mediated regulation of *Bok* has not been described previously. BOK was discovered in 1997 as a pro-apoptotic BCL2 protein, resembling BAK and BAX, and is mainly expressed in reproductive tissues [237]. It has been reported that *Bok* is regulated in a cell cycle-dependent manner, and suggested that BOK sensitizes dividing cells to stress [238]. In contrast to *Bok*, the expression of *Bcl2l1* has previously been reported to be regulated by PGR. In human breast cancer cells, *Bcl2l1* is regulated via PGR-A in an indirect fashion [101], suggesting mediating effects of other factors, perhaps via TGFB signaling [239].

Among the genes that were indicated to be related to apoptosis, we identified several candidates that seemed to be connected to TRP53 activity. Two such genes were crystallin alpha B (*Cryab*) and *S100b*. CRYAB is a small heat-shock protein that protects cells during stress. Cytosolic sequestration of TRP53 by CRYAB has been suggested to have a role in protection against apoptosis [240]. *Cryab* was one of the most clearly suppressed genes at 5 h following treatment with Org 31710. It is tempting to speculate that progesterone up-regulates the expression of *Cryab* as a protective agent during the inflammatory conditions of the periovulatory interval. S100B has not been previously described in ovarian tissues and is known mainly as a neural protein. The expression of *S100b* was very low early in the culture and

increased sharply at 22 h. This increase was suppressed by Org 31710. Its expression profile suggests that S100B could have a role late in the periovulatory interval or that its increase is an effect of the serum-free conditions. A neuroprotective function of S100B has been suggested to be related to activation of PI3K-signaling [241]. Interestingly, S100B has been reported to interact with, and inhibit, the actions of TRP53 in tumor cell lines [242]. To investigate a possible role for TRP53 activity in Org 31710-induced apoptosis, granulosa cells were treated with the TRP53 inhibitor pifithrin-α. This treatment strongly decreased caspase 3/7 activity, but Org 31710 still maintained a relative induction of apoptosis (Paper IV, Fig. 4). Therefore, it was concluded that TRP53 is involved in the induction of apoptosis caused by serum-free cell culture conditions, but is not involved in Org 31710-induced apoptosis.

In addition to TRP53, the influence of PI3K/AKT on Org 31710-induced apoptosis was also investigated. Both a PI3K inhibitor and an AKT inhibitor induced granulosa cell apoptosis (Paper IV, Fig. 5). Interestingly, when granulosa cells were co-treated with either of these agents and Org 31710, the effect was strongly potentiated. Granulosa cells deprived of these survival signals were apparently highly sensitive to a loss of progesterone signaling. This indicates that there could be a degree of redundancy associated with the cellular systems targeted by PGR and other factors. Interestingly, the PI3K pathway has been reported to modulate the activity of SMAD signaling [71, 72], and thus relates to the above discussion regarding TGFB. Further investigations will undoubtedly be required in order to elaborate on this matter.

Transcriptional regulation of reproductive processes

In agreement with expectations, the GO term “reproductive process” was high on the list of over-represented functional gene groups, following treatment with Org 31710 for 5 h. Related to this category, the microarray data confirmed the expected decrease of *Pgr* expression over time [132] and indicated a slight suppression of *Pgr* by Org 31710 at 5 h (Paper IV, Fig. 6). This pattern of *Pgr* expression has implications for interpreting the transcriptional patterns of other genes, many of which displayed a similar decrease in expression over time and a premature decrease after treatment with Org 31710. This indicates a direct dependence of transient target gene expression on PGR activity. Two examples of such regulatory patterns are the regulated expression of *Edn2* and *Adamts1* by Org 31710. As previously described, their regulation by PGR in periovulatory granulosa cells has been reported in previous studies [140, 145].

A number of regulated genes were associated with the regulation of steroidogenesis. Increased expression of aldo-keto reductase family 1 member C18 (previously known as *20α-HSD*) after treatment with Org 31710 supports a function for this enzyme in the proposed role of progesterone as a regulator of its own synthesis in the corpus luteum [243]. Also related to the control of steroidogenesis was the regulation of nuclear receptor subfamily 5, group A, members 1 and 2 (*Nr5a1* and *Nr5a2*, commonly known as *Sf-1* and *Lrh-1*). These genes encode two orphan nuclear receptors that in combination with other factors control the dynamics of progesterone and estrogen synthesis [244]. It is possible that they are involved in the regulation of steroidogenic activity occurring during the periovulatory interval. Interestingly, this implies that progesterone itself partakes in this process, considering its very potent suppression of *Nr5a2*.

Org 31710 appeared to cause a decrease in prostaglandin activity since it led to increased expression of the phospholipase A2-inhibitors secretoglobin, family 1A, member 1 (uteroglobin) and annexins 1 and 4, as well as hydroxyprostaglandin dehydrogenase 15-(NAD), which metabolizes prostaglandins. The prostaglandin levels have previously been reported to be decreased after treatment with PGR antagonists during the periovulatory interval [144]. These novel data support such an effect and elaborate on the possible transcriptional changes that mediate this effect.

In addition to the two major functional groups discussed above, genes involved in angiogenesis, cell adhesion, cell cycle regulation and negative regulation of transcription were also briefly investigated. As described in the introduction, these processes are integrated events of ovulation and luteinization. The microarray data presented in Paper IV suggest several progesterone-regulated genes of potential importance in these processes.

It is reasonable to assume that the effect of PGR antagonists on apoptosis in granulosa cells is explained not by a single regulated gene, but rather by the regulation of a complex network of cellular systems that together affect cell survival. The genes indicated as being related to apoptosis were often also related to the regulated genes found in other functional groups, including genes involved in cell adhesion, transcriptional modulation and cell cycle control. The literature analysis performed by the CoPub tool indicated more genes related to apoptosis than the strict GO analysis performed by DAVID, suggesting that many more of the regulated genes could have implications for apoptosis. The discussion in this section is inherently somewhat speculative in nature. This is the inevitable result of the large data sets obtained from multiple microarray comparisons. Further, more

focused investigations of the specific genes and signaling systems discussed here will be necessary to establish the connection between PGR and apoptosis.

Comparative transcriptome analysis

The results from the microarrays performed in Paper IV offer the opportunity to make a comparison to the microarray material in Paper I. When the DAVID and CoPub tools were applied to the microarray data from Paper I, two major functional groups were very prominent. The first group included genes involved in cholesterol, lipid and steroid synthesis, as we also identified manually at the time. The second group included genes involved in oxidative stress response, indeed also recognized in Paper I. Other functional groups of importance were "apoptosis", "drug metabolism", "reproduction", "ovulation" and "angiogenesis". While many of the genes in the latter groups were found in the Paper IV microarray as well, supporting their validity, the first two groups were not. The presence of the "oxidative stress response" group suggests that an increase in oxidative stress could contribute to the increased effect of the micromolar concentrations of Org 31710 used in the microarray experiments in Paper I. Three lines of reasoning support this hypothesis: 1) granulosa cells in serum-free culture are very susceptible to oxidative stress [245]; 2) the inflammatory process (i.e., ovulation) occurring during the periovulatory interval is characterized by increased oxidative stress and a concomitant increased requirement for anti-oxidants, possibly regulated by progesterone [88]; and 3) the metabolism of high concentrations of pharmaceuticals increases the risk for depletion of the cellular anti-oxidant systems [246, 247]. Indeed, there was a notable overlap between the genes found in the functional groups of "apoptosis", "oxidative stress response" and "drug metabolism".

Surprisingly, the clearly established effects on genes involved in cholesterol synthesis studied in Paper I were not found in the microarray data in Paper IV. Considering the discussion above, the physiological relevance of the PGR antagonist-mediated regulation of cholesterol synthesis can be discussed. PGRMC1 has recently been suggested to interact with the SREBP control system of cholesterol synthesis, and could function as an alternative mediator of the effects of high concentrations of Org 31710. Peluso reported in 2001, however, that RU 486 could not displace progesterone binding to SIGC, even at concentrations of 1 mM [223]. In contrast, binding of micromolar concentrations of RU 486 to PAQR 7 and 5 has been demonstrated [119], but to the best of our knowledge, there are no reports connecting the PAQR proteins to cholesterol synthesis. In two papers

published in 1996, Metherall et al. reported that progesterone and other steroids could inhibit cellular production of cholesterol [248, 249]. This ability only occurred at superphysiological concentrations (e.g., 5 μM for progesterone and higher), and correlated with the lipophilicity of the steroids. The authors reported that this property was due to direct binding of the steroids to multidrug resistance P-glycoproteins that function in the intracellular transport of cholesterol. These studies suggest that the effects of high concentrations of PGR antagonists could be due to similar interactions, based on their similar lipophilic properties. Metherall et al., however, described the inhibition of cholesterol production to be a result of defective intracellular transport rather than transcriptional regulation of the biosynthetic pathway. In fact, they noted increased general activity in total sterol production, consistent with the expected negative feedback.

CONCLUDING REMARKS

To summarize, the investigations reported in this thesis on isolated rat periovulatory granulosa cells have led us to conclude the following:

I High concentrations of PGR antagonists inhibit de novo cholesterol synthesis by transcriptional regulation. Furthermore, both treatment with PGR antagonists and pharmacologic inhibition of cholesterol synthesis lead to increased apoptosis in vitro.

II Cholesterol synthesis and protein isoprenylation are important factors maintaining granulosa cell survival in vitro. Decreased protein isoprenylation, however, cannot explain the detrimental effect of PGR antagonists on granulosa cell survival.

III The classical nuclear progesterone receptor, PGR, is the dominant mediator of the anti-apoptotic effects of progesterone in vitro. In contrast, we found no evidence to support a role for the proposed rapid progesterone response receptor PGRMC1 as a regulator of apoptosis in vitro.

IV Progesterone is a central regulatory factor during the periovulatory interval, involved in transcriptional regulation governing the major processes of ovulation and luteinization.

It is not trivial to translate our results showing increased apoptosis by PGR antagonist treatment in periovulatory granulosa cells in vitro into a physiologically relevant corollary. The model system used here results in increased spontaneous apoptosis due to the serum-free conditions and we essentially only detect the additional effect conferred by removal of progesterone signaling in that specific context. If only 2% fetal bovine serum is included in the culture, spontaneous apoptosis is reduced by approximately 50% but the addition of Org 31710 still induces apoptosis by 40% (unpublished observations). In a previous study in this group (Shao et al.), immature gonadotropin-primed mice treated with RU 486 in vivo at the time of hCG injection showed increased caspase 3 activity in periovulatory follicles [153]. The study by Shao et al. indicates that PGR mediates survival of periovulatory granulosa cells in vivo as well. The exact concentration of RU 486 present locally in the follicular fluid, however, was not assessed. Regardless, we find it plausible, considering all evidence, that progesterone is a mediator of the known survival-promoting effects of LH during the periovulatory interval.

The study of granulosa cell apoptosis has clinically relevant implications, described in [250]. For instance, the success rate of in vitro fertilization is clearly correlated with the degree of apoptosis in the granulosa-lutein cells of the aspirates. Ovarian apoptosis also poses a direct problem in female patients of fertile age given chemotherapeutic anti-cancer treatments. As a side effect, such treatments result in ovarian failure due to increased ovarian apoptosis. Direct targeting of the apoptotic pathways could preserve fertility in these patients.

The role of progesterone and the nuclear progesterone receptor in periovulatory granulosa cells is undoubtedly very complex. In this thesis, we performed investigations that identified a plethora of genes whose expression might be modulated after treatment with a selective PGR antagonist. These genes seem to be involved in many of the processes that are described to take place during ovulation and luteinization. Many of the genes are still poorly characterized, and although some intriguing genes have been described here, many remain to be further investigated. Consequently, much work remains before our understanding of the role of progesterone in periovulatory granulosa cells can be considered complete.

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