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Role of Progesterone Receptor Membrane Component 1 (PGRMC1) in controlling germinal and somatic cell division and function

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A.A
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La Natura compone alcune delle sue poesie più belle davanti al microscopio e al telescopio.

Theodore Roszak

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Riassunto

La sub-fertilità rappresenta uno dei principali problemi che l'industria lattiero-casearia si trova ad affrontare attualmente. Si tratta di un problema multifattoriale che dipende da diversi aspetti della funzione riproduttiva. La qualità dell'ovocita, determinata durante la follicologenesi, è sicuramente cruciale per ottenere risultati riproduttivi ottimali. Per migliorare la fertilità femminile nel bovino è fondamentale definire i fattori e i meccanismi che determinano la qualità del gamete femminile, in quanto il declino della funzione riproduttiva in questa specie è dovuto principalmente alla scarsa qualità degli ovociti. In particolare, è importante conoscere quali proteine regolano la meiosi, la fecondabilità e lo sviluppo embrionale dell'ovocita. In questo progetto, abbiamo considerato principalmente il ruolo del Progesterone Receptor Membrane Component-1 (PGRMC1) sia nell'ovocita che nel compartimento follicolare, considerato che il dialogo tra compartimento germinale e compartimento somatico è uno dei fattori chiave coinvolti nell'acquisizione della competenza allo sviluppo del gamete femminile.

Il PGRMC1 è una proteina multifunzionale ed altamente conservata in specie anche filogeneticamente distanti. Nei mammiferi è espressa in diversi sistemi, compresi gli organi riproduttivi, e localizza in diversi compartimenti subcellulari. Tuttavia, il suo ruolo e il preciso meccanismo d'azione in ogni sistema e/o compartimento subcellulare non sono ancora del tutto conosciuti.

La parte più considerevole di questo progetto di dottorato riguarda gli studi effettuati sul ruolo del PGRMC1 nella divisione cellulare, data la sua localizzazione a livello di fuso mitotico e meiotico. Abbiamo dimostrato che la deplezione del PGRMC1 determina difetti nella divisione cellulare e la sua interazione con l'Aurora chinasi B (AURKB) indica che potrebbe svolgere la sua azione durante la citodieresi, l'ultima fase della divisione. Durante la maturazione meiotica, abbiamo valutato anche il suo ruolo nel mediare l'azione del progesterone (P4) confrontando il suo effetto con quello del recettore nucleare del progesterone (nPGR). L'inibizione di entrambi i tipi di recettore determina lo stesso effetto sull'organizzazione della piastra metafasica e sulla competenza allo sviluppo dell'ovocita, ma in fasi differenti.

Inoltre, abbiamo testato l'ipotesi che il PGRMC1 possa modulare la funzione del nucleolo. Studi di immunofluorescenza hanno confermato la presenza del PGRMC1

nel nucleolo di cellule della granulosa (bGC) e negli ovociti di bovino, nonchè la colocalizzazione con la nucleolina, la proteina nucleolare più abbondante che svolge importanti funzioni in questo compartimento subcellulare. Inoltre, la down-regolazione del PGRMC1 determina uno spostamento della nucleolina dal nucleolo al nucleoplasma suggerendo un'associazione funzionale tra queste due proteine. Questa interazione è probabilmente mediata dalla presenza di ulteriori molecole in quanto successivi esperimenti di "in-situ proximity ligation" mostrano che le due proteine non interagiscono direttamente.

Oltre al tema principale di questo progetto, la divisione cellulare è uno dei processi più importanti anche nel cancro e l'espressione del PGRMC1 è elevata in molti tipi di tumore nell'uomo. Per questo abbiamo intrapreso uno studio preliminare per caratterizzare la sua espressione nei diversi tipi di tumore mammario di cane. In base ai nostri risultati, l'espressione del PGRMC1 diminuisce con l'aumentare della malignità del tumore e le diverse isoforme di PGRMC1 sono presenti sia nel tessuto sano che in quello neoplastico.

In conclusione, i nostri risultati suggeriscono che il PGRMC1 può avere un ruolo nel compartimento follicolare, in particolare sia nella mitosi che nella meiosi. La sua funzione potrebbe essere legata alla sua localizzazione nucleolare, nonchè alla sua capacità di mediare l'azione del P4 durante la meiosi dell'ovocita. Il suo ruolo come regolatore della divisione cellulare può essere rilevante anche in alcune patologie quali il cancro. Ulteriori studi saranno diretti a valutare i meccanismi di azione molecolari tramite i quali il PGRMC1 agisce in questi processi al fine di ampliare la nostra conoscenza di questa proteina poliedrica.

Abstract

Subfertility is one of the major problems that dairy industry is facing nowadays. Subfertility is a multifactorial issue depending on different aspects of reproductive function. Oocyte quality, which is determined during folliculogenesis, is certainly crucial for optimal reproductive outcome. Since declining fertility of cattle is mainly due to the poor quality of the oocytes, defining the factors and mechanisms that affect oocyte quality is essential to improve female fertility. In particular, basic knowledge of which proteins within the oocyte regulate meiosis, oocyte fertilizability and early embryonic development would be advantageous. In this project, we primarily considered the role of Progesterone Receptor Membrane Component-1 (PGRMC1) in both the oocyte and follicular compartment since the dialogue between the two compartments is one of the key factors involved in oocyte competence acquisition.

PGRMC1 is a highly conserved and multifunctional protein that is found expressed in multiple systems, including reproductive organs, and localizes in multiple sub-cellular compartments. However, its role(s) and precise mechanism(s) of action in each systems and/or subcellular compartment are not yet fully understood.

The more considerable part of this PhD project consists of studies on PGRMC1's involvement as a cell division regulator, according to its localization to the mitotic and meiotic spindle. We showed that PGRMC1 depletion leads to defective cell division in both oocyte and somatic cells and its action could be exerted during cytokinesis, the very last mitotic phase, as demonstrated by its association with Aurora kinase B (AURKB). During meiotic maturation, we also evaluated PGRMC1 involvement as a possible mediator of progesterone (P4) action, by comparing the effects of inhibiting its function with that of nuclear progesterone receptor's (nPGR) inhibition. Our data suggests that both receptors have an effect on meiotic progression but possibly at different stages of oocyte maturation.

Moreover, we tested the hypothesis that PGRMC1 might modulate the function of the nucleolus. Immunofluorescence experiments confirmed PGRMC1 nucleolar localization in bovine granulosa cells (bGC) and bovine oocytes and its co-localization with nucleolin, the most abundant nucleolar protein exerting important functions in this subcellular compartment. Moreover, a PGRMC1/nucleolin functional association is suggested by PGRMC1's downregulation determining nucleolin shift from the

nucleolus to the nucleoplasm. However, in situ proximity ligation assay did not detect a direct interaction between these two proteins, suggesting the involvement of additional molecules that could mediate PGRMC1/nucleolin interaction.

Besides the main theme of this project, cell division is one of the most important processes in cancer biology and PGRMC1 is known to be overexpressed in many types of tumors in humans. Therefore, we studied its expression in different types of canine mammary tumors. Our preliminary results showed PGRMC1 expression decreases with the malignancy of the tumor and different PGRMC1 isoforms are present both in normal and tumoral tissue.

To conclude, these findings suggest a role of PGRMC1 in the follicular compartment, being implicated in both meiotic and mitotic process of the germinal and somatic compartment, respectively. This action it is likely mediated by PGRMC1 located at a particular site of the spindle, i.e the midzone and /or the midbody of dividing cells. PGRMC1 also localizes at the nucleolus of both oocytes and granulosa cells where it likely exerts additional function(s) in mediating cellular stress and/or other nucleolar-relates process. Clearly these findings have major implication in the overall process of folliculogenesis. Moreover, PGRMC1's role as a regulator of cell proliferation could be relevant also in some diseases such as cancer. Further investigations will aim at investigating PGRMC1's molecular mechanisms of action in these processes to widen our knowledge on this multifaceted protein.

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List of abbreviations

A-TZP: Actin Transzonal Projections ()

AG205: cis-2-[[1-(4-Chlorophenyl)-1H-tetrazol-5-yl]thio]-1-(1,2,3,4,4a,9b-hexahydro-2,8-dimethyl-5H-pyrido[4,3-b]indol-5-yl)-ethanone,cis-5-({[1-(4-chlorophenyl)-1H-tetraazol-5-yl]sulfanyl}acetyl)-2,8-dimethyl-2,3,4,4a,5,9b-hexahydro-1H-pyrido[4,3-b]indole

AMH: Anti Mullerian Hormone

Ana/Telo I: Anaphase/Telophase I

ANOVA: Analysis Of Variance

APC: Anaphase Promoting Complex

AREG: Amphiregulin

ARTs: Assisted Reproductive Technologies

AURKB: Aurora Kinase B

BC: Breast Cancer

BCS: Body Condition Score

betaTUB: Beta Tubulin

BFGF*:* Basic Fibroblast Growth Factor

bGC: Bovine Granulosa Cells

BMP15: Morphogenetic Protein 15

BMPS: Bone Morphogenetic Proteins

BSA: Bovine Serum Albumin

BTC: Betacellulin

cAMP: Cyclic Adenosine Monophosphate

cGMP: Cyclic Guanosine Monophosphate

CK: Cytokeratin

CK2: Casein Kinase 2

CMT: Canine Mammary Tumors

CO: Carbon Monoxide

COCs: Cumulus Cell-Oocyte Complexes

CPC: Chromosomal Passenger Complex

CSF: Cytostatic Factor

CTRL: Control

Cyt b_5: Cytochrome b_5

DAB: Diaminobenzidine

DAP1: Damage Response Protein Related to Membrane Associated Progesterone Receptors 1

DAPI: 40,6-diamidino-2-phenylindole

DCIS: Comedo-Type Ductal Carcinoma In Situ

DEG: Degenerated

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

DOs: Denuded Oocytes

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

EGTA: Egtazic Acid

EREG: Epiregulin

ERK: Extracellular Signal-Regulated Kinase

ERK: Extracellular Signal-Regulated Kinases

FECH: Ferrochelatase

FGF: Fibroblast Growth Factors

FI: Fluorescent Intensity

FSH: Follicle-Stimulating Hormone

FSHR: Follicle Stimulating Hormone Receptor

G0/G1: Gap 0/Gap1 phases of the cell cycle

G2/M: Gap 2/ Mitosis phases of the cell cycle

G6PDH : Glucose-6-Phosphate Dehydrogenase

GAPDH: Glyceraldehyde-3-phosphate Dehydrogenase

GDF9: Growth Differentiation Factor 9

GDF9: Growth Differentiation Factor 9

GnRH: Gonadotropin-Releasing Hormone

GV: Germinal Vesicle

GVBD: Germinal Vesicle Break Down

HAS2: Hyaluronan Synthase 2

HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIST1H2A: Histone Cluster 1, H2ah

HPR6: Heme Progesterone Receptor 6

IGF-I: Insulin-Like Growth Factor-I

IGF: Insulin-like Growth Factors

INCENP: Inner Centromere Protein

INSIG1: Insulin-Induced Gene

IVA: In Vitro Meiotic Arrest

IVF: In Vitro Fertilization

IVM: In Vitro Maturation

IVP: In Vitro Embryo Production (IVP)

kDA: Kilodalton

KL: Kit Ligand

LH: Lutenizing Hormone

LIF: Leukemia Inhibitory Factor

M-Phase: Mitotic Phase

M-TZP: Microtubule Transzonal Projections

MAPR: Membrane Associated Progesterone Receptor family

MI and II: Metaphase I and II

MPF: Maturation-Promoting Factor

MPR: Membrane Progestin Receptors

mRNA: Messenger Ribonucleic acid

NCL: Nucleolin

NEB: Negative Energy Balance

NEFA: Non-Esterified Fatty Acids

NPBs: Nucleolar Precursor Bodies

nPGR: Nuclear Progesterone Receptor

NPPC: Natriuretic Peptide Precursor C

OSF: Oocyte-Secreted Factors

P4: Progesterone

PAIRBP1: Plasminogen Activator Inhibitor 1 Mrna-Binding Protein

PBI: First Polar Body

PBS/PVA: Phosphate-buffered saline/ Polivinilalcool

PGC: Primordial Germ Cells

PGRMC1: Progesterone Receptor Membrane Component 1

PGRMC2: Progesterone Receptor Membrane Component 2

PIPES: Piperazine-N,N′-bis(2-ethanesulfonic acid)

PLA: Proximity Ligation Assay

Pten*:* Phosphatase And Tensin Homolog

PTGS2: Prostaglandin-Endoperoxide Synthase 2

PTX3: Pentraxin-Related Protein

qRT-PCR: Quantitative Reverse Transcriptase-Polymerase Chain Reaction

RIPA: Radioimmunoprecipitation Assay

RNAi: Small Interfering RNA

ROI: Regions Of Interests

RPA: Replication Protein A

RU486: Mifepristone

S: Synthesis phase of the cell cycle

SAC: Spindle Assembly Checkpoint

SCAP: SREBP Cleavage Activating Protein

SDS-PAGE: Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis

SEM: Standard Error Of The Mean

SH: Src Homology

SIGCs: Spontaneously Immortalized Granulosa Cells

SOHLH2*:* Spermatogenesis And Oogenesis Specific Basic Helix-Loop-Helix 2

SREBP: Sterol Regulatory Element Binding Protein

STAR: Steroidogenic Acute Regulatory Protein

SULT1E1: Sulfotransferase Family 1E Member 1

SUMO: Small Ubiquitin-Like Modifier

TBS: Tris Buffered Saline

TBS/T: Tris Buffered Saline and tween

TGF-β: Transforming Growth Factor-β

TGF: Transforming Growth Factor

TGFBS: Transforming Growth Factors

TNFAIP6: TNF Alpha Induced Protein 6

TZP: Transzonal Projections

UBF: Upstream Binding Factor

ZP: Zona Pellucida

Foreword

This thesis is structured as follows.

The introduction consists of a wide overview about the infertility issue that is being faced by dairy industry nowadays, which has several implications at economical level and animal welfare. Infertility is a multifactorial problem and among the numerous factors that could be involved, we propose Progesterone Receptor Membrane Component-1 (PGRMC1) as one of the putative key factors. Therefore, introduction is followed by a state of the art about PGRMC1 focusing on its role in reproductive function.

The subsequent chapters represent the four different parts of this research project and each section is structured as a paper, each with its own bibliography at the end.

Chapter 5 and 7 are published papers. Chapter 6 describes results of a project shared with Prof. Trudee Fair, which were in part achieved during my PhD visiting period at University College of Dublin. Chapter 8 shows preliminary results obtained on PGRMC1 in canine mammary tumors that are to be submitted for publication. The original published papers of chapter 5 and 7 can be found in the appendix.

To conclude, a general discussion and future perspectives are shown to summarize and relate results from different PGRMC1 aspects studied during my PhD program.

Introduction

1. The contemporary issue of subfertility affecting dairy cows

Cow subfertility is one of the main problems that is being faced by dairy industry nowadays. Dairy cow lactation cycle and, therefore, milk production depends on female's ability to become pregnant and, therefore, fertility is one of the most important parameter to understand and improve in order to achieve high production rate [1].

Over the last 50 years there is evidence of a constant decline in worldwide dairy cow fertility, which could be expressed in different ways such as pregnancy rates to first insemination [2], calving interval, duration from calving to conception or number of insemination for conception [3]. Subfertility is defined as any condition leading to failure to establish a pregnancy following completion of uterine involution at 40–50 days post-partum [4]; being the major cause for culling animals it has a great impact on dairy economics. In addition, the lost income from milk sale, the cost of semen needed for repeated attempts to artificially inseminate cows and the replacement of culled animals are all factors contributing to economic loss. Furthermore, it also has obvious animal welfare implications [4].

High selection to achieve increased milk yields has been considered to negatively impact on reproductive efficiency. However, higher milk production does not seem to be the unique feature to blame and physiological adaptations to high milk production may explain only part of the reproductive decline [1]; indeed, some of the highest milk productive herds do not show poor reproductive efficiency maybe due to the better feeding, health and reproductive management [5, 6].

Hence, it is evident that subfertility is a complex and multifactorial issue; the exact reason for its decline is still not clear, but it is determined by a combination of genetic, physiological, environmental and managerial factors [1].

The main factors affecting fertility during dairy cow stages of reproductive life are shortly mentioned below and summarized in **Figure 1.1**.

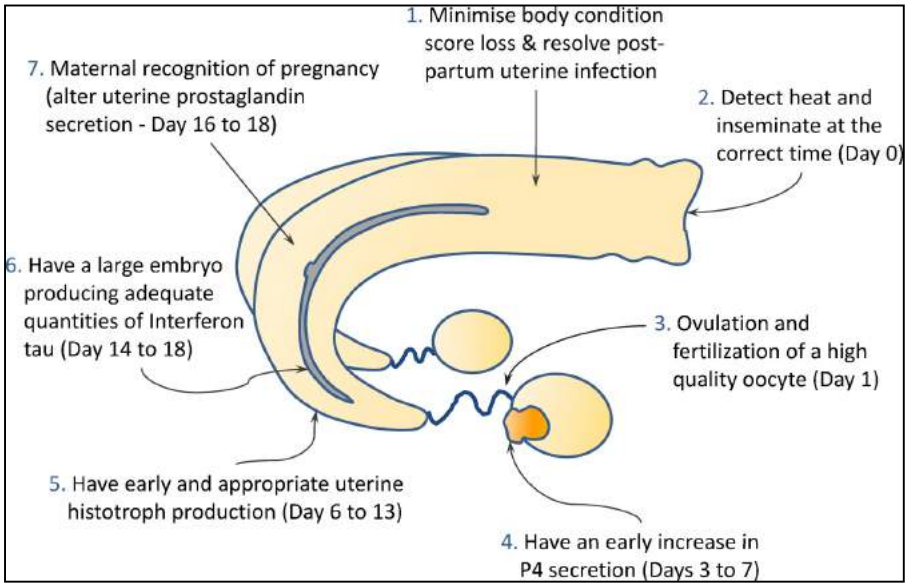

Figure 1.1 *From [7]. Summary of various aspects involved in the establishment of a pregnancy in dairy cows.*

1.1 Genetics, management and pre-partum period

During the last 30 years, genetic selection for increased milk production has been successfully conducted, in particular in the Holstein Friesian genotype. This is accompanied by a decrease in reproductive efficiency. However, the initial negative association between these two parameters has recently been reviewed and other factors are considered to be involved in subfertility besides genetic alone [7].

Poor management of dairy cows plays an essential role in reducing fertility, together with poor nutrition and environmental factors, which are all factors often not evaluated in studies considering only the genetic effect [8]. For example, reproductive management has to be adapted to new conditions such as bigger herd size which has impact on heat detection methods and on the incidence of some reproductive diseases (uterine and mammary infections) due to increased use of confinement houses, which affect reproductive efficiency [9, 10].

Moreover, also changing in global environment leading to heat stress could be partially a risk factor influencing fertility. Indeed, heat stressed animals may have extended interval from calving to first ovulation due to low lutenizing hormone (LH)

pulse amplitude and frequency, smaller dominant follicles and low oestradiol concentrations [11].

In addition, some production diseases that are caused by poor/incorrect management such as undernutrition, hypocalcaemia, mastitis or lameness are linked to reduced reproductive performance [7]. Nutritional and health status during productive cycle is visually monitored using the subjective parameter of body condition score (BCS) [12]. Energy requirements in high milk producing cows dramatically increases due to high daily milk yield, which peaks between 4 and 8 weeks post-partum. At the same time, the cow feed intake is reduced (limitations in the intake and less appetite) and inadequate to meet her maintenance and production requirements. Consequently, body reserves are mobilized and the animal enters negative energy balance (NEB). [13].

When NEB becomes severe, there is an increased risk of metabolic diseases, especially within the first month of lactation [12]. The most common metabolic diseases that could affect cows are acidosis, fatty liver disease, retained placenta and displaced abomasum, hypocalcaemia, hypomagnesaemia and ketosis. [14, 15].

NEB condition leads to elevated free fatty acid (NEFA) concentrations in maternal blood and this could alter follicular microenvironment. Several *in vitro* studies in bovine and murine models showed that especially long-term exposure to NEFAs impaired ovarian follicles growth and, consequently, reduced oocyte's developmental competence [16]. Blastocysts developing from these oocytes are characterized by a significant lower cell number and increased apoptotic cell index [17] and also their transcriptome and epigenome signature are altered [18, 19].

Moreover, metabolic changes in mothers negatively affect *in vitro* bovine oviduct epithelial cells; the oviduct likely modulate its microenvironment to safeguard the embryo from toxic metabolites, but *in vivo* the regulation of the oviduct embryonic milieu is not assured in females with metabolic disorders thus reducing embryo development and quality [20].

Finally, exposure to NEFAs during oocyte maturation may have long lasting effects influencing the later peri-implantation period, which is a very critical moment for the establishment of the pregnancy. Indeed, *in vitro* produced embryos exposed to NEFA and transferred to recipient cows were less developed and metabolically compromised when recovered after 7 days [21].

The above mentioned metabolic disorders could also worsen the physiological low degree of immunodeficiency during early lactation and increase the incidence of pathologies-such as: metritis, mastitis, lameness [14]. At parturition, bacteria normally contaminate the uterus and the majority of the cows successfully deal with the most common pathogens. However, few animals develop metritis within 21 days post-partum [22]. Clinical mastitis incidence is increased by reduced immune competence predisposing to the invasion of pathogens causing this infection. In animals affected by acidosis the release of endotoxins and histamine from the rumen destroy the microvasculature of the corium causing laminitis and consequent lameness; the same metabolites act at a neuroendocrine and ovarian level. It is clear that all these pathologies in the early post-partum have a detrimental effect on the subsequent fertility success of the animal, impairing different stages of reproduction Good management of the animals could improve the control of such adverse effects [1, 7].

1.2 Breeding season and insemination

In order to achieve a 365-day calving interval the breeding season should start 60 days postpartum and the establishment of the pregnancy should happen by 83 days postpartum, assuming a 282 days gestation length. Therefore, by this time all early post-partum/pre-partum disorders and diseases must resolve and the resumption of the normal oestrus cycle is essential for a cow to become pregnant again [7].

Up to 50% of modern dairy cows have abnormal oestrus cycles postpartum with a consequent increase in calving to first insemination interval and decreased conception rates [23]. Several risk factors have been identified for this delayed first ovulation and they include greater NEB in primiparous cows than multiparous cows [1].

Severe NEB together with low BCS reduce pulsatile LH secretion, ovarian responsiveness to LH and oestradiol production from the follicle, delaying ovulation [24]. Moreover, periparturient disorders, season of calving, wrong management and diseases listed above are all factors impairing the resumption of ovulation [23, 25].

In order to inseminate cows at the correct time of ovulation, together with the resumption of cyclicity overt signs of oestrus must be clearly present. Over the past 50 years the duration and the intensity of detected oestrus has reduced [26]. Poor expression of oestrous behaviour renders it less easy to be visually detected, but

luckily there are methods such as pedometers assuring 80-100% of oestrus detection rate [1]. Risk factors for poor expression are both cow-specific (silent/anovulatory anoestrus, parity, milk production, health) or environmental-related (nutrition, housing, season, number of mates simultaneously in oestrus) [27].

Clearly, one of the most important factors to ensure optimal ovulation rates is a correct folliculogenesis, which is extensively dealt with in the next chapter. Ovarian follicle development also affects fertilization success and the consequent establishment of pregnancy.

1.3 Fertilization, embryo development and pregnancy

Assuming that all possible issues and paraphysiological statuses of post-partum are resolved or absent and that fertilization is done at the correct timing with the appropriate technique, reproductive failure could depend on other two major factors: fertilization failure and embryonic mortality/poor embryo survival after fertilization [3, 7].

High fertilization failure is observed especially in high producing cows with fertilization rates dropping from 95% to 83% during the last 40 years [28]. Fertilization and the consequent development of a viable embryo depend on the intrinsic quality of the two gametes; the oocyte and the sperm. Sperm characteristic affecting fertilization are: viability, morphology and functional and molecular traits, inability to reach the site of fertilization, to penetrate the oocyte, to prevent polyspermy or to initiate fertilization itself. When fertilization is achieved through artificial insemination, also technicians performing the procedure could impact the fertilization rate [29].

Heat stress, physiological status (lactating and non-lactating) and factors listed previously such as diseases or metabolic disorders could affect oocyte quality during follicle development and have detrimental effects on the female gamete also after months from the insult [7, 30]. Oocyte quality is poorly defined and is strictly dependent on the follicle, which represents its developmental niche; a wider discussion on this topic is made in the next chapter.

In Holstein dairy cows, also calving rates have declined and are close to 35-40% [1]. After fertilization of the oocyte in the oviduct, the resulting embryo undergoing the first mitotic cleavage divisions is transported towards the uterus, where it reaches

the 16-cell stage at day 4 of pregnancy. Then it becomes a morula, a compact ball of cells and at day 7 of pregnancy it forms a blastocyst characterized by an inner cell mass, which will form the embryo, while the placenta will rise from the trophectoderm. Until this stage the embryo is almost autonomous and the contact with maternal reproductive tract is not mandatory, but already affects its quality [3]. On days 9 to 10 the blastocysts undergo hatching from the zona pellucida and its morphology changes from spherical to a final filamentous form on days 16-17 that occupies one of the uterine horns; here the elongated conceptus begins implantation on the endometrium [31]. Post-hatching the interaction between the conceptus and endometrium is essential for the conceptus development and the establishment of the pregnancy. Thus, a healthy endometrium and its correct function are essential [32].

Embryo mortality can happen in three periods. Very early mortality occurs between days 0 and 7, early mortality between days 7 and 24 and late mortality from days 24 to 45. After day 45 it is considered fetal mortality. The majority of embryonic mortality occurs within 3 weeks of gestation (early embryonic mortality) [7, 33]. Very early embryo mortality is mainly due to poor oocyte quality [34] or suboptimal uterine environment [35]. Moreover, an early rise in progesterone between days 4 and 7 after insemination alters endometrial secretions that stimulates embryo development after day 7, leading to larger embryos more prone to stimulate maternal recognition of pregnancy [33].

Also during early embryo mortality uterine microenvironment and function together with inadequate levels of circulating progesterone are risk factors leading to failure of maternal recognition of the conceptus and mortality [7].

Late embryo and fetal mortality are due to genetic, physiological, endocrinological, environmental factors and pathogenic agents [33, 36].

1.4 Oocyte quality

In mammalian fertility, the good quality of the female gamete is essential to have a successful reproductive outcome. As already mentioned, oocyte quality is poorly defined but is commonly expressed as the oocyte capability to complete meiosis (meiotic competence), being fertilized (fertilization competence) and develop into a viable preimplantation embryo (embryonic developmental competence), either in vivo

or following in vitro embryo production (IVP) procedure. While the spermatozoon makes a complementary genetic contribution to the zygote, the oocyte is obviously the major cytoplasmic donor, contributing nearly all the organelles and nonchromosomal molecules needed for early embryonic development, up to the stage in which embryonic genome if activated. The oocyte grows and differentiates within the ovarian follicle; thus, folliculogenesis is the process leading to oocyte growth, maturation and ovulation, therefore determining also its quality [37].

The criteria to evaluate oocyte quality comprise morphological and cellular/molecular predictors (reviewed in [38]) which are continuously under investigation to find the most objective and noninvasive ones to be used in different species.

Morphological parameters include scoring system and classification of cumulus-oocyte complexes (COCs) evaluating compactness of the cumulus investment and ooplasm characteristics [39]. Moreover, data in humans suggest that evaluation of polar bodies (PB) morphology and meiotic spindle characteristics such as its presence, location and length, which are essential for chromosome alignment and segregation in meiosis, can be used in living oocytes to predict oocyte developmental competence [40, 41].

While intrinsic predictors, which could be evaluated with more invasive techniques, include the activity and the organization of mitochondria, essential for cytoplasmic maturation, or glucose-6-phosphate dehydrogenase (G6PDH) activity which is associated with high rate of fertilization when it is low in mature oocyte [42, 43].

Finally, follicular fluid and serum components would represent the most suitable non-invasive predictors to be used. Some examples of these extrinsic factors are steroid hormones, the transforming growth factor (TGF)-β superfamily and insulin-like growth factors (IGFs) and their ligands, controlling cellular proliferation and differentiation [44-46]. Evaluation of the presence/concentration of these molecules would aid indirect assessment of oocyte quality. As already stated above, follicular cells and especially cumulus cells are regulator of oocyte development, maturation and fertilization and are currently subjects of considerable research as a possible source of material to be used to non-invasively assess oocyte quality.

In conclusion, multiple factors are involved in fertility decline of dairy cows and there is still a lot to investigate to resolve this issue. On the other hand, correct management of the animals including a correct nutrition, efforts to limit known risk factors at different stages of reproduction and developing strategies in order to enhance fertility such as improving genetic selection, ameliorating oocyte quality and *in vitro* embryo production together with basic research on reproductive physiology could all hopefully invert this negative trend.

In this project, we have focused our studies in the **folliculogenesis** process that is fundamental for the production and ovulation of a good quality female gamete, on which depend the subsequent reproductive steps to achieve a viable calf. Oocyte quality and key factors affecting it are still poorly understood and, therefore, our aim is to investigate on this topic.

2. Oogenesis is deeply rooted in folliculogenesis

The lifelong relationship between the oocyte and follicular cells starts very early during fetal development of an individual. Prymordial germ cells (PGC) are the embryonic precursor of the female gametes, they are located outside the embryo, in the yolk sac, and migrate along the hindgut reaching the genital ridge [47, 48]. When PGC reach the ovary they start undergoing mitosis, increasing in number and are called oogonia [49]. Oogonia switch from mitotic to meiotic division, replicating their DNA, thus becoming primary oocytes. During fetal life DNA is exchanged between homologous chromosomes through homologous recombination. This process is critical for pairing between homologous chromosomes. The paired and replicated chromosomes are called bivalents or tetrads, which have two chromosomes and four chromatids, with one chromosome coming from each parent.

Meanwhile, primordial flattened granulosa cells start surrounding some of these oocytes hence forming primordial follicles. The transition from a mitotic to a meiotic program is regulated by signals from somatic cells [50, 51]. The origin of granulosa cells is still not clear and they could derive from mesonephros and/or from the coelomic epithelium [52] or from the mesenchyme of the ovary [53].

In the primordial follicle, the oocyte continues its meiotic division going through the longest meiotic phase, that is prophase I, undergoing leptotene, zygotene, pachytene stages and finally arrests at diplotene (or dictyate) stage until puberty. These meiotic arrested oocytes represent the definite pool of oocytes of an individual and wait to be recruited for development, that could happen months or even years later [49, 54]. After puberty, few of the arrested primordial follicles at a time enter the nonreversible growing phase during which granulosa cells become cuboidal and restart to proliferate.

The growing follicles pass through the stages of primary and secondary follicles, characterized by the presence of two layers of granulosa cells, the initial deposition of zona pellucida (ZP) material around the oocyte and the beginning of theca cell layer formation. When the secondary follicle becomes finally a tertiary follicle, two types of granulosa cells differentiate: mural granulosa cells, lining the follicle wall and cumulus granulosa cells, surrounding the oocyte and forming the cumulus cell-oocyte complex (COC). Moreover, an antral cavity with follicular fluid forms among these cells. Finally,

the outer part of the follicle is delimited by layers of interstitial cells that differentiates into internal and external theca cell [55-57].

During follicular growth the oocyte, still arrested at diplotene stage undergoes a growth phase, which consists of a period of hypertrophy due to massive synthesis of cytoplasmic components. The storage of organelles and macromolecules that will be inherited by the embryo requires the chromosomes to decondense and the chromatin to become transcriptionally active; indeed, in the nucleus intense RNA synthesis is detectable until it stops at the end of the growth phase, which is species-dependent [58]. For example, in the early antral follicle mouse oocytes growth is already complete [59], while bovine oocytes are still growing [60].

The systemic LH surge determines the selection of just one large antral follicle (or more in polyovulatory species) that continues growing until reaching ovulation, while the other follicles will be eliminated through atresia. Only at this time, the oocyte(s) within the selected pre-ovulatory follicle(s) is able to resume meiosis and mature. What is generally called ‘oocyte maturation’ ends when the oocyte complete meiosis I and part of the DNA is extruded with the first polar body, and the oocyte’s chromosomes arrange in the metaphase II plate (MII stage). After first PB emission, the majority of the cytoplasm is retained in the oocyte [61]. Meanwhile, the granulosa cells are responsible for the process of cumulus expansion, producing a muco-elastic extracellular matrix, leading the COC to detach from the follicle wall [62]. Then the follicle wall breaks and ovulation takes place: the expanded COC is extruded from the ovary and moves to the oviduct where fertilization will take place. The meiosis process will complete after fertilization and extrusion of the second polar body. The remaining peripheral granulosa cells and the theca cells form the corpus luteum responsible for the production of high levels of progesterone required for successful implantation of the embryo and maintenance of the pregnancy [49] (**Figure 2.1**).

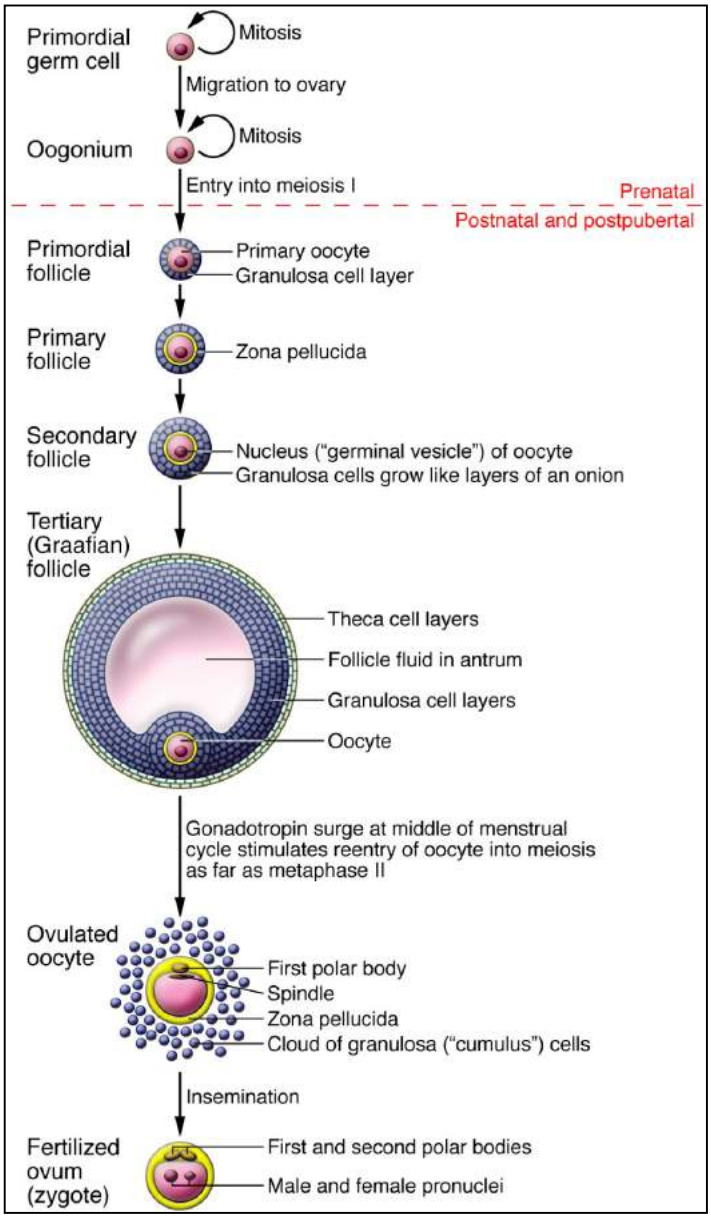

Figure 2.1 Schematic representation of oocyte and follicle maturation stages from fetal life to fertilization. From [37].

It is clear that folliculogenesis and oogenesis are two strictly interconnected events and their correct coordination determines the quality and competence of the female gamete [56, 57]. Follicles, and especially granulosa cells, are an essential niche for growing oocyte survival, nourishment and regulation [37].

The communication between these two compartments is bidirectional and it is mediated by a complex junctional communication system and several paracrine factors (reviewed in [61]).

Prominent extensions, called transzonal projections (TZP), on the surface of granulosa cells terminate on the oocyte plasma membrane interacting through gap junctions and adherens junctions. TZPs may be formed by F-actin (A-TZP) or microtubule –rich structures (M-TZP) and allow the transport of ions, metabolites, regulatory molecules. Even transport of RNA transcripts through micro-vesicles has been recently proposed [61, 63]. As the oocyte volume increases, TZP complexity evolves to assure correct support to the gamete [61]. Gap junctional mediated communication system is proved to be essential for correct oocyte maturation and developmental competence in cattle [64], as well as in other mammals [65, 66].

Paracrine factors are mainly oocyte-secreted factors (OSF) including members of the transforming growth factor- family, such as bone morphogenetic protein 15 (BMP15) and growth differentiation factor 9 (GDF9), and fibroblast growth factors (FGF) (reviewed in [67]). OSF alone are needed for cumulus expansion in mice [68], while in cattle, pig and rat is suggested that OSF alone are less influential than in the mouse [69]; indeed, the interaction between OSF and cumulus cells derived factors, such as FGF2 and kit ligand (KL), is essential for COC maturation [70, 71].

Moreover, both paracrine factors and ZTP are important to control meiosis. In fact, they regulate signaling of natriuretic peptide precursor C (NPPC), secreted by granulosa cells, which is responsible for maintaining meiotic arrest in mutant mice and in bovine oocytes *in vitro* [72, 73]. NPCC stimulates cGMP production, which is transferred to the oocyte through gap junctions and keeps cAMP levels required to prevent the synthesis of maturation promoting factor (MPF). NPPC production is inhibited by LH and cGMP flow to the oocyte is decreased after reduced gap junctions' functionality, thus promoting meiosis resumption [74]. Interestingly, NPPC is also regulated by intrafollicular steroids, including estradiol and progesterone. In cattle, steroids enhance NPCC action slowing nuclear maturation and increasing gap junctions mediated cumulus-oocyte communication in bovine COC. Their combination in pre-IVM culture also enhanced embryo quality [75, 76].

2.1 Cell division during folliculogenesis

During folliculogenesis cell proliferation and cell division are two fundamental processes; indeed, folliculogenesis involves a huge rate of cell proliferation making

granulosa cells mitosis a fundamental event in the follicle. On the other hand, oocyte meiosis prepares a haploid gamete ready for fertilization and production of a viable embryo. Mitosis is the process leading to faithful replication of somatic cellular contents, including DNA, and the production of two daughter cells with identical genetic and cellular composition. By contrast, meiosis is exclusive of germ cells and ultimately results in the production of gametes with half the DNA content so that, after fertilization, the zygote will contain the full complement of genetic material from the female and the male counterparts [56, 77].

Mitosis is “cyclical” in nature since the two daughter cells are diploid (2n) and ready to start forthcoming cycles of DNA replication and cell division, while meiosis is more unidirectional as the two gametes need to undergo fertilization to achieve the diploid status and be able to proliferate through mitosis for embryogenesis [77]. Even if they are two different types of cell division they share some common features, especially during the last phase of division, and their correct completion is important to avoid mistakes leading to cell defects and/or cell death.

2.1.1 Oocyte maturation

After LH surge, a mammalian oocyte that has terminated the growth phase and is arrested at prophase I, initiates the so-called meiotic maturation that encompasses the period in which the oocyte progresses from the prophase I stage to the Metaphase of the second meiotic division (MII stage), when meiotic progression stops again until fertilization occurs. As such, oocyte maturation involves both a nuclear and a cytoplasmic maturation. Cytoplasmic maturation involves cytoplasmic changes required to prepare the cell for fertilization and embryo development. The oocyte’s cytoplasm contains a variety of organelles typical of most cells such as mitochondria, Golgi apparatus and endoplasmic reticulum, but also some oocyte-specific organelles as, cortical granules, annulate lamellae, acidic granules. During maturation only some organelles undergo transformation (cytoskeletal elements, cortical granules and endoplasmic reticulum) while others remain stable. Cytoplasmic maturation remains poorly understood but has been shown to be fundamental for the completion of meiosis, the prevention of polyspermy and early embryonic development [51, 61].

Nuclear maturation consists in chromosome segregation and formation of the metaphase plate of the second meiotic division leading to the MII stage oocyte. Before meiotic resumption the oocyte contains a large nucleus, called Germinal Vesicle (GV), in which chromosomes are decondensed and transcriptionally active during the oocyte growth phase. Transcription is globally silenced toward the end of the oocyte growth phase and transcription is in fact already silenced when the chromosomes condense and the GV breaks down (GVBD).

At GVBD the meiotic spindle starts to assembly; the microtubule spindle assembles around the paired homologous chromosomes. This spindle segregates half of the homologous chromosomes in a small cell, that is the first polar body through an asymmetric division. The remaining chromosomes are captured by a second meiotic spindle and the oocyte arrests at this stage until fertilization. Only after fertilization the oocyte eventually resume and complete meiosis II undergoing a rapid anaphase II and telophase II with the elimination of another half of the genetic material in the second polar body, The chromatids remaining in the oocyte decondense and form a pronucleus that will fuse with the male pronucleus, giving birth to the zygote which will start dividing by mitosis to form the blastocyst [47, 77-79] (**Figure 2.2**).

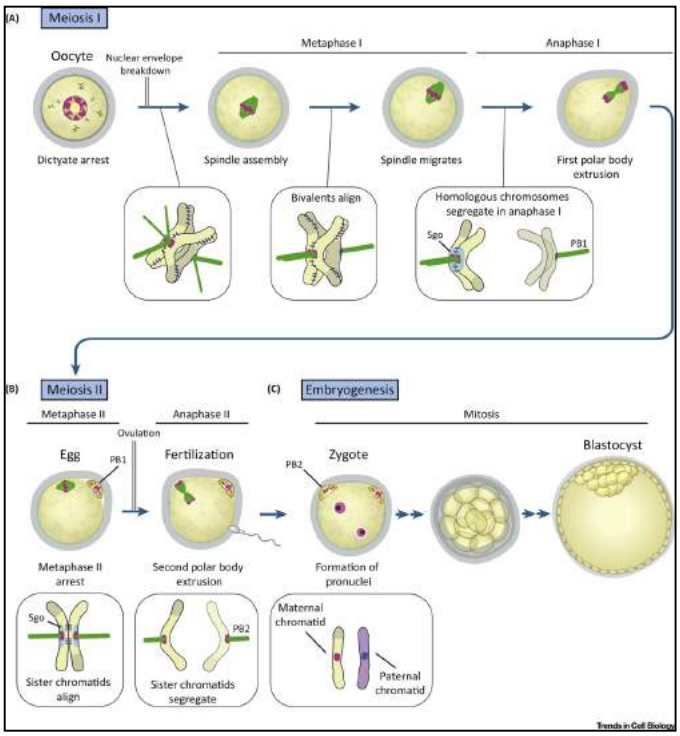

Figure 2.2 Schematic representation of the two meiotic divisions leading to an oocyte ready to be fertilized and progress into an embryo. From [79].

2.1.2 Mitotic cellular division

The cell cycle of somatic cells is characterized by two phases: interphase and mitotic phase (M-Phase). Interphase includes an initial growth phase (called Gap1 phase, G1) and a DNA synthesis phase (S), during which the genomic DNA is replicated, followed by a G2 phase, in which the cell ultimate preparatory events that would later allow the cell to produce daughter cells with enough organelles and properly replicated genetic material. The M-phase is generally divided into four stages: prophase, metaphase, anaphase, telophase and the last cytokinesis stage giving birth to the two daughter cells. During prophase, loose replicated chromatin condenses in individual chromosomes and the nuclear envelope starts to fragment. Chromosomes align along the mitotic plate during metaphase and microtubules attach to the duplicated centrosomes. This phase is the most important and needs to be strictly spatiotemporally regulated to assure the proper chromosomal positioning and number before segregation. During anaphase, the two centrosomes reposition to the opposite spindle poles thanks to motor proteins and sister chromatids segregate. Finally, during telophase each of the new complete DNA genomic set is surrounded by a newly formed nuclear envelope. Cytokinesis, leads to the division of the cytoplasmic compartment and actually starts at ana-teplophase, being highly coordinated with caryokinesis (i.e. segregation of the genetic material in two nuclei). Cytokinesis is ultimately responsible for the division of the two daughter cells through the formation of an initial cleavage furrow by an actin-myosin contractile ring, which halts when it encounters the midbody. During final abscission, the opposing bridge membranes fuse and the two cells finally divide [80] (**Figure 2.3**).

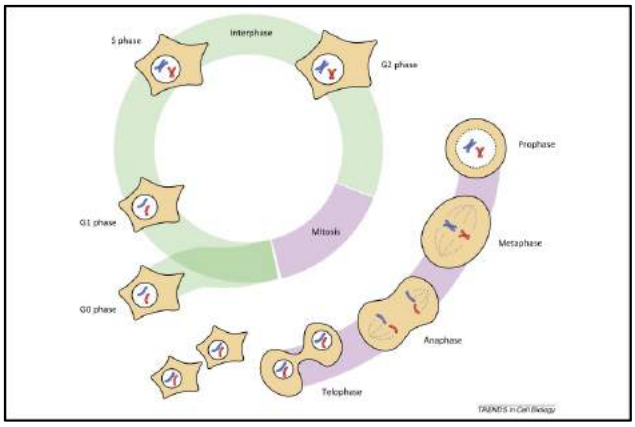

Figure 2.3 From [80]. Schematic representation of mitotic division stages.

2.1.3 The mitotic and meiotic spindles

Even if there is a great difference between mitotic and meiotic division, in both types of cellular division faithful chromosome segregation and completion of division in the last stages through cytokinesis and the extrusion of the polar body, respectively, are essential to achieve healthy new daughter cells. To this aim spindle made up of microtubules is the major structure playing an important role in both systems, even if with some differences, to orchestrate and spatiotemporally drive chromosomes movement during last stages of cell division and in the physical separation of the cells through the formation of the actin-myosin contractile ring [81].

In mitosis, the structure of the spindle at each mitotic phase has been well described. At anaphase two microtubule populations form the spindle: astral microtubules that radiate from the centrosomes toward the cell cortex and the midzone microtubules that form an antiparallel array between the separating chromosomes. The microtubule attachment site of chromosomes are the kinetochores, which enable the two sister chromatids of a chromosome to be attached to opposing spindle poles and to be pulled to opposite sides during cell division. This anaphase spindle determines the position at which the contractile ring, and subsequently the cytokinetic furrow forms. The cleavage furrow together with the central spindle microtubules forms the midbody that controls the correct cell division. The division plane usually bisects the anaphase spindle and, consequently, the cell. [81, 82] (**Figure 2.4**).

In the oocyte, the meiotic spindle lacks the centrosomes and the astral microtubules, while spindle midzone microtubules induce contractile ring constriction but do not define the site of polar body extrusion (the division site in the oocyte), which instead is a singular location on the cortex where the spindle is anchored and is determined by chromatin itself and not by the microtubules. In the mouse, it has been shown that the meiosis I spindle forms approximately at the center of the oocytes and then moves eccentrically toward the oocytes periphery. I other mammals such as cows, the GV is already found eccentrically in the fully-grown oocytes, thus the spindle migration toward the oocytes periphery is less probable in this species. Nevertheless, at the oocyte periphery the meiotic spindle is found perpendicularly oriented to the adjacent cortex. Its migration and rotation depend on microtubule/actin filament cytoskeleton. The meiosis II spindle then forms already off center [81] (**Figure 2.5**).

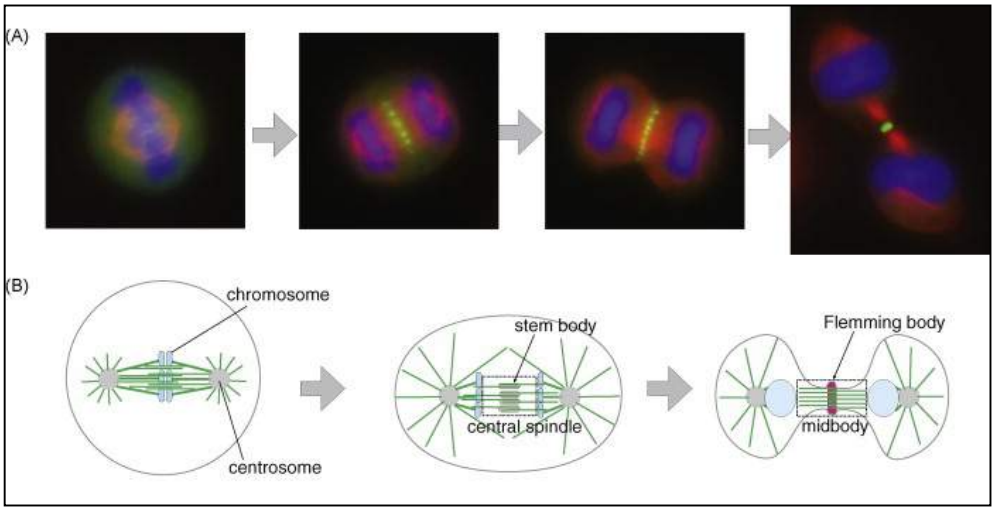

Figure 2.4 Adapted from [82]. Microtubules (red) in dividing Hela cells (A) and representation of microtubule spindle during anaphase and telophase of somatic cells (B).

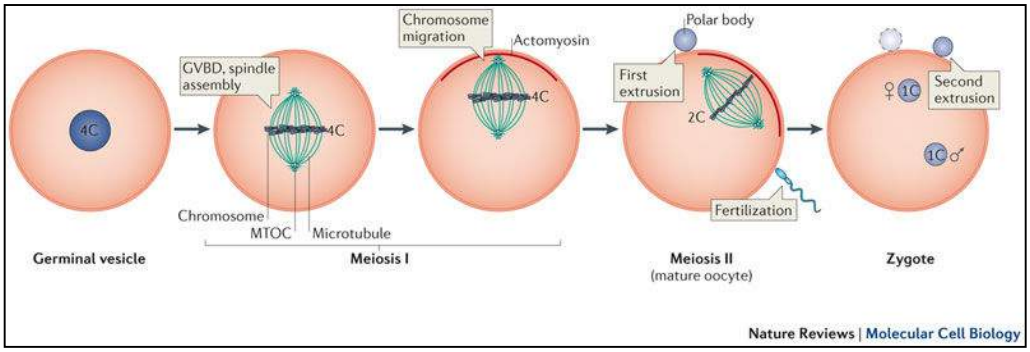

Figure 2.5 Representation of tubulin meiotic spindle (green) during maturation of a mammalian oocyte [61].

The spindle functioning is orchestrated and strictly controlled by several molecules. The best characterized of these controllers during mitosis is the chromosomal passenger complex (CPC). The CPC is part of the bigger spindle assembly checkpoint (SAC) and is composed by Aurora kinase B (AURKB), the inner centromere protein (INCENP) and the chromatin-targeting subunits Survivin and Borealin. This complex is dynamic throughout the mitotic division and it localizes from the centromeric region of the chromosome to the midzone during the last phases of mitosis [83, 84]. AURKB acts as an error-correction machinery allowing for

kinetochore-microtubule interactions to create and destroy, phosphorylating microtubule-interacting proteins, until the chromosomes are correctly oriented and stabilized. The other three members of CPC control its targeting, enzymatic activity and stability. The SAC complex acts as a messenger for CPC information for the onset or the delay in chromosome segregation at anaphase thus assuring the correct timing for division [85]. The CPC complex is described to be conserved also in meiotic spindle [86, 87], but with some differences in its dynamical localization. Indeed, in meiosis I it does not transfer to central spindle microtubules but remains at the centromeres of the segregating bivalents until late anaphase I to preserve centromere cohesion, while it starts to accumulate at the spindle midzone, as in mitosis, during metaphase II-anaphase II transition [88, 89]. Importantly, in mouse AURKC regulates oocyte meiosis, while in bovine this function is exerted by AURKB [90].

The major consequence of missegregation of chromosomes occuring during cell division is aneuploidy, i.e an abnormal number of chromosomes in the daughter cells [91]. Missegregation during meiosis causes aneuploidy in progeny or fertilized eggs, leading to infertility, miscarriages and birth defects [79, 92]. Errors during meiosis are caused by age-related changes to structure responsible for chromosome attachment to tubulin; one hypothesis is cohesin complex deterioration which is evident in mouse studies, but still to be proven in human [93, 94]. There are also age-independent pathways causing errors during meiosis such as defects in homologous recombination, insensitivity to meiotic checkpoint activation or instability of the meiotic spindle [79]. While during mitosis, aneuploidy is considered the consequence of failure in CPC and SAC checkpoints signaling [84] and it is a feature of tumoral cells, hence might facilitate tumorigenesis [95, 96].

2.2 In vitro maturation (IVM) value as commercial tool and experimental model

Oocyte maturation, intended from meiotic resumption and progression to the mature stage (MII), can be achieved *in vitro*. The fundamental difference between *in vitro* and *in vivo* oocyte maturation lies in the fact that in the follicle oocytes are maintained under meiotic arrest and the maturation of selected oocytes can start only

after hormonal stimulation (LH-dependent). While *in vitro*, fully-grown oocytes isolated from mid-large antral follicles and placed under suitable culture environment undergo spontaneous (LH-independent) meiotic resumption and progress to MII phase with polar body I extrusion [97]. In vitro, oocytes resume meiotic resumption generally earlier when compared to the in vivo counterpart. Thus, generating an asynchrony between oocyte nuclear and cytoplasmic maturation, which is generally considered one of the drawback of IVM [98].

Nevertheless, *in vitro* maturation (IVM) was first established in rodents [99] and then progressively extended to many other species such as pig, sheep, goat, horse and cow being used for both scientific and commercial interests [100].

In particular, IVM of bovine oocytes is being greatly exploited by bovine industry and in research field since cow oocytes mature *in vitro* with a very high efficiency (over 90%). Indeed, in the last century IVM and consequent in vitro fertilization (IVF) to achieve in vitro embryo production represents a significantly growing field [101]. Assisted Reproductive Technologies (ARTs) are extensively used in bovine industry for high quality embryo production and to quickly propagate genetics, thanks to the continuous improvement of culture media [101].

On the other hand, for research purposes IVM represents a good accessible investigation strategy to easily observe the process of maturation. Scientists could also test the action of hormones, growth factors and other biologically active molecules in this simplified system, rapidly and without confounding influence of in vivo elements such as the follicle, the ovary and the organism [102].

Finally, being a monovular species, bovine is a suitable model to study woman reproduction and to ameliorate ARTs that are extensively used in human medicine and IVM has been introduced as an alternative to minimize or eliminate the risks and disadvantages associated with intense gonadotropin stimulation of the ovary [100].

2.3 Mammalian folliculogenesis and oogenesis regulation

Folliculogenesis and oocyte growth and maturation toward developmental competence acquisition are gradual processes strictly regulated by checkpoints to assure the correct timing for every stage. Every checkpoint is controlled by numerous molecules and hormones, which are shortly listed here (reviewed in [30]).

2.3.1 Molecular regulation

Until puberty, follicle activation is repressed by several factors such as *spermatogenesis and oogenesis specific basic helix-loop-helix 2* (*Sohlh2)* [103], anti mullerian hormone (AMH) [104] and *Phosphatase and tensin homolog* (*Pten)* [105]. In mice, the transition from primordial to primary follicle is mainly driven by the oocyte, that is characterized by upregulated expression of members of the transforming growth factor (TGF)-β superfamily, including *Bone morphogenetic proteins* (Bmp5, Bmp6, Bmp15), Growth differentiation factor 9 (Gdf9), Transforming growth factors (Tgfb2, Tgfb3), as well as other growth factors such as *Basic fibroblast growth factor* (bFgf) and *Leukemia inhibitory factor* (Lif) regulated by several master transcription factors (reviewed by [106]).

These events occur also in other species and, for example, it is postulated that changes in the oocyte transcriptome in cattle happens during the following passage from primary to secondary follicle, which takes longer than in mouse, and is characterized by nucleolus reorganization and activation determining the beginning of RNA synthesis [107]. Moreover, at this point bidirectional communication between oocyte and granulosa cells through gap junctions initiates and the regulatory loop regulating signaling and metabolic pathways between the two compartments is probably mainly driven by Gdf9 and Bmp15, which have key roles especially in glycolysis and cholesterol biosynthesis of cumulus cells [108].

In cattle Follicle stimulating hormone receptor (FSHR) mRNA expression and a progressive responsiveness to gonadotropins is observed during the secondary follicle stage [109-111]. The transition to tertiary follicle is characterized by intensive mRNA and rRNA transcription. Locally produced factors, such as insulin-like growth factor-I (IGF-I) and members of the TGF-β superfamily, still play an important role in bovine preantral follicle development [106].

In cattle, the development of the antral follicle is completely dependent on gonadotropins; indeed, the growing follicles express transcripts encoding steroidogenic enzymes, gonadotropin receptors and local regulatory factors together with their receptors, meanwhile the oocyte transcriptome becomes quiescent and the nucleolus inactivate [107]. During the last stages of follicular growth, the follicle responds to increasing LH levels, the resumption of meiosis in the oocyte is likely promoted by epidermal growth factor (EGF)-like factors *Amphiregulin* (Areg),

Epiregulin (Ereg) and *Betacellulin* (Btc) released by granulosa cells, since the oocyte does not express LH receptors. In addition, meiotic resumption requires activation of maturation-promoting factor (MPF) resulting in the germinal vesicle breakdown (reviewed in [112]).

The subsequent stages of meiotic maturation are regulated by the Anaphase Promoting Complex (APC) and other key factors previously described. The arrest at metaphase II is under the control of cytostatic factor (CSF). All these regulatory complexes are in turn regulated by kinases, phosphates, polyadenilation [113]. Finally, after cumulus expansion, the LH surge determines follicle rupture and ovulation. Also these events are strictly coordinated by different extracellular signal-regulated kinase (ERK1/2) target genes, as shown in vivo in ERK1/2 depleted mice; for example, cytochrome P450 Cyp19a1 and Sulfotransferase Family 1E Member 1 (Sult1e1), which regulate oestradiol biosynthesis and activity, Steroidogenic Acute Regulatory Protein (Star) and cytochrome P450 Cyp11a1, which are associated with granulosa cell luteinisation, the EGF-like factors Areg, Ereg and Btc and the cumulus expansion factors Prostaglandin-endoperoxide synthase (Ptgs2), TNF Alpha Induced Protein 6 (Tnfaip6), Hyaluronan Synthase 2 (Has2), Pentraxin-related protein (Ptx3) [114].

2.3.2 Hormonal regulation and progesterone (P4) role in the follicle development

The main hormones involved in folliculogenesis are Gonadotropin-releasing hormone (GnRH), secreted by the hypothalamus, which stimulates the release of Follicle-Stimulating Hormone (FSH) and LH from the anterior pituitary gland. FSH is responsible for follicle development, promoting antral follicles growth and differentiation of somatic cells, ensuring they acquire sensitivity to LH. LH is then responsible for triggering ovulation and oocyte maturation.

Estrogen and progesterone (P4) also play key roles. Estrogen is a steroid hormone produced by the granulosa cells of the developing follicle exerting a negative feedback on LH production until the oocytes is ready for ovulation, it then leads to the LH surge. P4 is a steroid hormone synthesized by the ovary at different levels depending on gonadotropin stimulation and the physiological status of the ovary [115, 116]. During the oestrus cycle of cattle, P4 circulating levels regulate pulsatile secretion frequency of gonadotrophin-releasing hormone (GnRH), which in turn regulates LH

pulse frequency. The ovulation of a dominant follicle is driven by LH pulse frequency: high P4 concentrations determine low LH pulse frequency and the dominant follicle undergoes atresia; on the contrary, low P4 concentration after luteolysis leads to ovulation of the dominant follicle after production of oestradiol and inhibin [117, 118].

The P4 well known effects on reproductive function include also the formation and maintenance of the corpus luteum [116], the effects on reproductive tissues, especially the uterus and mammary gland [119] to prepare them for the pregnancy, and the placenta formation [120, 121]. It is also involved in regulating the mating behavior [122]. It is demonstrated that it has a role also during rodents ovulation since blocking P4 leads to anovulation [123-125] and more recent studies using P4 receptor knockout mice demonstrate that this action is mediated, at least in part, through the nuclear Progesterone Receptor (PGR-A and PGR-B) [126, 127].

2.3.3 Insights into P4 and P4 receptors' role in folliculogenesis

The role of P4 before ovulation is still poorly understood even if some indications suggest its contribution during folliculogenesis and oocyte maturation. Indeed, P4 levels in the follicular fluid of preovulatory follicles between LH surge and ovulation are very high [128]. Moreover, cumulus cells exhibit receptors for P4 and secrete P4 during oocyte maturation suggesting a role in oocyte competence. It has been demonstrated that inhibiting P4 production *in vitro* leads to reduced embryo development in cattle [129]. Additionally, *in vivo* studies showed elevated P4 during development of the ovulatory follicle is associated with improved pregnancy rates in lactating dairy cattle [130].

There is evidence that P4 potential actions are exerted both on follicular cells and on the oocyte. In the follicular compartment, periovulatory P4 surge is responsible for the inhibition of follicular growth, in particular *in vivo* studies showed that it decreases the rate of granulosa cells mitosis [131] during the oestrus cycle and pregnancy of different species including hamster [132, 133], rabbit [134], rats [135], mice [136] and also in primates [137]. Strikingly, this role appears independent of its ability to influence gonadotropin levels, so it is likely that it acts directly on granulosa cells. This hypothesis is further confirmed by *in vitro* studies on granulosa cell cultures [138-142]. While in luteal cells after ovulation, P4 promotes its own synthesis and

prevents apoptosis to maintain luteal cells. This happens also in rat luteal cells, which do not express PGR receptors. This further suggests some signaling pathway other than the classical genomic one.

The role of progesterone in inducing oocyte maturation in frog and fish oocytes is well known [143-146], while in mammalian oocyte its role is still debated. Despite in rodents there is little evidence of a role in oocyte maturation [147], in other species there is growing evidence of its contribution. In in vitro culture of bovine and porcine oocyte and its surrounding cumulus cells, inhibiting P4 synthesis or blocking PGR receptor, which is not expressed in the mouse counterpart, impairs cumulus expansion [129, 148, 149]. Moreover, recent studies have shown that oocyte meiotic and developmental competence are affected by P4 as well as by both classic and non-genomic receptors [129, 150, 151].

Besides the classical nuclear Progesterone Receptor (A and B) [152], which mediates P4 signaling regulating genes cascade, over the last years other putative non-genomic P4 receptors have been investigated. The presence of non-genomic P4 receptors could justify some rapid responses to P4, not compatible with genomic signaling transduction, which is slow and complex. Moreover, studies in PGR-A/PGR-B null mice showed a normal follicular growth and, since granulosa cells of immature follicles respond to P4, these newly proposed receptors could be mediating P4 actions before gonadotropin surge [153, 154]. Finally, luteal cells of short luteal phase species, as mice and rats, do not express PGR and, therefore, P4 luteotropic action may be transduced by other receptors [155, 156].

The putative non-genomic, membrane-bound P4 receptors are found to be expressed also in mammalian ovaries and include:

- A family of three Membrane Progestin Receptors (MPR) (isoforms mPRα, mPRβ and mPRγ). Based on amino acid sequence homology, the mPRs belong to a larger family of 11 highly conserved mammalian paralogs termed the PAQR (progestin and adipoQ receptors). They were first identified in fish oocytes [157, 158] and then also being expressed in rat ovary [159] and in bovine [129] and porcine oocytes [160].
- Progesterone Receptor Membrane Components (PGRMC1 and PGRMC2) expressed in both follicular cells and oocyte of different species indicating a possible role in mediating P4 action in this system (see PGRMC1 overview chapter). Importanly,

it must be underlined that both PGRMC1 and PGRMC2 also exert P4 independent function in mammalian cells [161].

From this premise, it is clear that many factors and molecules are involved in reproductive function and the complex folliculogenesis regulation is continuously under investigation. Nonetheless, several mechanisms of action and molecules are still poorly understood or unknown. Hence in this study we focused on Progesterone Receptor Membrane Component 1 (PGRMC1) as a putative key factor involved in oocyte competence acquisition and oocyte maturation having a role in regulating folliculogenesis at different stages in both the somatic and germinal cells.

3. PGRMC1 overview

3.1 Cloning and nomenclature

Progesterone Receptor Membrane Component 1 (PGRMC1) is a small heme-binding protein, which is expressed and conserved in several animal tissues and characterized by a wide variety of biological functions [161]. PGRMC1 protein was first discovered and described in 1996 by two independent groups. *Selmin et al*. cloned PGRMC1 cDNA as a gene product responsible for reducing dioxin toxicity in rats; the 25 kDa dioxin-upregulated protein was named *25-Dx* [162]. While *Meyer* group purified this protein from swine liver membrane as a component of a membrane associated P4-binding activity and therefore named it membrane progesterone receptor (mPR) [163]. In 1998, the same group cloned by homology to its porcine counterpart the human PGRMC1 gene, on chromosome X, which they firstly called *Heme Progesterone Receptor 6* (Hpr6) [164].

Since then PGRMC1 has been found and reported in different areas of biological research in mammalian tissues (see 3.4 paragraph) under different synonyms, until it was officially and univocally referred to as Progesterone Receptor Membrane Component 1 (PGRMC1), approved by HUGO Gene Nomenclature Committee (HUGO Gene Nomenclature Committee ID HGNC:16090).

3.2 Lower eukaryotic homologues

PGRMC1-like proteins have been described also in non-mammalian species. In 2003, *Craven* group cloned a *Saccharomyces cerevisiae* yeast member of the MAPR gene family and the encoded protein was called *Damage Response Protein Related to Membrane Associated Progesterone Receptors* (DAP1). DAP1 shows similarity to PGRMC1 [165] and in yeast it mediates resistance to azole antimycotics [165], regulates cytochrome P450 proteins [166] and mediates iron homeostasis [167].

In 2004 *Runko et al.* published the discovery of *C.elegans* protein VEM-1 as the homologue of PGRMC1 they found in rat neurons [168], sharing 37% amino acid identity. This protein is involved in neuron guidance and axon formation in a variety of neurons in the nematode ventral midline [168].

Moreover, *Song et al.* [169] described an ortholog called AT2G24940 in *Arabidopsis* which NMR structure revealed a cytochrome b5 domain highly homologous and conserved between this and the mammalian PGRMC1 protein [170, 171]. Nonetheless, little is known about its functions in plants since no functional studies are available.

3.3 PGRMC1 structure

PGRMC1 is a 194-aminoacid long protein and has a predicted molecular weight of 21 KDa in most mammalian species. According to electrophoretic mobility the molecular weight is of 25-28 kDa. Frequently, electrophoretic analysis followed by Western blotting detects a second band of double molecular weight. This band was thought to be a disulfide link dimer, since it disappeared after treatment with reducing agents [172]. However, in other studies treatments with reducing agents failed to disrupt this putative dimeric form and described this as a not membrane-bound PGRMC1 with high molecular weight, since it did not disappear when detergents were omitted from the extraction buffer. On the contrary, the 25Kd resulted the membrane bound form [173].

The existence of a PGRMC1 dimer is further confirmed by recent studies by *Kabe et al.* that showed that PGRMC1 dimer formation is heme-dependent [174]. Moreover, additional bands of >50 and < 50 kDa molecular weight are frequently detected in western blots at long exposures (depending on cell types) and PGRMC1 siRNA treatment depletes these higher forms as well as the lower band, confirming that PGRMC1 exits in multiple molecular weight forms [175].

PGRMC1 is a member of the Membrane Associated Progesterone Receptor (MAPR) family, which is a sub-family of proteins sharing a basic cytochrome b_5 ($Cytb_5$) domain fold [176]. In mammals, MAPR has four family members: PGRMC1 (the archetypal member of the family), PGRMC2 (strictly related to the previous one), Neudesin and Neuferricin [176].

Human PGRMC1 is composed of 194 aminoacids in a single chain, is predicted to be anchored to the cell membrane through a N-terminal transmembrane domain and has a putative cytoplasmic C-terminal Cytb5-like domain based on sequence analysis [177, 178] (**Figure 3.1**).

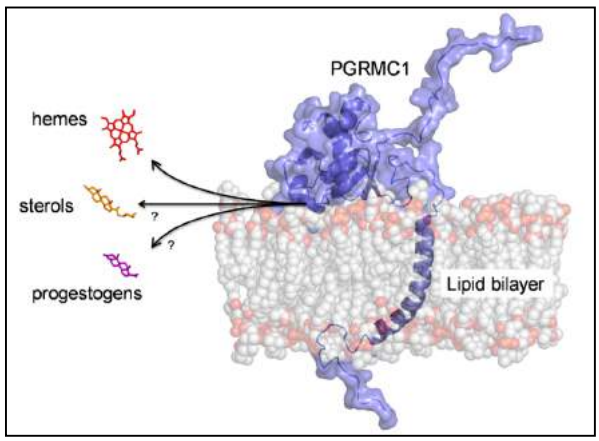

Figure 3.1 Graphical representation of PGRMC1 structure. In particular its proposed localization to the membrane of cellular organelles, potentially binding small hydrophobic ligands. From [179]

Kabe et al. recently published the NMR and crystal structure of PGRMC1 and they showed for the first time that after binding to heme PGRMC1 forms a stable dimer that is required for binding and activation of CyP450 enzymes and epidermal growth factor receptor (EGFR). The dimer is dissociated into monomers by Carbon Monoxide (CO) that interferes with the heme-stacking interface [174] (**Figure 3.2**).

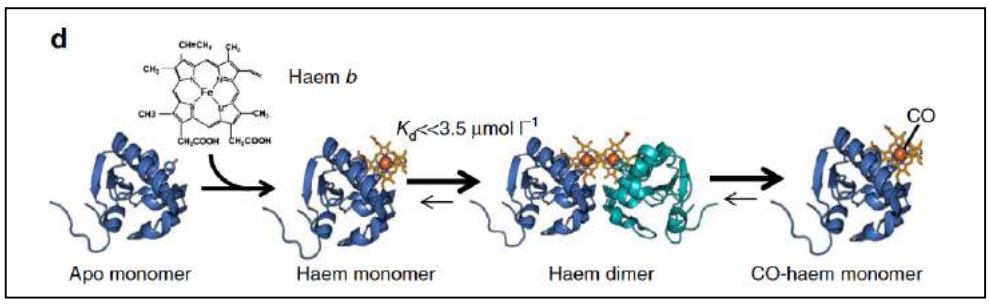

Figure 3.2 Model of PGRMC1 structural regulation from monomer to dimer in response to haem and CO. From [174].

This study also confirmed some of the previous predictions made on PGRMC1 structure, such as the presence of different peptide sequences that might be involved in its interaction with other cellular proteins or molecules for signal transduction. In

particular, the consensus motifs Src homology 3 (SH3) target sequence at P63 and the Src homology 2 (SH2) target sequences centered at Y139 and Y180. They are adjacent to one another and lie on the opposite surface of the heme-binding site. Tyrosine phosphorilation of SH2 Y139 and Y180 could be necessary to recruit proteins to PGRMC1 surface and, on the other hand, SH3 and SH2 on Y181 are probably negatively regulated by constitutive phosphorylation of adjacent Casein Kinase 2 (CK2) consensus motifs at S57 and S181 [174]. Therefore, this further suggests that phosphorylation status seems to have an important role in orchestrating PGRMC1 function as Cahill stated after describing that *in vivo* PGRMC1 is differentially phosphorylated [180, 181] (**Figure 3.3**).

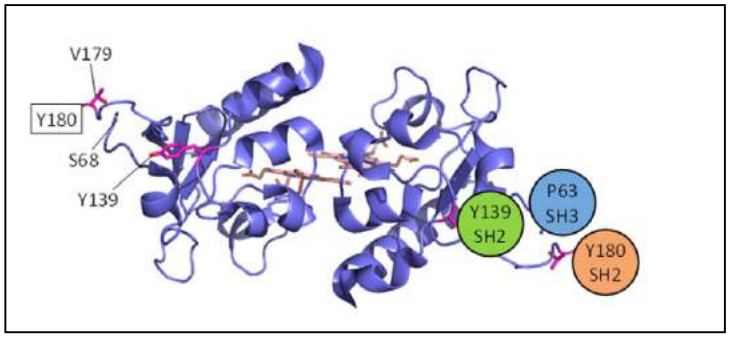

Figure 3.3 PGRMC1 heme dimer structure highlighting important peptide sequences possibly involved in its interaction with different proteins and molecules. From [180].

Moreover, in silico analysis shows that PGRMC1 presents also 3 sumoylation sites at lysine residues 136, 187, and 193 (http://sumosp.biocuckoo.org/online.php) [182]. Sumoylation, which involves the rapid and reversible covalent binding of small ubiquitin-like modifier (SUMO) proteins, is another process that is involved in regulating PGRMC1 function [173].

3.4 PGRMC1 localization in mammalian tissues

From its first description in rat and swine liver [162, 163] PGRMC1 has been found to be expressed in a very wide variety of mammalian organs and tissues, and in several tumors as well (see chapter 8).
It was described in the rat adrenal cortex [183], in rat nervous system [184-188] and in several reproductive organs.

In particular, PGRMC1 is expressed in rat and human uterus and placenta [189], in macaque endometrium [190], in bovine [151, 191-193] and canine reproductive systems [194]. Specifically, PGRMC1 has been described also in the ovary [151, 191, 195]. In the **ovarian follicle** it is found in the **somatic compartment**, where it is expressed in granulosa and luteal cells of rodent, bovine and canine ovaries [129, 191, 194, 196-198], and also in bovine and rat **oocyte** [150, 151, 195].

From a **subcellular** point of view, PGRMC1 is expressed in many cellular compartments. Indeed, PGRMC1 has a transmembrane domain so it is a membrane bound protein found in several membranous compartments: the nuclear and plasma membranes [189, 195, 199], the endoplasmic reticulum [163, 183, 188], the Golgi apparatus [188], the endosomes [200] and the secretory vesicles [201]. Interestingly, it localizes also to the mitotic spindle, interacting with microtubules [202-204], and then it associates to the midzone and the midbody during cell division [204]. Similarly, in meiotic spindle PGRMC1 associates to condensing chromosomes and then to centromeric region of metaphasic chromosomes during metaphase I and metaphase II, while during ana/telophase I it concentrates between separating chromosomes [150, 151].

This protein was also described within the nuclear compartment [173, 175, 189, 205-207] in which it localizes also to the nucleoli both in somatic cells [208-210] and in nucleolar precursor bodies of the zygote [151] (**Figure 3.4**).

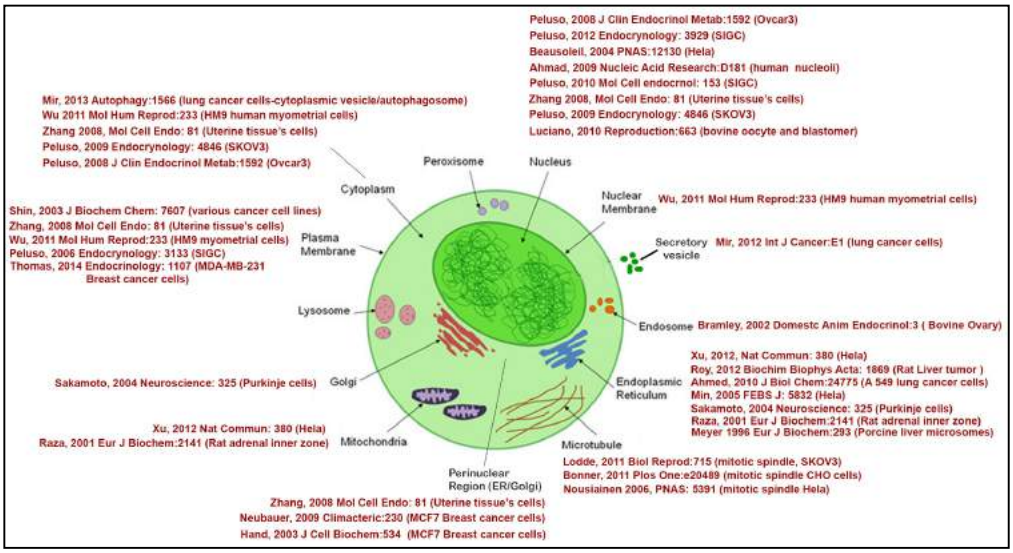

Figure 3.4 Image showing the plethora of PGRMC1 subcellular localizations. Data from: [151, 163, 173, 175, 183, 188, 195, 199-208, 211-220].

It is clear from the disparate multiplicity of PGRMC1 localizations that its function(s) may vary according to the organ/tissue considered. Moreover, its high subcellular compartmentalization suggests that at each site PGRMC1 might participate in the control of precise and diverse cellular processes, depending on the protein(s) with which it interacts at each sub-cellular site. In fact, this is reflected by a very wide range of proposed functions for PGRMC1, the majority of those is currently under investigation. Our main hypothesis is that in every specific subcellular localization PGRMC1 interacts with different proteins and, consequently, it is involved in diverse pathways resulting in distinctive functions.

3.5 Putative PGRMC1 ligands and functions

Although PGRMC1 is a member of MAPR protein family, it has no homology with the nuclear or membrane-associated steroid receptors [176] but it is more similar to Cyt b_5 heme binding proteins, sharing key structural motifs. Indeed, the best known PGRMC1 biochemical function is binding to heme and, consequently, binding and activating a wide range of cytochrome P450 proteins; thus, regulating in an heme-dependent manner drugs, hormones and lipids metabolism [161, 178].

For example, PGRMC1 regulates cholesterol synthesis interacting with CYP51/lanosterol demethylase [221], which catalyzes the essential reaction from lanosterol to cholesterol in the sterol synthetic pathway. Moreover, PGRMC1 interacts with INSIG1(insulin-induced gene)/SCAP (SREBP cleavage activating protein) /SREBP (sterol regulatory element binding protein) complex that regulates sterol precursors synthesis, but its role in modulating this complex is still unknown [222]. It also binds several drug metabolizing P450s, including CYP2C2, CYP2C8 [223] and CYP3A4, a major enzyme responsible for the metabolism and clearance of almost 50% of all known drugs, CYP7A1, an important enzyme involved in bile acid synthesis and CYP21A2, a progesterone 21-hydroxylase required for production of glucocorticoids and mineralocorticoids [221].

PGRMC1 is also involved in embryogenesis; in fact, both mammalian PGRMC1 and its nematode homologue VEM-1 regulates the fidelity of nerve cord axonal guidance along the ventral midline of nematodes and the spinal cord of rats [168, 184, 185]. What's more, this function relies on the vesicle trafficking properties of PGRMC1 to expose specific cell surface receptors required during axonal guidance, indicating another possible function of this protein [161].

Moreover, for its localization to the outer mitochondrial membrane, PGRMC1 interacts with Ferrochelatase (FECH), the final enzyme in the heme synthetic pathway [224].

PGRMC1 effect on cell proliferation and division is another attested PGRMC1 function as suggested by its localization to the meiotic and mitotic spindle in both somatic cells and oocytes and its co-localization with AURKB in oocytes [150, 204]. Studies conducted in bovine oocytes (which represent the base knowledge for the present PhD research) demonstrated that injecting an antibody against PGRMC1 into immature oocytes arrested the majority of the oocytes at prometaphase I during bovine oocyte meiosis and prevented completion of meiosis I [151]. Moreover, several studies inhibiting PGRMC1 function with different techniques impaired cell proliferation in rat granulosa cells, ovarian, breast and lung cancer cell lines. The same results were observed injecting PGRMC1 depleted-SKOV3 human cancer cell lines into athymic nude mice, where these cells failed to proliferate and form tumors when compared to the wild type counterpart. [204, 207, 211, 225]. Moreover, studies have shown that in spontaneously immortalized granulosa cells (SIGCs) and ovarian cancer cells SKOV-3, PGRMC1 interacts with β tubulin microtubules as assessed by in situ proximity

ligation assay. The stability of the microtubules could be regulated by P4-PGRMC1 interaction consequently affecting the rate of mitosis; the presence of P4 causes slow cell proliferation and delayed mitotic progression [204].

Finally, there is strong evidence of PGRCM1 role in cancer biology. Indeed, PGRMC1 is overexpressed in a wide range of tumors, compared to corresponding normal tissues, in which it promotes tumor growth and chemoresistance. *Kabe* and colleagues recently described PGRMC1 heme-dependent dimer formation as essential for interaction of this protein with cytochrome P450 proteins but also with EGFR, respectively enhancing chemoresistance and proliferation of cancer cells [174].

3.6 Last but not least - Is PGRMC1 a Progesterone Receptor?

PGRMC1 was initially cloned in search of membrane receptors for P4 that were distinct from the classical nuclear progesterone receptor (nPGR). So, even if PGRMC1 structure shares no homology with steroid receptors, researchers have always wondered if it could also exert some P4 related functions.

In 2015, *Kaluka et al.* definitely demonstrated by ultraviolet–visible and resonance Raman spectroscopies that P4 can interact with heme bound to an N-terminally truncated bacterially expressed PGRMC1. Thus providing the strongest evidence for PGRMC1 binding P4 [177]. Nevertheless, P4 anti-apoptotic and anti-proliferative action on rat granulosa cells and rat spontaneously immortalized granulosa cells (SIGCs) is the only demonstrated P4 action mediated by PGRMC1 [154, 226] and a multiplicity of PGRMC1 function not related to P4 have been reported.

It is clear that more studies are needed to understand the precise mechanism of action through which PGRMC1 mediates P4 signaling. The current hypothesis is that for this process both a cytoplasmic and a nuclear component are required. P4 binds to cytoplasmic PGRMC1, which is likely on plasma membrane, and three cytoplasmic events could be involved in P4's anti-apoptotic action: the induction of protein kinase G activity [227] and the suppression of Erk1/2activity [228], together with intracellular free calcium levels [229].

In addition, also a genomic function for PGRMC1 has been described, in which PGRMC1 mediates gene expression of certain genes. The genomic component of PGRMC1's action, according to *Peluso* group studies, seems to involve PGRMC1

dimer in the nucleus, responsible for changes in gene expression profile to finally promote cell survival [175]. This signaling pathway could involve PGRMC1 binding to plasminogen activator inhibitor 1 mRNA-binding protein (PAIRBP1) [226], which is considered essential since P4 increases this interaction and depleting PAIRBP1 attenuates P4's ability to inhibit apoptosis [230].

From this overview, the multifunctional protein PGRMC1 is proposed to be a signaling nexus hub protein in the middle of a network of different functions, modulating several pathways in distinct organs and subcellular compartment according to different interactions with other molecules. Moreover, PGRMC1 seems to be relevant to multiple diseases processes, making it an interesting protein to study in all its multiplicity and being considered a potential target for therapy/biological marker in disparate contexts [231] (**Figure 3.5**).

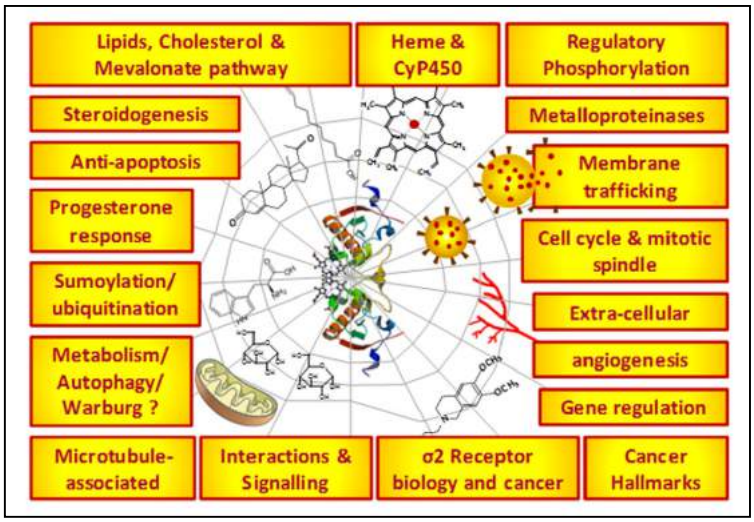

Figure 3.5 PGRMC1 variety of functions supporting the hypothesis of a multifunctional protein changing its role according to its localization, structure and interaction with different molecules. From [231].

4. Objectives of the thesis and significance

The main aim of this thesis project is to study the putative role of PGRMC1 as one of the key molecules involved in reproductive function. In particular, given the wide variety of localizations even within the same system and its numerous functions, there is the need to study its contribution relatively to each cell type and subcellular compartment to ultimately unravel PGRMC1's role.

We propose that PGRMC1 has a role in bovine ovary during folliculogenesis, both in follicular cells compartment and in the oocyte. Our hypothesis is based on strong evidence supporting this protein being expressed in follicular cells and oocytes of many mammalian species (see PGRMC1 overview chapter).

Hence, in this PhD project firstly we focused on PGRMC1 putative contribution to cellular division, both in follicular granulosa cells and in the oocyte, as suggested by the dynamic localization of PGRMC1 in mitotic and meiotic spindle indicating a possible common function. We especially evaluated its role during the last phases of division including chromosome segregation, as this is one of the most important stages as seen before.

Since PGRMC1 could have a role in mediating P4 action both in follicular cells and in the oocyte, more experiments were conducted on investigating its role as a mediator of P4 action specifically during meiotic maturation and comparing its effect to nPGR putative non-genomic role in the oocyte.

In a second part of the project we extended our research to add more insight into the putative mechanism of action of PGRMC1 in cell division studying its possible role and mechanism of action in a specific nuclear region, the nucleolus, in bovine granulosa cells and in growing and fully grown oocytes.

Finally, since PGRMC1 role in cell proliferation could be relevant also in cancer biology we performed some preliminary studies on its role in tumors, considering the growing evidence and interest of its involvement in this disease. We described for the first time its expression in normal canine mammary gland and then its expression in three main different types of canine mammary cancer.

The importance of studying factors involved in reproduction and in particular in the quality of the gamete is relevant for dairy industry. PGRMC1 could be one of the key molecules to be used to overcome the infertility issue reviewed in the introduction,

which leads to economic losses and has an impact also on animal welfare. Moreover, besides likely improving assisted reproductive technologies in livestock, the results of our studies could be translated also for human reproduction unraveling causes and solutions for women infertility since PGRMC1 is already one of the proposed proteins involved in this problem. Finally, as PGRMC1 is becoming an interesting putative biomarker in cancer biology and our preliminary studies could add some new insight in this field as well.

5. PGRMC1 participates in late events of bovine granulosa cells mitosis and oocyte meiosis

Foreword:
This chapter is a published article. The original full paper is provided in the appendix.
***Terzaghi L**, Tessaro I, Raucci F, Merico V, Mazzini G, Garagna S, et al.*
PGRMC1 participates in late events of bovine granulosa cells mitosis and oocyte meiosis. Cell Cycle. 2016;15(15):2019-32.

In this paper, we evaluated PGRMC1 role in meiotic and mitotic division. Our experimental approach mainly involved interfering with PGRMC1 expression and evaluating the effect on mitotic and meiotic division. On a primary cell line of bovine granulosa cells, we used small interfering RNA (RNAi) gene silencing technique and evaluated the effect on cell proliferation, further assessing the precisely mitotic phase which was most affected by using flow cytometry analysis and time-lapse imaging. While in *in vitro* matured bovine oocytes we impaired PGRMC1 function through RNAi downregulation and pharmacological inhibition using AG205. To further assess the involvement of PGRMC1 in the correct completion of division we evaluated its relationship with regulating proteins such as AURKB using immunofluorescence in both systems.

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Conflict of interest: Authors declare no conflict of interest.

Author's role

L.T performed all experiments related to bovine granulosa cells, participated in oocyte collection and RNAi procedures and contributed to the writing of the manuscript. I.T. carried out RNAi microinjections in COC and contributed to the writing of the manuscript; F.R. performed the Western blot analysis; F.F. contributed to microinjections procedures optimization, to data analysis and interpretation and contributed to the writing of the manuscript; V.M., S.G., M.Z contributed in time-lapse and fluocytometry analysis; G.M contributed to flow cytometry analysis. V.L performed AG205 experiments, qRT-PCR, WB and immunofluorescence analysis; V.L conceived

the project, designed the experiments, analyzed the data and wrote the paper. All authors read and approved the final manuscript.

5.1 Abstract

Progesterone Receptor Membrane Component 1 (PGRMC1) is expressed in both oocyte and ovarian somatic cells, where it is found in multiple cellular sub-compartments including the mitotic spindle apparatus. Strikingly, PGRMC1 localization in the maturing bovine oocytes mirrors its localization in mitotic cells, suggesting a possible common action in mitosis and meiosis. To test the hypothesis that altering PGRMC1 activity leads to similar defects in mitosis and meiosis, PGRMC1 function was perturbed in cultured bovine granulosa cells (bGC) and maturing oocytes and the effect on mitotic and meiotic progression assessed. RNA interference-mediated PGRMC1 silencing in bGC significantly reduced cell proliferation, with a concomitant increase in the percentage of cells arrested at G2/M phase, which is consistent with an arrested or prolonged M-phase. This observation was confirmed by time-lapse imaging that revealed defects in late karyokinesis. In agreement with a role during late mitotic events, a direct interaction between PGRMC1 and Aurora Kinase B (AURKB) was observed in the central spindle at of dividing cells. Similarly, treatment with the PGRMC1 inhibitor AG205 or PGRMC1 silencing in the oocyte impaired completion of meiosis I. Specifically the ability of the oocyte to extrude the first polar body was significantly impaired while meiotic figures aberration and chromatin scattering within the ooplasm increased. Finally, analysis of PGRMC1 and AURKB localization in AG205-treated oocytes confirmed an altered localization of both proteins when meiotic errors occur. The present findings demonstrate that PGRMC1 participates in late events of both mammalian mitosis and oocyte meiosis, consistent with PGRMC's localization at the mid-zone and mid-body of the mitotic and meiotic spindle.

5.2 Introduction

Progesterone Receptor Membrane Component 1 (PGRMC1) is a multi functional protein that plays important roles in regulating mammalian ovarian function [1, 2]. Within the ovary, PGRMC1 is expressed and exerts a function in both somatic and germ cells [3-5]. Its clinical relevance is indicated by studies showing that altered PGRMC1 expression correlates with defective follicular development and infertility in women [6-8].

Primary evidence that PGRMC1 has a fundamental role in ovarian somatic cells comes from *in vivo* studies in mice, in which conditional knockout of PGRMC1 in granulosa cells impairs antral follicle development [2, 9]. Accordingly, *in vitro* studies using different ovarian cell lines have shown that depleting PGRMC1 expression suppresses cell proliferation [10-12]. However, the mechanism of action by which PGRMC1 controls ovarian cell proliferation is poorly understood.

So far, PGRMC1 is known as a mediator of progesterone's antiapoptotic action in ovarian cell lines [3, 13, 14]. When apoptosis is induced by serum starvation in rat spontaneously immortalized granulosa cells (SIGCs), PGRMC1 mediates progesterone's anti-apoptotic function, at least in part, through the regulation of the expression of apoptosis-related genes [13, 15]. This genomic action is exerted by high molecular weight forms of PGRMC1 that localize to the nucleus of interphasic cells [13, 15]. However, PGRMC1 is also found in other sub-cellular compartments where it probably exerts additional functions. In particular PGRMC1 associates to the mitotic spindle [11, 16, 17], where it directly interacts with beta tubulin [11] suggesting a role in the regulation of mitosis. Immunofluorescence studies have shown that PGRMC1 changes its localization dynamically during mitosis: it associates with the spindle apparatus in metaphase, while it localizes to the midzone and the midbody in anaphase and telophase/cytokinesis [11]. These studies indicate an involvement in mitosis, however the molecular mechanism by which PGRMC1 regulates mitosis has not been fully characterized and further studies are needed to better understand its function.

PGRMC1 is also expressed in oocytes of several mammalian species [3-5]. Previous experimental evidence obtained in *in vitro* matured bovine oocytes supports the hypothesis that PGRMC1 regulates meiotic chromosome segregation during meiosis I [5, 18]. In the period that spans from meiotic cell cycle reentry to metaphase II, also known as oocyte maturation, PGRMC1's localization dramatically changes.

Specifically, PGRMC1 begins to associate with the condensing chromosomes after nuclear envelope break down and localizes to the centromeric region of the metaphasic chromosomes at Metaphase I (MI) and MII stage, while at Anaphase/Telophase I (Ana/Telo I) it concentrates between the separating chromosomes [5]. Interestingly, in an oocyte model characterized by increased aneuploidy and embryonic developmental failure, PGRMC1 fails to properly associate with the MII chromosomes [18]. Remarkably, PGRMC1 co-localizes with phosphorylated (active) form of AURKB in all the different stages of maturation [5], suggesting an interaction between the two proteins. However, as in the case of somatic cells mitosis, the precise role of PGRMC1 during oocyte meiosis is not known.

Strikingly, PGRMC1 localization in the maturing oocytes mirrors its localization in ovarian mitotic cells, suggesting a possible common function in both mitotic and meiotic cell division. The present study investigates the hypothesis that interfering with PGRMC1 function leads to similar defects in mitosis and meiosis in primary culture of bovine granulosa cells (bGC) and maturing bovine oocytes respectively. bGC were cultured in the presence of serum to stimulate cell growth and PGRMC1 function was altered using small interfering RNA (RNAi) mediated gene silencing approach. Bovine oocytes were in vitro matured and PGRMC1 function was impaired by using either a known PGRMC1 inhibitor (AG 205) [19] or RNAi. In addition, a possible relationship between PGRMC1 and AURKB has been investigated in both systems.

5.3 Materials and Methods

5.3.1 Reagents

All the chemicals were purchased from Sigma-Aldrich (St. Louis, MO) except for those specifically mentioned. Gene silencing was performed by Stealth RNAi™ siRNA technology from Life Technologies (https://www.lifetechnologies.com/it/en/home/life-science/rnai/synthetic-rnai-analysis/stealth-rnai-technology.html?icid=cvc-invivo-sirna-c2t2). The 'BLOCK-iT™ RNAi Designer' tool from Life Technologies (https://rnaidesigner.lifetechnologies.com/rnaiexpress/) was used to design PGRMC1 Stealth RNAi (PGRMC1 RNAi) within the coding region of the bovine PGRMC1 sequence (NM_001075133). Sequence of the PGRMC1 RNAi used was: (RNA)-GAG

UUG UAG UCA AGU GUC UUG GUC U. Negative control (cat n. 12935-200) was chosen among the Stealth RNAi negative control (CTRL RNAi) duplexes available from Life Technologies,. Stock solution of AG205 (16 mM) was prepared in Dimethyl sulfoxide (DMSO) and stored at -20C°. Primary antibodies were: rabbit polyclonal anti-PGRMC1 (Sigma, cat. HPA002877, lot number: A01099 and A27579), mouse monoclonal anti beta tubulin (Sigma, cat. T8328, clone AA2) and mouse monoclonal anti AURKB (BD transduction Laboratories, cat. 611082, clone 6/AIM-1). Primer pair sequences were synthetized from Primm s.r.l. (Milan, Italy).

5.3.2 Sample collection

COCs were collected from pubertal Holstein dairy cows recovered at the abattoir (INALCA S.p.A., Ospedaletto Lodigiano, LO, IT 2270M CE, Italy) as previously described from of 2-6 mm ovarian antral follicles [5]. Only medium-brown in color COCs, with five or more complete layers of cumulus cells and oocytes with finely granulated homogenous ooplasm were used.

After COC retrieval, the bGC were collected, washed and plated. Cells were cultured in growth medium, which was Dulbecco's modified medium supplemented with 10% bovine calf serum, 100 U/ml of penicillin G, 100 µg/ml of streptomycin and 1U/ml Glutamax (Gibco,Thermo Scientific), in humidified air at 37°C with 5% CO_2. After 24 h cells were washed with PBS, cultured in growth medium for 6-7 days until confluent, and then used according to the experimental design.

5.3.3 RNAi treatment

bGC RNAi treatment

2 X 10^5 bGC were plated in 35-mm culture dishes. For immunofluorescence studies cells were plated on cover glasses, while for time-lapse experiments, glass bottom dishes (CELLview, Greiner bio-one) were used. After 24 h of culture (50-70% confluence), bGC were transfected with 6 µl of 20 µM PGRMC1 Stealth RNAi or CTRL RNAi combined with 10 µl of Lipofectamine® RNAiMAX Transfection Reagent (Thermo Fisher Scientific) in a final volume of 2 ml Opti-MEM® Reduced Serum Medium (Gibco, Thermo Fisher Scientific). Transfection efficiency, evaluated using the BLOCK-iT™ Alexa Fluor® Red Fluorescent Control (Invitrogen, Thermo Fisher

Scientific) and calculated as the number of red fluorescent cells on the total number of DAPI stained nuclei, was 80% after 24 h treatment and remained constant up to 72 h.

Oocyte RNAi treatment

COCs were collected in medium supplemented with the 3-isobutyl-1-methyl-xanthine (IBMX) at the final concentration of 0.5 mM, as previously described [20]. Groups of 20-30 COCs were maintained meiotically arrested by adding 10 μM cilostamide as before described [21, 22] until microinjection (minimum 30 minutes). A microinjection apparatus (Narishige Co. Ltd.) mounted on an inverted microscope (Nikon Diaphot; Nikon Corp.) and a Femtojet microinjector (Eppendorf, Hamburg, Germany) were used to inject 10 pl of 20 μM PGRCM1 RNAi or CTRL RNAi into the ooplasm of COC. After injection, COCs were cultured with cilostamide overnight (the total treatment with cilostamide lasted 18 hours). COCs were then washed and in vitro matured for 24 h as previously described [23].

5.3.4 Treatment with AG205

Groups of 15-20 COCs or denuded oocytes (DOs) were in vitro matured for 24 h with or without 10, 20 or 40 μM AG 205 [19]. To obtain DOs, oocytes were mechanically separated from cumulus cells as previously described [23, 24]. The control group (0 μM AG205) was cultured with an equivalent amount of DMSO that was used to dissolve AG205.

5.3.5 Quantitative Reverse Transcriptase-polymerase chain reaction (qRT-PCR)

Oocyte's total RNA was extracted with the Pico-Pure RNA Isolation Kit (Applied Biosystems) following the manufacturer's protocol and including DNase treatment (Qiagen) on the purification column, while bGC total RNA was extracted with the RNeasy Mini Kit (Qiagen).

Total RNA was retro-transcribed (RT) with random hexamers using the First-strand Synthesis System for RT-PCR System (Invitrogen, Life Technologies). 1 μg of bGC RNA or cDNA equivalent to 0.3 oocytes per reaction were used. Primer pairs are shown in supplemental table 1. SYBR green (Bio-Rad) was used according to the manufactures instructions and reactions were developed in an iQ5 PCR Thermal Cycler (Bio-Rad). Primer pairs specificity was assessed using standard RT-PCR and sequencing analysis. PGRMC1 relative expression level was analyzed with the ΔΔ-ct

method [25] using the HIS1H2A [26, 27] and/or GAPDH [28] as reference genes with the Biorad iQ5 Software. Data from 3 independent experiments were imported and grouped into a single 'Gene Study'.

5.3.6 Western blot analysis

SDS-PAGE of bGC lysates was conducted as previously described [15]. Briefly, PGRMC1 or CTRL RNAi treated bGC were lysed in radioimmunoprecipitation assay (RIPA) buffer Total amount of protein was determined using the Qubit® Protein Assay Kit and Qubit® fluorometer (Thermo Fisher Scientific). 20 µg of total protein/lane were used for Western blotting.

SDS-PAGE of oocytes was optimized to detect protein expression in small samples as previously described [29, 30]. RNAi treated oocytes were denuded, washed and collected in 2 µl of PBS supplemented with proteases and phosphatase inhibitor cocktails. Samples were than mixed with 2 µl of 2X SDS–Laemmli loading buffer (Bio-Rad), boiled for 5 min and stored at –80°C until essayed. After thawing, samples were boiled for additional 5 minutes and resolved on a 10% SDS-PAGE with a 4% stacking gel. Micro-wells in the stacking gel were 1mm in width, 0.5 mm in thickness and 10 mm height, contains a maximum volume of 5 µl. The comb for these micro wells was made with a 3-D printer in William Kinsey laboratory and kindly provided by Lynda McGinnis, University of Kansas Medical Center.

Run transfer and immunoblotting were performed as previously described [11] using the anti PGRMC1 rabbit polyclonal antibody (1:200) . PGRMC1 was revealed using a goat anti rabbit IgG peroxidase conjugated antibody (1:1000, Thermo scientific, cat.32460) and detected using the Super Signal West Dura Extended Duration Substrate (Thermo scientific, cat.37071). The nitrocellulose membrane was stripped and re-probed with the anti beta tubulin antibody (1:1000) as before described [11], which was revealed using a stabilized goat anti mouse IgG peroxidase conjugated antibody (1:1000, Thermo scientific, cat.32430). Relative amount of protein was quantified on scanned films [31] using Image J software. The intensity of each PGRMC1 band was firstly normalized for the corresponding beta tubulin band and than the ratio between PGRMC1 RNAi and CTRL RNAi corresponding bands were calculated. PGRMC1 expression in PGRMC1 RNAi treated oocytes was expressed as a percentage of the corresponding CTRL RNAi treated group. These values were pooled for statistical analysis.

5.3.7 Assessment of bGC growth, effect on cell cycle and mitosis

After treatments, cells were collected after trypsinization. Care was taken to avoid loss of cells during collection and to ensure complete detachment of the cells from the plate by looking at the culture dishes under the microscope after trypsinization. Total cell number was counted with a Neubauer chamber. Cell growth rate was calculated as the ratio of the total number at each time point on the total cell number at the time of plating. Flow cytometry analysis was conducted in order to evaluate the percentage of cells at each cell cycle phase as previously described [32]. Cells were collected, fixed in 70% cold EtOH and kept at +4°C until essayed.

For time-lapse analysis CTRL and PGRMC1 RNAi treated bGC were stained with 0.05 μg/ml of Hoechst 33342 for 30 minutes and then substituted with fresh medium and imaged on a biostation IM (Nikon). Images were captured every 5 minutes for 6 h after 30 h from treatment. Each time 6 different fields were captured at a 20X magnification.

5.3.8 Assessment of PBI extrusion and meiotic progression of bovine oocytes

After IVM, oocytes were denuded and examined under the stereomicroscope at the highest magnification (50 X) to assess complete extrusion of the PBI in the perivitelline space. Then, oocytes were fixed in 4% paraformaldehyde in PBS, permeabilizaed in 0.1% Triton-X 100 in PBS for 10 minutes and washed in 0.1% PVA in PBS (PBS/PVA). Samples were mounted on slides with the anti-fade medium Vectashield (Vector Laboratories) supplemented with 1 μg/ml 40,6-diamidino-2-phenylindole (DAPI) to visualize DNA. Samples were analyzed on an epifluorescence microscope (Eclipse E600; Nikon Corp.) equipped with a digital camera (DS-Fi2; Nikon Corp.) to assess the stage of meiotic progression as previously described [18].

5.3.9 Immunofluorescence

Immunofluorescence analysis of PGRMC1 and AURKB localization were conducted as previously described on bGC grown on cover glasses [11] or denuded oocytes [5, 18] with minor modification. Briefly, after culture, washing and fixation procedures were conducted with pre-warmed media. Paraformaldehyde fixed samples were incubated overnight at 4°C with a combination of the rabbit anti-PGRMC1 (dilution 1:50) and anti AURK B (dilution 1:50) antibodies. Secondary antibodies used

were: TRITC-labeled donkey anti-rabbit antibody (dilution 1:100; Vector Laboratories, Inc.) and Alexa Fluor 488-labeled donkey anti-mouse antibody (dilution 1:500; Invitrogen, Life Technologies) for 1 h at room temperature. The samples were mounted on slides in the antifade medium Vecta Shield (Vector Laboratories) supplemented with 1 µg/ml 40,6-diamidino-2-phenylindole (DAPI). Samples were analyzed on an epifluorescence microscope (Eclipse E600; Nikon) equipped with a 60X objective, a digital camera, and software (NIS elements Imaging Software; Nikon). Immunofluorescence controls, which were performed by omitting one of the 2 primary antibodies while both fluorescently labeled secondary antibodies were present in all reactions, did not show any staining.

5.3.10 In situ Proximity Ligation Assay (PLA)

The interaction between PGRMC1 and AURKB was assessed using in situ PLA (Duolink II; OLINK Bioscience, Uppsala, Sweden http://www.olink.com/) in *in vitro* cultured bGC following the manufacturer protocol. Primary antibodies for PGRMC1 and AURKB were the same used for immunofluorescence while anti-rabbit PLUS and anti-mouse MINUS PLA probes were used as secondary antibodies. Negative controls were performed omitting one of the two primary antibodies. Cells were mounted with Duolink mounting medium (OLINK Bioscience). Samples were analyzed as described for immunofluorescence and ImageJ software was used to calculate the total area of the fluorescent signal corresponding to proteins interaction during the different mitotic phases.

5.3.11 Statistical analysis

Experiments were run in triplicates, unless otherwise specified. All statistical analysis was done using GraphPad Prism software (GraphPad Prism v. 6.0e, La Jolla, CA, USA). Data from the replicate experiments were pooled and the data expressed as a mean ± SEM. Student’s t test was used to determine differences between two groups. When more than two groups were compared, one-way ANOVA followed by Tukey's Multiple Comparison test or two way ANOVA followed by Bonferroni post test were used. Fisher’s exact test was used to analyze percentage data. Details on the statistical analysis are indicated for each experiment in the figure caption.

5.4 Results

5.4.1 PGRMC1 silencing affects bovine granulosa cells (bGCs) proliferation

To determine the effect of PGRMC1 silencing on the proliferation of cultured bGCs, cells were treated with PGRMC1 small interfering RNA (RNAi) or control (CTRL) RNAi and cultured for 24, 48 or 72 h in serum-supplemented medium to stimulate cell growth. Over the course of 72 h, treatment with PGRMC1 RNAi significantly reduced *PGRMC1* mRNA levels compared to CTRL RNAi treated group, as assessed by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR, **figure 5.1 A**). Western blot analysis confirmed the presence of multiple PGRMC1 bands, similar to what is observed in human and rat granulosa cells [15]. All the PGRMC1 band decreased at 72 h after PGRMC1 RNAi treatment (**figure 5.1 B**). Quantification of band revealed a significant overall 52.7 ± 10.9% reduction of PGRMC1 protein in RNAi treated cells compared to control cells (one sample t-test, $p< 0.05$).

To evaluate the effect of reduced PGRMC1 expression on bGC growth, cells were harvested at each time point and total cell number was counted. There were significanlty less cells in the RNAi PGRMC1 depleted group compared to CTRL RNAi treated group (**figure 5.1 C**). Moreover, flow cytometry analysis revealed that the decrease in total cell number after 72h of culture in PGRMC1 RNAi-treated bGCs was accompanied by a decrease in the frequency of G_0/G_1 stage cells and an increase in the frequency of cells arrested at G2/M phase of the cell cycle (**figure 5.1 D and supplemental figure 5.1**).

The decreased cell number and parallel increase in G2/M rates in PGRMC1 RNAi treated bGC suggests a defective proliferation, which can be due to an arrested or prolonged M-phase of the cell cycle. This hypothesis was confirmed using time-lapse imaging, in which PGRMC1 and CTRL RNAi treated bGCs were stained with the supravital Hoechst 33342 fluorochrome and imaged every 5 minutes, for a total of 6 h. In these experiments, morphological evaluation of dividing nuclei throughout the course of mitosis revealed that cells treated with CTRL RNAi progressed from prophase to telophase giving rise to two daughter nuclei (**figure 5.2 A and movie 5.1**). In contrast, cells exposed to PGRMC1 RNAi started to divide but their further progression through the cell cycle was impaired and three main phenotypes were observed. In the first phenotype, cells undergo prophase/metaphase but do not

progress beyond the Ana/Telo phase, reforming a single nucleus (**figure 5.2 B and movie 5.2**); in the second phenotype, cells undergo prophase/metaphase and progress through the following stages in an irregular manner that lead to the formation of aberrant nuclei (**movie 5.3**), in which small clumps of DNA remain excluded from the reforming nuclei while, in the third phenotype, cells undergo prophase/metaphase but DNA remains interconnected through the following stages leading to incomplete karyokinesis (**movie 5.4**). Collectively, we defined these phenotypes as 'abnormal mitosis' since they differed from the phenotype observed in CTRL RNAi treated bGC. As shown in **figure 5.2 C** nearly 70% of the PGRMC1 RNAi treated cells that started dividing showed these morphological alterations of mitotic progression**.** We can assume that errors leading to the first phenotype occurred earlier during meiotic progression when compared to the second and the third phenotypes. Interestingly, the sum of phenotype 2 and 3 represented the majority of 'abnormal' mitosis (78.9%) suggesting that PGRMC1 RNAi treatment mostly affected events occurring during late mitosis.

Moreover, the duration of cell cycle progression, from the beginning of prophase to the formation of two daughter nuclei, was significantly increased (**figure 5.2 D**). As expected, under these experimental conditions (i.e. culture in serum), cellular death of non-mitotic cells was low in both CTRL and PGRMC1 RNAi treated bGC (2.37 ± 0.42 and 2.41± 0.65 % on a total of 1585 and 1209 observed cells, respectively). Therefore, apoptosis does not likely account for the lower cell number observed in PGRMC1 depleted cells.

5.4.2 PGRMC1 co-localizes and interacts with Aurora kinase B during bGC mitosis.

Since cell division is dependent on Aurora kinase B (AURKB) [33-35], we performed double immunofluorescence staining to examine the relationship between the localization of AURKB and PGRMC1 during bGC mitosis. As shown in **figure 5.3,** the two proteins colocalize, particularly during telophase. Furthermore, in situ Proximity Ligation Assay (PLA) was conducted to test whether PGRMC1 directly interacts with AURKB during the different stages of mitosis. This technique uses a pair of oligonucleotide-labeled secondary antibodies (PLA probes), which generate a signal only when the two probes have bound in close proximity. The signal from each

detected pair of PLA probes is visualized as an individual fluorescent spot. As shown in **figure 5.4 A**, PLA revealed an interaction between AURKB and PGRMC1. Moreover, the degree of interaction, evaluated as the mean of the total fluorescence area in each mitotic cell, was low from prophase to anaphase and then increased significantly at telophase (**figure 5.4 B**).

5.4.3 Pharmacological inhibition of PGRMC1 function during bovine oocyte meiosis impairs karyokinesis and polar body emission

During meiosis I, homologue chromosome segregation and cytokinesis are spatiotemporally coordinated in order to ensure the proper ploidy but asymmetric cytoplasmic division between the MII oocyte and the first polar body (PBI) (reviewed in [36]). To determine whether PGRMC1 plays a role in PBI cytokinesis, cumulus cell-oocyte complexes (COCs) and denuded oocytes (DOs) were cultured in in vitro maturation (IVM) medium in the presence or absence of increasing concentrations of the PGRMC1 inhibitor AG 205 [19]. After culture, the effect on PBI emission was evaluated under bright field microscopy, while the effect on chromosome segregation was assessed under epifluorescence microscopy after fixation and DNA staining.

As shown in **figure 5.5**, PBI extrusion rate decreased when COCs or DOs were cultured with 20 and 40 μM AG205 compared to the control group (0 μM, $P<0.05$). This effect was more pronounced in DOs, where PBI formation was already lower with 10 μM AG205. As shown in **figure 5.6**, AG205 treatment also impaired chromosome segregation determining a decrease in the percentage of oocytes that reached the MII stage and an increase in oocytes showing aberrant meiotic figures. In particular, these aberrancies included the presence of DNA clumps scattered within the ooplasm (**figure 5.6 A, aberrant**) as well as the coexistence of two metaphase plates or telophases (these examples of aberrant meiotic figures observed after AG205 treatment are shown in **supplemental figure 5.2**). Oocytes in which the DNA was collapsed into a single clump, or in which DNA was not detectable within the ooplasm, were classified as degenerated. Sample observation under bright field combined to UV light illumination confirmed the correct analysis of DNA clumps localization within the ooplasm (**supplemental figure 5.3**). The effect of AG205 appeared more pronounced in DOs (**figure 5.6 C**).

Finally, in order to assess whether AG205 treatment affected AURKB localization, PGRMC1 and AURKB localization was assessed in AG205 treated COCs by double immune fluorescence experiments. PGRMC1 and AURKB localization in the oocytes was classified as regular or irregular according to the previously published criteria [18]. Localization was judged as regular when the proteins localized at the centromeric region of each chromosome or irregular when one or more of the following configurations was observed: more than one point on a chromosome, shape different from the punctuated, not in the centromeric region, and/or lack of signal. As shown in **figure 5.7** aberrant meiotic figures showed irregular AURKB and PGRMC1 localization, while both PGRMC1 and AURKB showed a focused centromeric localization in oocytes with MII plate.

5.4.4 RNAi mediated PGRMC1 silencing during oocyte maturation mirrors AG205 effects impairing karyokinesis and polar body emission

PGRMC1 function in oocytes was also assessed by microinjecting RNAi into germinal vesicle (GV) stage oocytes surrounded by cumulus cells. After microinjection with either CTRL or PGRMC1 RNAi and culture for 18 h under *in vitro* meiotic arrest conditions (IVA), allowing sufficient time for the RNAi to effectively deplete the gene of interest, oocytes were cultured in IVM medium for further 24 h. Preliminary optimization experiments in which a fluorescent tracker was used to assess microinjection efficiency indicated that ≈80% of the oocytes were successfully microinjected. Moreover, survival rate, assessed as the percentage of viable COCs after IVA, did not differ in CTRL and PGRMC1-RNAi microinjected COCs (96.06±2.10 and 96.23±2.73% respectively, $p>0.05$ t-test, n=11 independent experiments)

As reported in **figure 5.8A-C**, PGRMC1 mRNA and protein were reduced approximately by 40% as assessed by qRT-PCR and Western blotting, respectively. Depleting PGRMC1 significantly reduced the percentage of oocytes that extruded the PBI (**figure 5.8 D**). Moreover, fluorescence microscopy analysis revealed that even though the formation of MII plate was not blocked in PGRMC1 RNAi-treated oocytes, there was an increase in the frequency of oocytes with aberrant meiotic figures (**figure 5.8 E and F),** which were similar to that observed after AG205 treatment. Additional

examples of aberrant meiotic figures upon PGRMC1 RNAi treatment are shown in **supplemental figure 5.4**.

5.5 Discussion

Proper completion of cell division is an essential process in both somatic and germ cells. In somatic cells, after DNA replication and sister chromatids separation, cytokinesis ensures that the genetic material as well as the cytoplasmic organelles are evenly distributed into two daughter cells [37]. In contrast, cell division in oocytes allows for the elimination of half of the genetic material through PB emission while retaining most of the cytoplasm to support the early stages of embryo development. Extrusion of the PB is essential for the formation of the mature egg [36, 38]. Nonetheless late mitotic and oocyte meiotic division share many similarities [36]. In both systems, microtubules of the anaphase spindle play important roles in the formation of the actino-myosin contractile ring and the formation of the cytokinetic furrow at this site, allowing the physical separation of the two daughter cells (reviewed in [36]). Moreover, the essential role of the chromosomal passenger complex (CPC) in orchestrating late mitotic events [33, 34] is likely conserved in meiosis [39-42].

The present study adds new insights into the role of PGRMC1 as a regulator of both mitotic and meiotic cell division. Our data indicate that when PGRCM1 function is impaired both somatic cells and oocytes fail to divide properly. Specifically, knocking down PGRMC1 by RNAi in cultured bGC results in the inability to successfully complete mitosis and/or to form two normal nuclei in dividing cells. Similarly, in the maturing oocytes, perturbing PGRMC1 function by using pharmacological or RNAi approaches results in the impairment of polar body emission together with the formation of aberrant meiotic figures. As a result, a second metaphasic plate and/or scattered chromatin are usually observed.

Previous works demonstrate that PGRMC1 specifically localizes to the mitotic spindle apparatus of different cell lines [11, 16, 17], where it directly interacts with beta tubulin and controls spindle microtubule stability [11]. The present study demonstrates also that PGRMC1 directly interacts with AURKB and that the extent of this interaction is highest during the final phase of granulosa cell division. This is important since events occurring at the central spindle during the formation of the midbody are crucial for proper cell division. For example, the central spindle plays an important role in

keeping separated chromosomes apart prior to cytokinesis completion, because when microtubules are depolymerized in late anaphase, the nuclei collapse back together [43, 44]. Thus our findings may suggest that an additional mechanism by which PGRMC1 controls mitosis involves a direct interaction with AURKB at the mid-body. This concept is consistent with the finding that interfering with PGRMC1 function affects the localization of AURKB in oocytes that failed to properly complete meiosis. Furthermore, previous studies in bovine oocytes reveal that altering AURKB function by using the AURKB inhibitor ZM447439 also alters PGRMC1 localization, which was associated with meiotic defects [18]. Thus PGRMC1 and AURKB functional association seems to be reciprocal, at least in the oocyte. However, the precise mechanism of action by which PGRCM1-AURKB interaction affects AURKB and CPC function remains to be elucidated. PGRMC1 could act as an adaptor protein in many different biological processes as proposed by Aizen et al [45]. If this hypothesis were confirmed, PGRMC1 action would depend on the proteins with which it interacts in different cellular and subcellular systems. In this view, altering PGRMC1 function in our studies could have had an effect on different effector proteins, such as AURKB and other CPC components, in the spindle midzone/midbody, thereby determining kariokinesis/cytokinesis disturbance and/or failure. However, this hypothesis remains to be confirmed.

Most probably, the fate of PGRMC1 depleted granulosa cells that do not properly complete cell division would be cellular death, which would contribute to the lower proliferation observed in PGRMC1 RNAi treated cells. This is consistent with effects of depleting PGRMC1 in SIGCs, where cells accumulate in metaphase and then undergo cell death [12]. Another possible outcome of cytokinesis failure is the formation of bi- or multi-nucleated cells. This is not the case for bGCs since multinucleated granulosa cells were not observed in PGRMC1 RNAi treated cells (data not shown).

Cell death resulting from aberrant mitosis is generally referred to as 'mitotic catastrophe' [46]. Interestingly, this phenomenon has been functionally re-defined as an '*atypical mechanism that sense mitotic failure and respond to it by driving the cell to an irreversible fate, be it apoptosis, necrosis or senescence*' [47, 48]. Thus, it has been proposed that mitotic catastrophe can be considered as an '*onco-suppressive mechanism for the avoidance of genomic instability'* [47]. Our time-lapse fluorescent microscopy experiments suggest that mitotic catastrophe might occur in PGRMC1

depleted bGC. This finding is in accordance with the experimental evidences that PGRMC1 depletion suppresses cancer cells proliferation in vitro and tumor growth in vivo in all the types of cancers studied so far [10, 19, 49-51]. Moreover, the observation that PGRMC1 is overexpressed in a wide range of tumors when compared to corresponding normal tissues [19, 52-55] further support this hypothesis. In this view PGRMC1 overexpression would sustain propagation of abnormal cancer cell, helping them to escape mitotic catastrophe. However, further analyses are required to confirm this hypothesis.

It is possible that, in previous studies, the involvement of PGRMC1 in mitotic catastrophe events has been underestimated. In these studies, indeed, end-point methods have been used to assess the effect of PGRMC1 silencing. Clearly, end-point methods are inappropriate to detect this phenomenon, as they do not take into account the 'history' of the cell death [47]. On the contrary, time lapse-imaging used in the present study overcomes this limitation giving experimental evidences for the first time of a possible involvement of PGRMC1 in mitotic catastrophe events, which must be deeply investigated in future studies.

Similarly, it is likely that oocytes that fail to extrude PBI and form aberrant meiotic figure would not undergo fertilization and/or second meiotic division, thus degenerate. In a previous study, injecting a PGRMC1 antibody into the ooplasm of immature bovine oocytes revealed a role for PGRMC1 in bovine oocyte maturation [5] impairing the transition from pro-MI to MI stages with the majority of the oocytes arresting at the pro-MI stage. Only a very small percentage of oocytes reached MII, thus obscuring a putative PGRMC1 function during the final phase of oocyte maturation. In the present study the use of RNAi gene silencing and a specific PGRMC1 inhibitor, AG205 reveals a role for PGRMC1 during the final phase of maturation (MI to MII transition). In particular, the RNAi study clearly indicates that PGRMC1 is required to allow proper PBI emission. That PGRMC1 RNAi was effective also suggests that PGRMC1 is translated during this period. Moreover, it is known that PGRMC1 can undergo post-translational modifications such as phosphorylation and sumoylation [15, 53]. Whether translational regulation and/or post-translational modifications are involved in the mechanism that ensures a proper localization of PGRMC1 during oocyte maturation remains to be established.

AG205 experiments have been conducted on both COCs and DOs because cumulus cells also express PGRMC1 [4, 56, 57]. The observation that AG205 was more

effective on DOs than COCs confirms PGRMC1's specific role within the oocyte. AG205 is a small aromatic compound that acts as a PGRMC1 ligand. It has been used to functionally assess PGRMC1 activity in several biological systems including mammalian cells with effective concentrations in the μM range [19, 45, 58]. It was originally identified as a ligand for AtMAPR2 of the Arabidopsis thaliana [59], which shares homology with the cytochrome b5/heme binding domain of PGRMC1 [1, 19, 59]. Thus, although the precise biochemical mechanism through which AG205 inhibits PGRMC1 activity is not known, it likely acts by binding to PGRMC1's heme-binding domain, thereby disrupting PGRMC1's ability to interact with yet to be identified heme proteins. This hypothesis is supported by the experimental evidence that AG205 alters the spectroscopic properties of the PGRMC1-heme complex [19]. Thus, it is likely that the PGRMC1 heme binding domain is an important component in the mechanism by which PGRMC1 regulates meiosis.

In conclusion the present findings reveal a new role of PGRMC1 in late stages of the mitotic division and oocytes meiosis. This function is consistent with the localization at the mid-zone and mid-body of the mitotic and meiotic spindle. To the best of our knowledge this is the first study that reveals a role of PGRMC1 during late mitotic and meiotic events and represents an advancement of the state of the art in the field by giving information of a precise stage of cell division in which PGRMC1 exerts a function. Furthermore, PGRMC1's action possibly involves a direct interaction with AURKB, as revealed by PLA studies. Importantly this function seems to be conserved in mammalian cell mitosis and oocyte meiosis. Thus this observations provides a strong rationale for future studies on the precise mechanism of PGRMC1's action in the female gamete, in which technical limitation do not allow mechanistic conclusions, especially in large animal species. For example, since PGRMC1 exists in multiple forms (monomer and specific higher molecular weight forms and post translational modifications, [15, 53]) in future studies it will be important to assess which of these modifications have a predominant role in regulating PGRMC1 function during final karyo/cytokinesis and are important in targeting PGRMC1 to specific sites within the different subcellular domains. Moreover, since PGRMC1 is a mediator of P4 actions, further studies would be needed to address whether and how PGRMC1 function at this particular site of action is mediated by P4.

5.6 Figure and supplemental movies

5.6.1 Figures

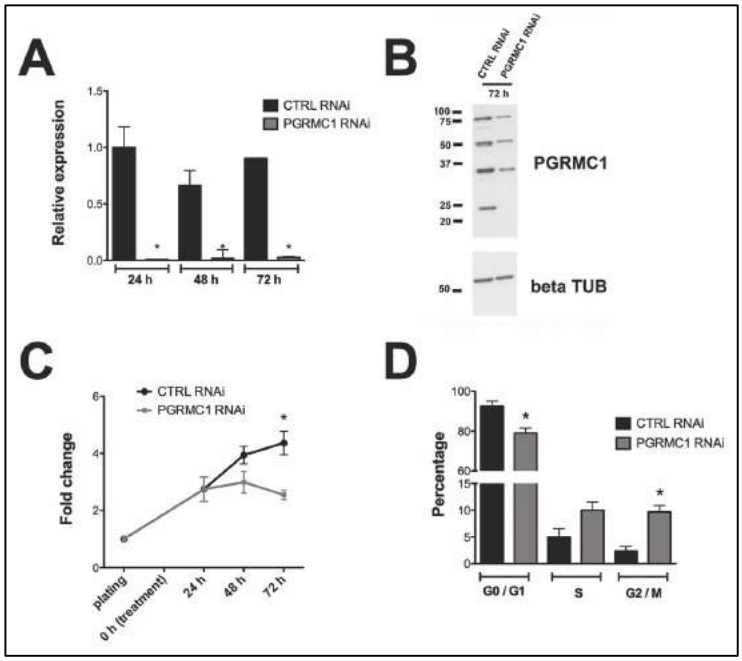


Figure 5.1: Effect of RNAi mediated PGRMC1 silencing on bGC growth. (**A**) Graph showing PGRMC1 mRNA silencing in PGRMC1 RNAi treated bGC as assessed by qRT-PCR. PGRMC1 expression level was normalized using GAPDH as reference gene. Data were analyzed by one way ANOVA, followed by Tukey's Multiple Comparison Test, Values are means ± SEM (n=3). * indicates significant differences between groups ($P<0.05$). (**B**) Representative Western blot showing the decreased expression of all PGRMC1 bands at 72 h in PGRMC1 RNAi treated bGC; beta tubulin was used as loading control. (**C**) Graph showing the effect of PGRMC1 down regulation on cell growth. Data were analyzed by two way ANOVA followed by Bonferroni post-hoc test. Values are means ± SEM (n=3). * indicates significant differences between groups $P<0.05$. (**D**) Graph showing the increase in the percentage of cells arrested at G2/M phase after 72 h from transfection, as assessed by flow cytometry analysis. Data were analyzed by unpaired Student's t-test, Values are means ± SEM (n=3). * indicates significant differences between groups ($P<0.05$).

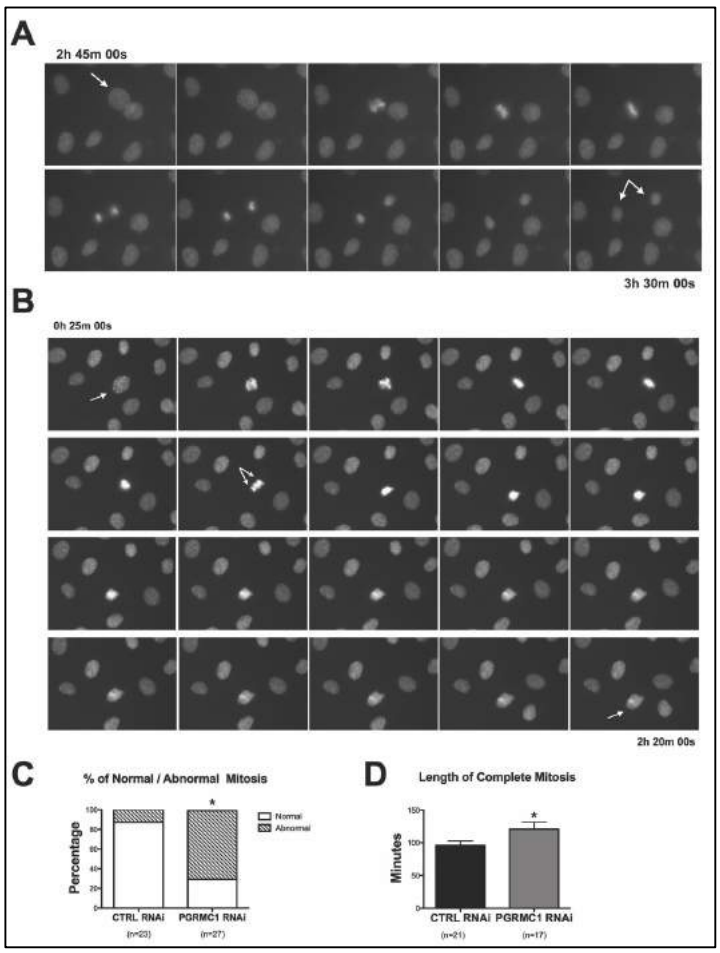


Figure 5.2: Effect of PGRMC1 silencing on bGC mitosis by time-lapse analysis of transfected bGC. (**A**) Example of normal mitosis occurring in bGC transfected with CTRL RNAi. The cell goes through all the mitotic phases from prophase to telophase giving rise to two daughter nuclei (**see supplemental movie 5.1**). (**B**) Example of abnormal mitosis in bGC transfected with PGRMC1 RNAi, in which the cell starts the division process undergoing prophase but then fails to proceed beyond the Ana/Telophase (see **supplemental movie 5.2**). Additional examples of abnormal mitosis occurring in PGRMC1 RNAi treated bGC are shown in **supplemental movies 5.3 and 5.4**. (**C**) Graph showing the frequency of normal and aberrant mitotic events assessed in CTRL and PGRMC1 RNAi treated cells. Data were analyzed by Fisher exact test. * indicates significant differences between groups P< 0.05. (**D**) Graph showing the time to complete mitosis in CTRL and PGRMC1 RNAi treated cells. Data were analyzed by unpaired Student's t-test, * indicates significant differences between groups (P<0.05). This experiment was replicated four times; the total number of cells analyzed is shown in brackets.

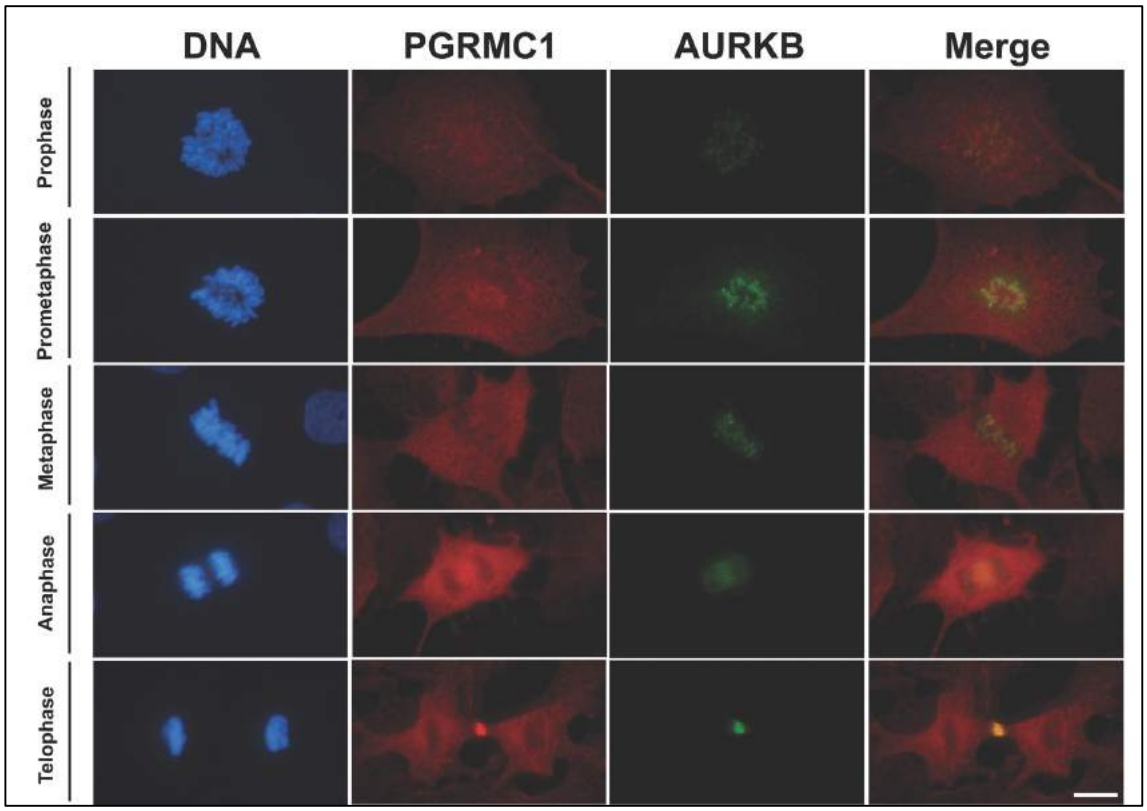


Figure 5.3: Colocalization PGRMC1-AURK B in bGC. Representative images showing PGRMC1 (red) and AURKB (green) colocalization during the different mitotic phases of cultured bGC; DNA was stained with DAPI (blue). PGRMC1 and AURKB start to colocalize to the mitotic spindle region during prophase and the colocalization is more pronounced in the central spindle during ana/telophase. Scale bar is 10µm.

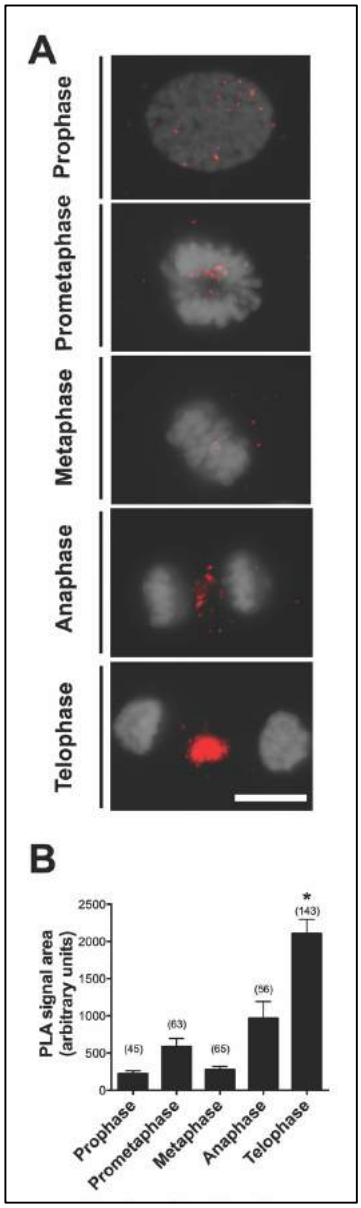


Figure 5.4: Interaction between PGRMC1 and AURKB during mitosis in bGC. (**A**) Representative images of bGC showing PGRMC1 - AURKB interaction as assessed by In situ proximity ligation assay (PLA) from prophase until telophase. DNA was stained with DAPI (white). The red spots indicate PGRMC1-AURKB interactions. Scale bar is 10 µm. (**B**) Graph showing the increased interaction between these two proteins during telophase. Data were analyzed by one way ANOVA followed by Tukey's Multiple Comparison Test. Values are means ± SEM; * indicates significant differences between groups ($P<0.05$). This experiment was replicated three times with the total number of cells analyzed shown in brackets.

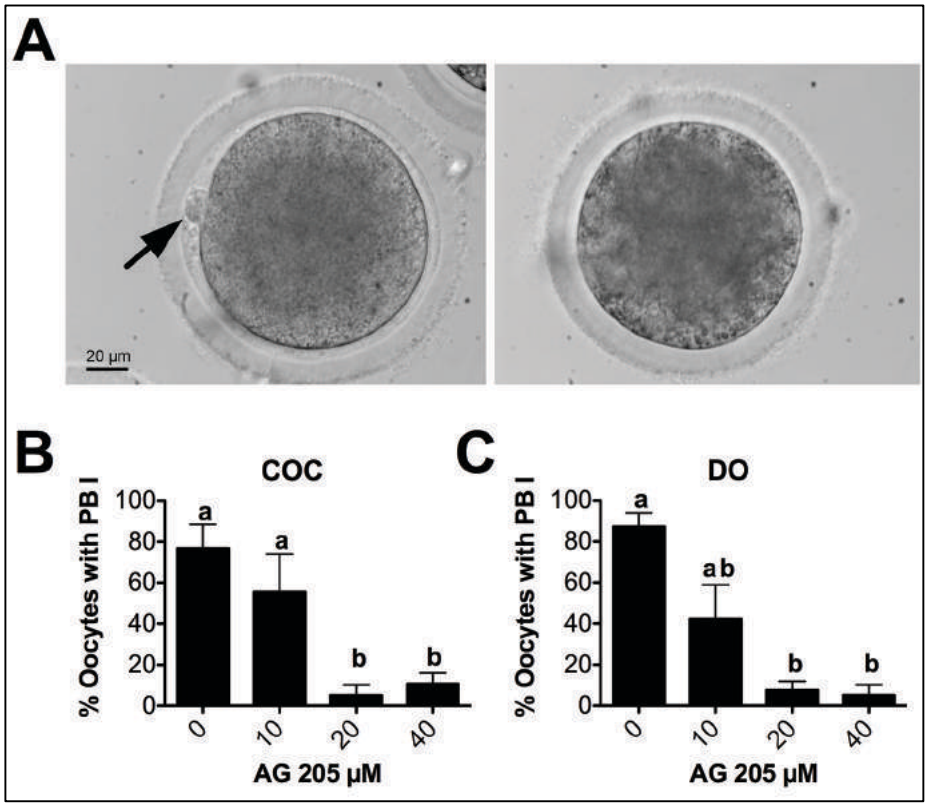


Figure 5.5: Effect of AG205 treatment on PBI emission. (**A**) Representative images showing in vitro matured oocytes with (left, arrow) or without (right) extruded PBI. (**B**, **C**) Graphics showing the effect of AG205 treatment on the percentage of oocytes that extruded the PBI in COC and DO, respectively. Data were analyzed by one way ANOVA followed by Tukey's Multiple Comparison Test. Values are means ± SEM (n=3); a,b different letters indicate significant differences between groups ($P < 0.05$).

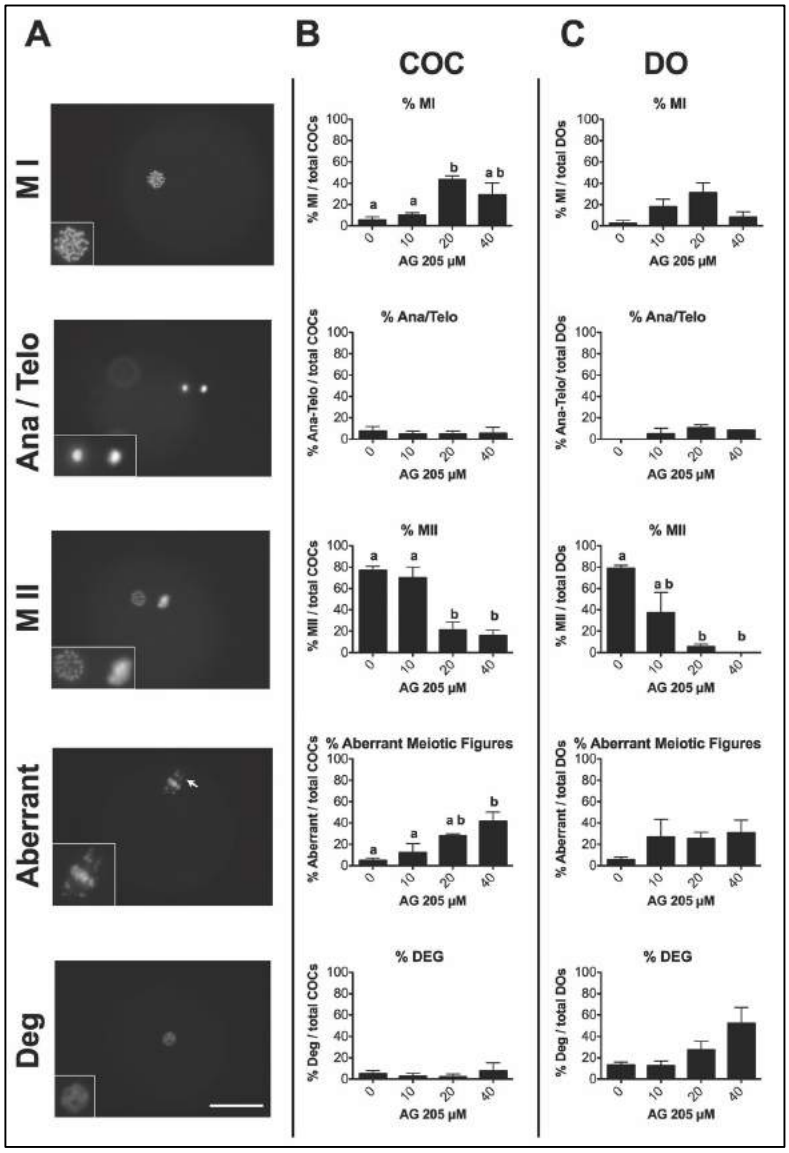


Figure 5.6: Effect of AG205 treatment on meiotic progression. (**A**) Representative images showing oocytes classified as MI, Ana/Telophase, MII, aberrant or degenerated after AG205 treatment. Aberrant mitotic figure shown in this figure is characterized by the presence of DNA scattered within the ooplasm (arrow), while additional examples of aberrant meiotic figures observed after AG205 treatment are shown in **supplemental figure 5.1.** DNA was stained with DAPI (white). Scale bar is 50 µm. Insets show the DNA at 2X magnification. Graphs in (**B**) and (**C**) show the percentages of oocytes at each stage of the first meiotic division (shown in A) in COC and DO respectively. Data were analyzed by one way ANOVA followed by Tukey's Multiple Comparison Test. Values are means ± SEM (N=3); a,b different letters indicate significant differences between groups ($P < 0.05$).

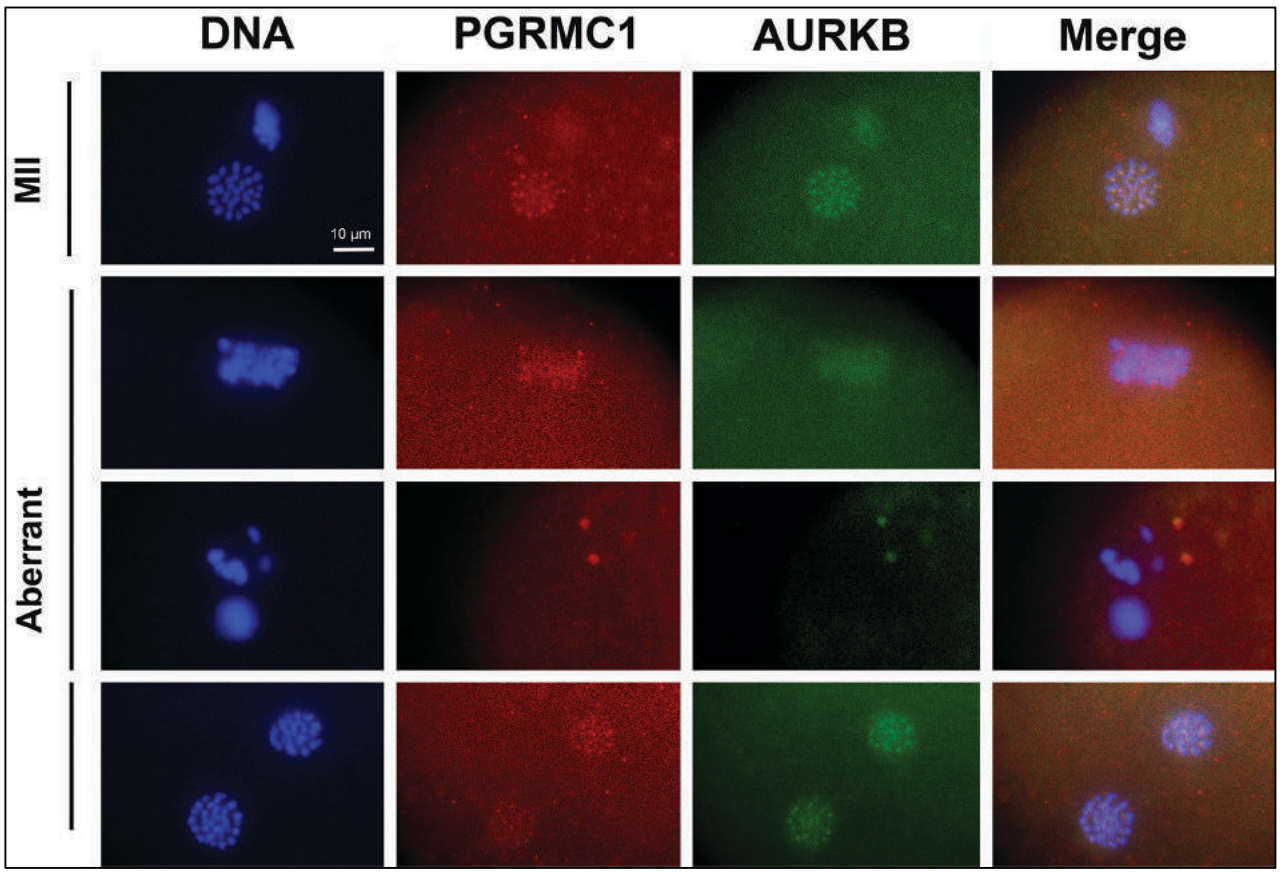


Figure 5.7: Effect of PGRMC1 AG205 on PGRMC1 and AURKB localization. Representative images showing PGRMC1 and AURKB localization in MII plates of matured oocytes or in oocytes showing aberrant meiotic figures. COC were treated with 0 or 20 µM AG205 for 24 h. After AG205 treatment oocytes were fixed, immunostained with anti- PGRMC1 (red) and AURKB (green) antibodies; DNA was stained with DAPI (blue). A total of 97 oocytes from 2 independent experiments were analysed. Both PGRMC1 and AURKB showed a focused centromeric localization in oocytes with MII plate, while they often showed a more diffused localization in aberrant meiotic figures with scattered chromosomes. When clumps of chromatin were present within the ooplasm, none of them were associated with AURKB and / or PGRMC1. Finally, when double meiotic plates were present, both AURKB and PGRMC1 showed a focused localization on metaphasic chromosomes of both plates.

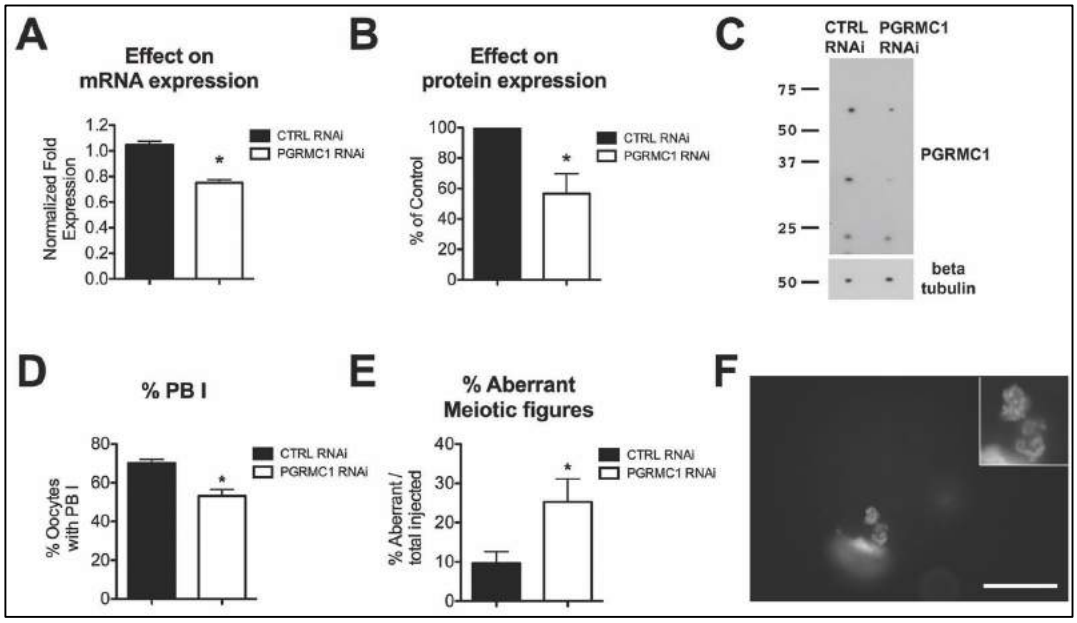


Figure 5.8: Effect of PGRMC1 RNAi treatment on PGRMC1 expression level, PBI extrusion and meiotic progression. (**A**) Graph showing *PGRMC1* mRNA expression level in CTRL and PGRMC1 RNAi treated oocytes by RT-qPCR; PGRMC1 expression level was normalized using *GAPDH* and *HIST1H2A* as reference genes and differences in gene expression levels were assessed with the Delta-Delta Ct method. Data were analyzed by Student's t-test. Values are means ± SEM (N=3); * indicates significant differences between groups ($P < 0.05$). (**B and C**) graph and representative western blotting showing PGRMC1 protein expression levels in CTRL and PGRMC1 RNAi treated oocytes; beta tubulin was used as loading control. PGRMC1 protein expression in PGRMC1 RNAi treated oocytes is expressed as a percentage of the CTRL RNAi treated group. Data were analyzed by one sample t-test. Values are means ± SEM (n=6). * indicates significant differences ($P < 0.05$). (**D**) Graph showing the percentage of oocytes that extruded the PBI after CTRL or PGRMC1 RNAi treatment and IVM. Data were analyzed by t-test. Values are means ± SEM (n=14); * indicates significant difference between groups ($P < 0.05$). (**E**) Graph showing the percentage of oocytes showing aberrant meiotic figures. Data were analyzed by t-test. Values are means ± SEM (n=6); * indicate significant differences between groups ($P < 0.05$). Image in (**F**) is representative of an aberrant meiotic figure observed after PGRMC1 RNAi treatment, in which chromosomes and DNA clumps are dispersed in the ooplasm and not organized in a MII plate (additional examples of aberrant meiotic figures are shown in supplemental figure 3). Scale bar is 50 µm. Insets show the DNA at 2X magnification.

5.6.2 Supplemental movies

All the movies can be downloaded at their corresponding links:

Supplemental Movie 5.1: time lapse of normal mitosis occurring in bGC transfected with CTRL RNAi shown in figure 5.2A. Cell progress from prophase to telophase giving rise to two daughter nuclei.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4968956/bin/kccy-15-15-1192731-s002.mov

Supplemental Movie 5.2: time lapse of abnormal mitosis occurring in bGC transfected with PGRMC1 RNAi, shown in figure 5.2B. Cell undergoes prophase/metaphase but does not progress beyond the Ana/Telo phase, reforming a single nucleus
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4968956/bin/kccy-15-15-1192731-s003.m4v

Supplemental Movie 5.3: time lapse of abnormal mitosis occurring in bGC transfected with PGRMC1 RNAi. Cell undergoes prophase/metaphase and progressed through the following stages in an irregular manner leading to the formation of aberrant nuclei, in which small clumps of DNA remained excluded from the reforming nuclei while
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4968956/bin/kccy-15-15-1192731-s004.mov

Supplemental Movie 5.4: time lapse of abnormal mitosis occurring in bGC transfected with PGRMC1 RNAi. Cell undergoes prophase/metaphase but DNA remained interconnected through the following stages leading to incomplete karyokinesis.
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4968956/bin/kccy-15-15-1192731-s005.mov

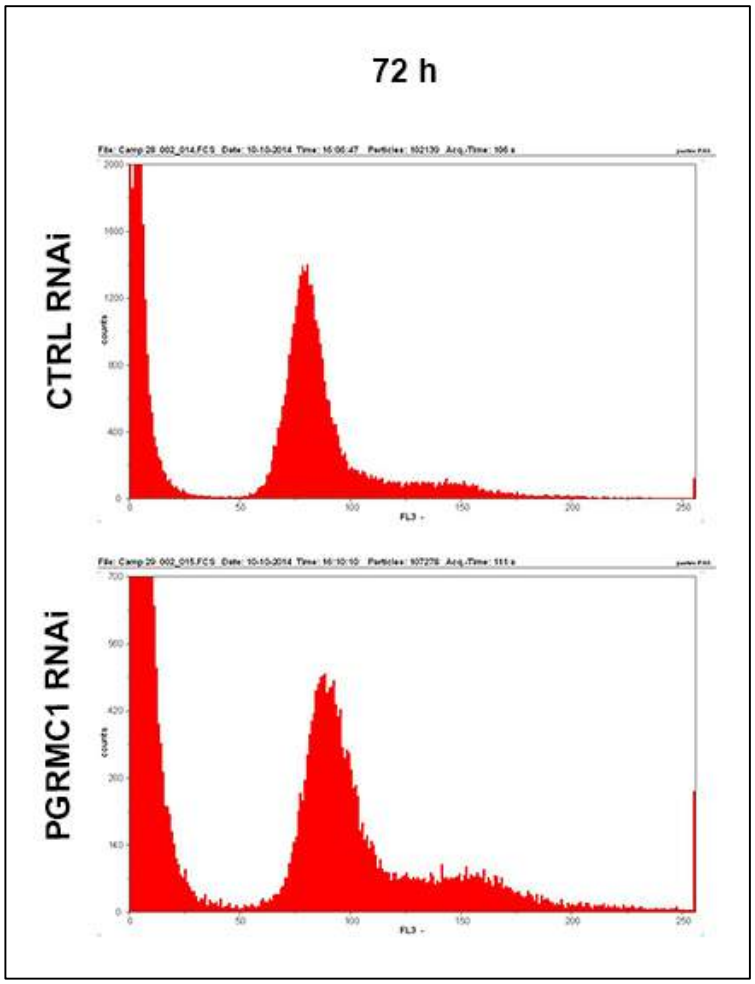
5.7 Supplementary material

5.7.1 Supplemental tables

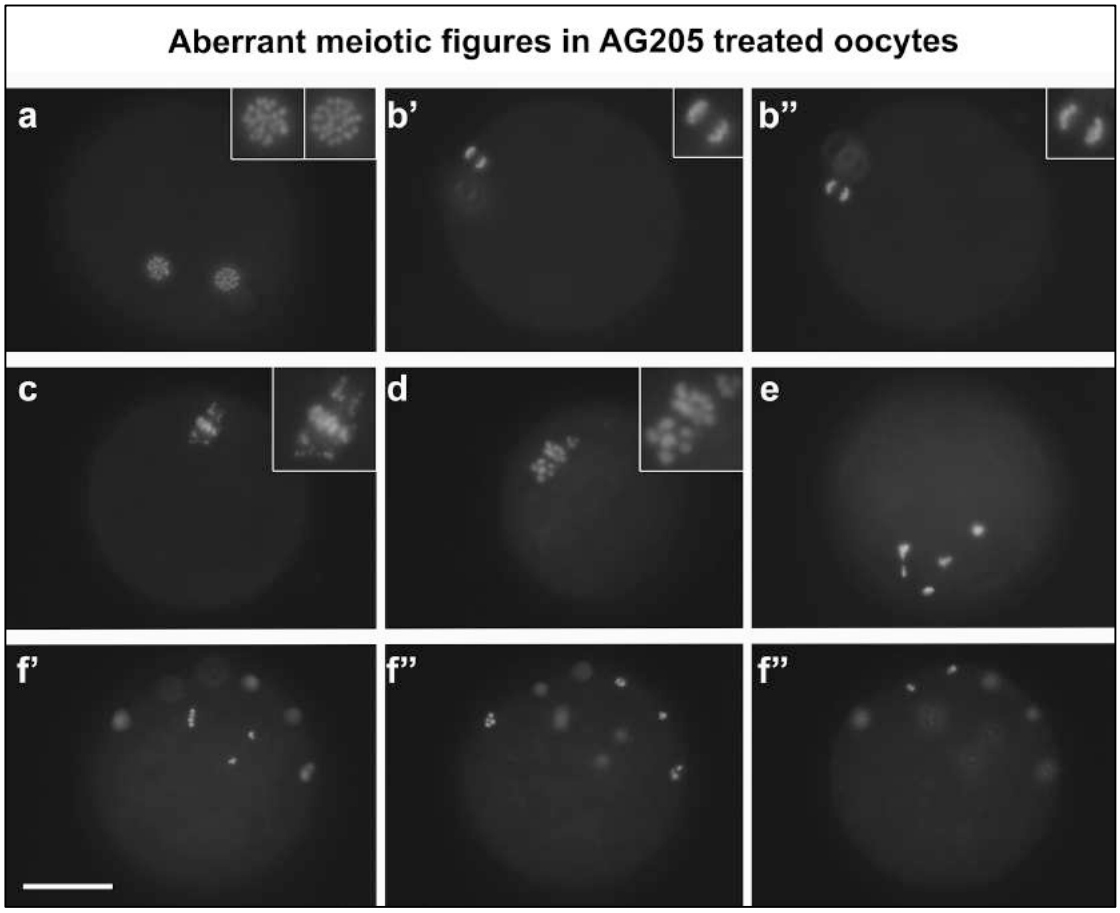
Supplemental table 5.1: Primer pair sequences used in q-RT PCR experiments

Gene	Oligo Sequence (5'-3')	Prod. size (Bp)	PCR Efficiency and r^2	Reference
PGRMC1	GCCTTTGCATCTTTCTGCTC TGGTTACGTCGAACACCTTG	195	E=102.2 %; r^2= 0.98	Primer 3 design; NM_001075133
GAPDH	CCAACGTGTCTGTTGTGGATCTGA GAGCTTGACAAAGTGGTCGTTGAG	218	E=100.2 %; r^2= 0.99	Assidi et al., BOR 2008 79:209; NM_001034034
HIST1H2A (variant H, C, G)	GTCGTGGCAAGCAAGGAG GATCTCGGCCGTTAGGTACTC	182	E=109.7 %; r^2= 0.97	Robert et al., BOR 2002 76:1465; Vigneault et al., MRD 2007 74:703; LOC616634,LOC506900; LOC616790

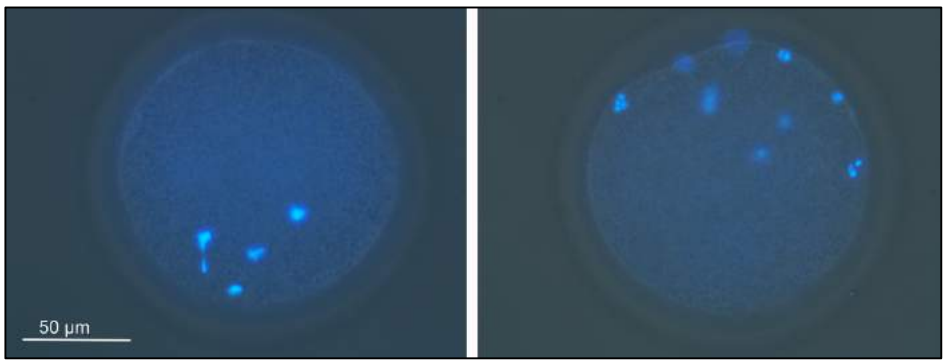
5.7.2 Supplemental figures



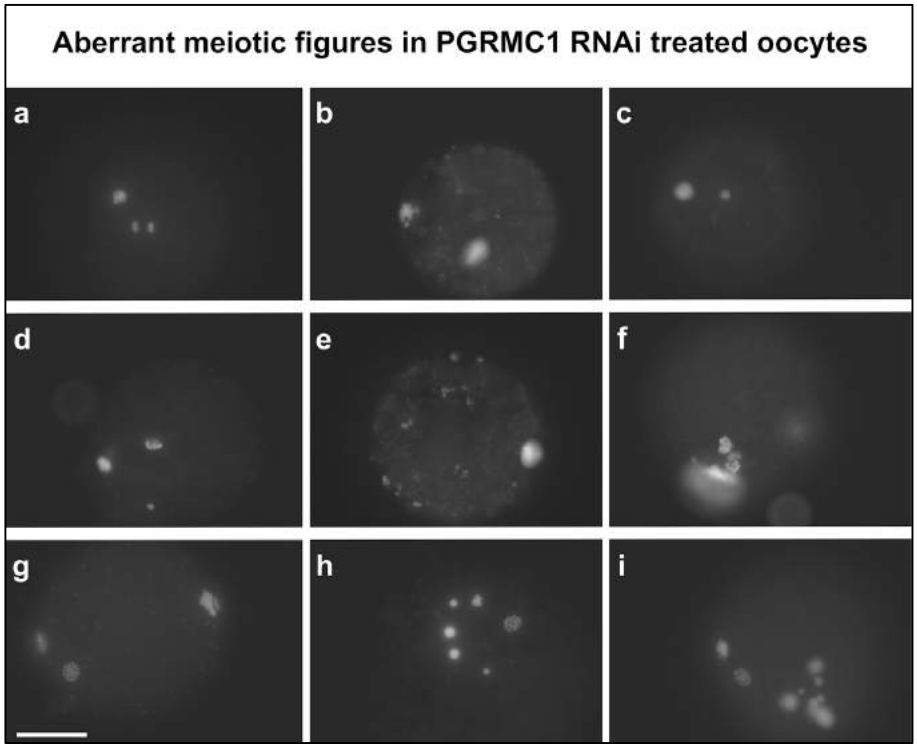
Supplemental figure 5.1: Representative DNA histograms generated by flow cytometric analysis of PGRMC1 and CTRL RNA1 treated bGCs after 72 h of culture. The difference in the G2/M phase is clearly appreciable. Note that the presence of debris at the left of the histogram in both groups is due to the nature of the sample analyzed.



Supplemental figure 5.2: Aberrant meiotic figures in AG205 treated oocytes Images show examples of aberrant meiotic figures observed after AG205 treatment. Images in **a** and **b** represent oocytes showing double Metaphase (a) or Telophase (b, note that b' and b'' are different focal planes of the same oocyte). Images c, d, e and f show oocytes with DNA clumps scattered within the ooplasm (note that f', f'' and f''' are different focal planes of the same oocyte and lighter stained areas are out of focus clumps of chromatin). Scale bar is 50 μm. Insets show the DNA at 2X magnification.



Supplemental figure 5.3. Representative images of DAPI stained oocytes under bright field. Samples were concomitantly illuminated with white and UV light using the DAPI filter, in order to ensure correct analysis of DNA clumps localization within the ooplasm.



Supplemental figure 5.4: Aberrant meiotic figures in PGRMC1 RNAi treated oocytes Images show examples of aberrant meiotic figures observed after PGRMC1 RNAi treatment. Image a shows an oocyte with a Telophase and additional DNA clumps. Images b to i show oocytes with DNA clumps scattered within the ooplasm. In some of these oocytes (g, h, i) the occurrence of scattered DNA was concomitant to the presence of a metaphase plate. Scale bar is 50 µm.

5.8 References

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6. Role of Progesterone and its receptors Nuclear Progesterone Receptor (nPGR) and Progesterone Receptor Membrane Component 1 (PGRMC1) in oocyte meiosis

Foreword

This study was in part conducted during my period of PhD externship in Prof. Trudee Fair laboratory at University of Dublin. In this study, we compared PGRMC1 and nPGR action in oocyte meiosis through their pharmacological inhibition using AG205 and Aglepristone, respectively, evaluating the effect on meiotic MII plates and tubulin spindle morphology.

6.1 Introduction

P4 has a role in both follicular cells and in the oocyte during maturation. Yet its mechanism of action is still unknown and some receptors other than the classical nPGR have been described. Specifically, in bovine both oocyte and cumulus cells express nPGR as well as the non-genomic receptors (PGRMC1, PGRMC2 and Membrane Progestin Receptors) in a cell-dependent way (oocyte-cumulus cells) and they dynamically change in *in vitro* maturation and after LH, FSH or P4 supplementation [1-3].

The functional importance of P4 and its receptors has started to be elucidated by experiments inhibiting cumulus cells P4 synthesis and blocking nPGR signaling using Trilostane and RU486, respectively, resulting in a significative reduction of blastocyst developmental rate [1, 4, 5]. Previous studies also demonstrated that RU486 impaired oocyte maturation in a dose-dependent manner [6]. On the other hand, PGRMC1 appears to be involved in meiotic maturation since intracytoplasmic injection of oocytes with an antibody against PGRMC1 affected chromosome segregation during oocyte meiotic maturation [2], while adding an antibody specific to mPRα during IVM reduced the percentage of oocytes progressing through the early cleavage stages [1]. However, the precise mechanism through which these receptors could mediate P4 actions remains to be elucidated together with their relative contribution during oocyte maturation and the consequent stages until embryogenesis, considering their function could be species-specific or be relative to different levels of P4 and combined expression of its receptors.

Both PGRMC1 and nPGR localize to chromosomes in Metaphase II plate of bovine oocytes and according to previous studies they could have a role predominantly in meiotic maturation and on subsequent embryo development, respectively [1, 2].

Therefore, the main aim of this study is to decipher the role of progesterone receptors in meiotic maturation of bovine oocytes disrupting their function through inhibition of nPGR and PGRMC1. To inhibit nPGR we used both RU486 and Aglepristone. The PGRMC1 function was inhibited by AG205, as previously described [7].

Aglepristone blocks nPGR-mediated P4 effects and has a relative binding affinity equal to that of RU486 for the receptor, but has >9 times greater affinity than

that of P4 [4], while AG205 interacts with cytochrome b5/heme-binding domain of PGRMC1 which is the site interacting with various proteins and possibly also P4 [8].

6.2 Material and methods

6.2.1 Samples collection and in vitro maturation

Bovine ovaries were collected from a local abattoir in Ireland or Italy and immature COCs were collected from 2-6 mm antral follicles and in vitro matured as previously described [2, 7]. Groups of 20-25 COCs were matured for 24 hours in IVM medium supplemented with different concentrations of Aglepristone – (RU534/Alizin virbac, 0,1 – 1 – 10 – 25 and 50 μM concentrations), RU486 – (SIGMA, 1 – 10 - 25 and 50 μM) or AG205 - (SIGMA, 20μM final concentration) or without the inhibitors. We also included a solvent control group adding only DMSO (10 μM concentration).

6.2.2 Assessment of meiotic progression of bovine oocytes

The oocytes were denuded, fixed in 4% paraformaldehyde in PBS and permeabilized in 0.1% Triton-X 100 in PBS for 10 minutes and washed in 0.1% PVA in PBS (PBS/PVA). Samples were mounted on slides with the anti-fade medium Vectashield (Vector Laboratories) supplemented with 1 mg/ml 4',6'-diamidino-2-phenylindole (DAPI) to visualize DNA. Samples were analyzed on an epifluorescence microscope (Eclipse E600; Nikon Corp.) equipped with a digital camera (DS-Fi2; Nikon Corp.) to assess the stage of meiotic progression as previously described [7].

6.2.3 Meiotic spindle and metaphase II plate morphological assessment

For tubulin spindle length analysis oocytes were fixed in microtubule-stabilizing buffer (100 mM PIPES, 5 mM MgCl2, 2.5 mM EGTA, 2% formaldehyde, 0.1% Triton-X-100, 1 mM taxol, 10 U/ml aprotinin and 50% deuterium oxide) for 30 min at 37°C and stored in blocking solution (0.2% sodium azide, 2% normal goat serum, 1% BSA, 0.1 M glycine and 0.1% Triton X-100 in PBS) at 4° C until further processing for immunostaining [9]. Oocytes were incubated in a solution of mouse anti-tubulin (SIGMA T6074) in PBS with 1% BSA (dilution 1:50) at 4°C overnight. After extensive washing, the oocytes were incubated in a solution of INVITROGEN Alexa Fluor 488-conjugated goat anti-mouse IgG (dilution 1:500 in PBS with 1% BSA) from for 1 h at room temperature in the dark, then washed again and mounted on slides in the anti-

fade medium VectaShield. Oocytes were analyzed using the confocal laser scanning microscope (FLUOVIEW FV1000 model, Olympus, xxx) and in all lateral view plates the pole-to-pole tubulin spindle distance was measured (Figure 6.4).

Morphometric analysis of the meiotic spindle and plate was performed using ImageJ software and all measurements are expressed in microns. Plate diameter was calculated in polar view plates of oocytes treated as described above and expressed as the mean of 3 different diameters measurements (see **Figure 6.4 A-B**).

6.2.4 Statistics

The analysis of inhibitor effects was carried out by evaluating 15-20 oocytes for each concentration/independent run. Experiments were repeated at least three times. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test or t-student test using Graph Pad Prism version 6.0 h accordingly to the experimental design. Data are presented as mean ± SEM. P values < 0.05 were considered as statistically significant. For each experiment, the specific test used is indicated in the figure legend.

6.3 Results

6.3.1 Dose response curve for Aglepristone and RU486 treatment during IVM

The administration of Aglepristone in increasing concentrations showed that the percentage of oocytes able to reach MII phase decreased significantly already after 1μM concentration of the inhibitor. At the same time, we observed an increase of the percentage of oocytes showing aberrant meiotic figures. Aberrant plates were mainly characterized by scattered chromatin or misaligned chromosomes and the absence of the polar body. No statistical differences were observed between standard control (basic maturation medium without DMSO) and solvent control (**Figure 6.1 A**).

Analogously, the administration of RU486 was effective in affecting the capability to reach MII-stage of meiotic division starting from 1 μM the percentage of oocytes able to reach MII-stage of meiosis decreased significantly. However, an increase of aberrant meiotic figures was observed only at the maximum

concentration tested (50μM) while the critical effect was a significant increase of oocytes arrested at MI stage of meiosis (**Figure 6.1 B**).

The following experiments were conducted evaluating the effects of using Aglepristone at a 1μM concentration.

6.3.2 Comparison of the effect of AG205 and Aglepristone treatment during in vitro maturation

The results indicate that disrupting PGRMC1 function has a more detrimental effect on meiotic maturation. Indeed, inhibiting PGRMC1 using AG205 had a dramatic effect on the percentage of oocytes reaching MII meiotic stage, leading to a very high percentage of aberrant figures and confirming what was previously described [7]. By contrast, disrupting nPGR function with Aglepristone caused a lower percentage of aberrancies concomitant to a higher percentage of oocytes reaching MII phase (**Figure 6.2**). As to the aberrant figures, in AG205 treated oocytes half of the aberrancies was represented by double plates has already previously shown [7] (**Figure 6.3**), while in Aglepristone treated oocytes no double plates were detected and the aberrancies were represented by arrested or scattered plates (**Figure 6.3**).

Due to this difference, we decided to evaluate if the apparently normal MII plates in Aglepristone treated oocytes were equal to the control group from a morphological point of view.

6.3.3 Effect of Aglepristone treatment on meiotic plate and tubulin spindle morphometry

Aglepristone treatment affected both MII plate diameter and tubulin spindle length; indeed, overall the diameter and the tubulin spindle were significantly bigger and longer compared to the control (**Figure 6.4**).

6.4 Discussion

These data add new insights on P4 and their receptors role in oocyte meiosis. They suggest a possible P4 action mediated by both PGRMC1 and nPGR receptors affecting different meiotic maturation stages. Indeed, PGRMC1

might mediate an effect mainly on chromosome segregation since the effects of its inhibition strongly impairs MII plates morphology and maturation rate, while impairing nPGR has a mild effect on MII plate morphology and spindle formation that, however, could explain consequent reduced rate in blastocysts development observed in previous studies inhibiting P4 production with Trilostane and RU486 nPGR inhibitor [1, 5].

Moreover, rescue experiments with P4 after inhibition of nPGR confirmed the receptor was mediating a P4 signaling pathway [1]. While for PGRMC1, rescue experiments with P4 are still ongoing. However, our previous AG205 studies showed that the detrimental effects of the inhibition on correct chromosome segregation were more pronounced on denuded oocytes compared to COC. This indicates that P4 could be part of the effect mediated by this receptor, since it is known that cumulus cells produce P4 during in vitro culture [10]. Therefore, we could assume that in the previous study [7] higher P4 concentration was present in COC than DO culture. Thus, the presence of P4 in the COC culture could have attenuated the effect of AG205. This hypothesis is currently under investigation.

Since little is known on the mechanism of action of these receptors further studies are foreseen to understand P4 nPGR non genomic-mediated effect, since transcription in the oocyte is silenced, as well for PGRMC1 only some hypothesis have been proposed (see chapter 3.6).

In conclusion, P4 function in the ovary is complex and discordant or different results on its action could be due also to species-specific differences. Moreover, P4 levels and its binding affinity to each receptor, together with different expression patterns of its receptors in the follicle might be responsible of divergences in its action. However, the growing evidence of its role during oocyte maturation deserves more investigations.

6.5 Figures

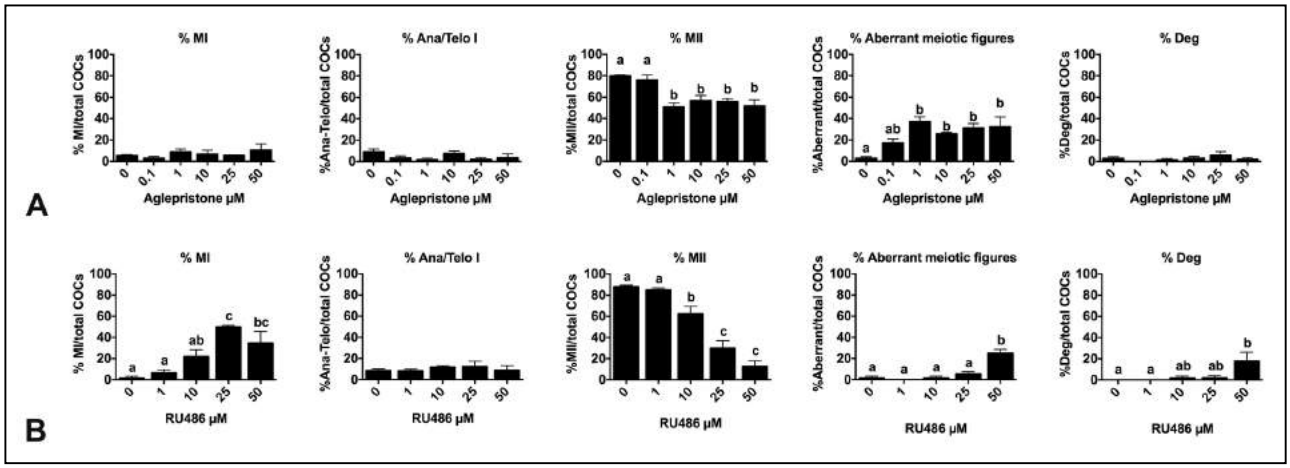

Figure 6.1 Dose response curve for Aglepristone treatment (A) and RU486 treatment (B) on meiotic progression. Graphs show the percentage of oocytes at each stage of the first meiotic division and of aberrant meiotic figures.

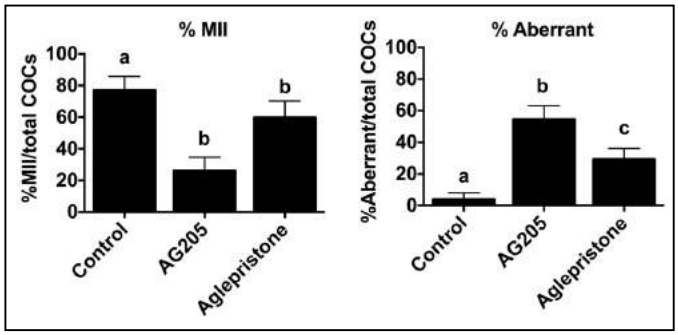

Figure 6.2 Effect of AG205 and Aglepristone treatment during in vitro maturation showing the percentage of oocytes reaching MII phase and oocytes showing aberrant plates. a,b,c indicates significant difference (one-way ANOVA followed by Tukey's multiple comparisons test, P < 0.05).

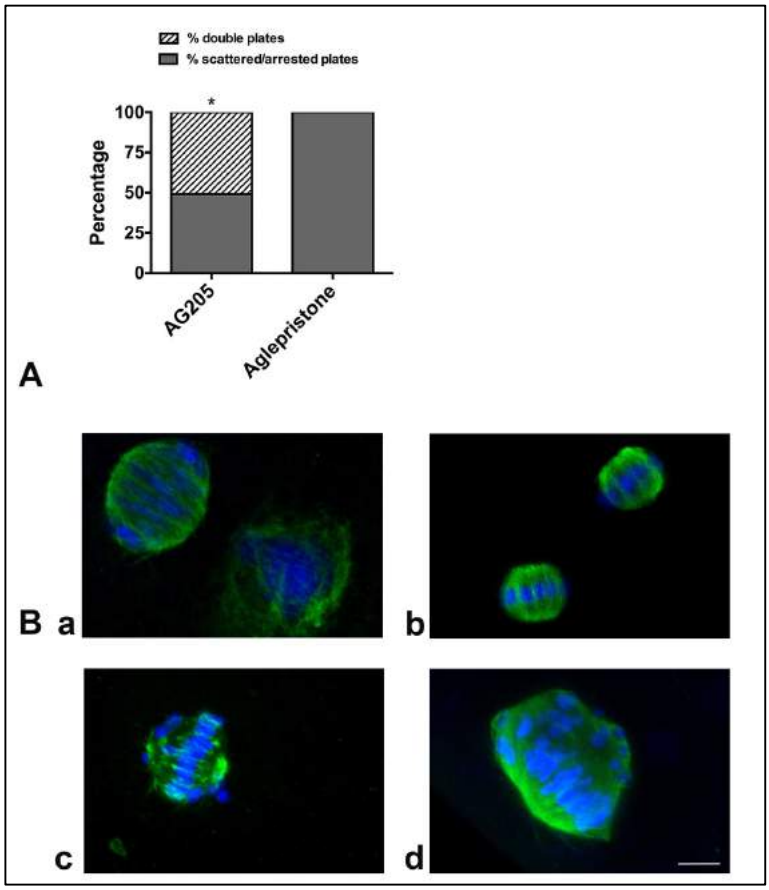

Figure 6.3
A. Graph showing distribution of different types of aberrancies in AG205 and Aglepristone treated oocytes expressed on the total of aberrancies.
B. Representative images of aberrant meiotic figures in AG205 and Aglepristone treated oocytes showing an oocyte with a MII plate with I polar body (a), a double MII plate (b), a large plate with scattered chromatin (c) and an oocyte with DNA clumps scattered within the ooplasm (d). Scale bar is 10 µm.

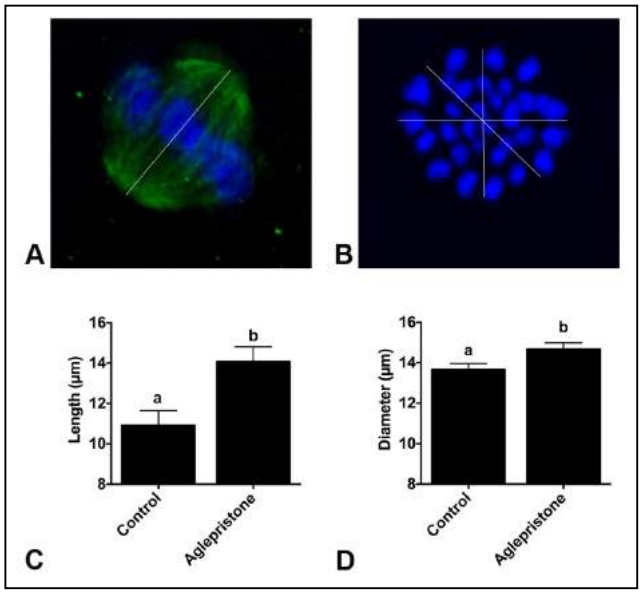

Figure 6.4 Sample figures for plate diameter and spindle length measurement. Representative images showing tubulin (green) and DNA (blue) in MII oocytes after in vitro maturation. The tubulin image (A) shows the axis used for spindle length measurement in lateral view, while polar view plates (B) were used for diameter measurements calculating the media of the 3 axes shown.
The bar graphs represent the measurement of the spindle lenght in MII-stage oocytes after in vitro maturation (mean± s.e.m.) (C) and of the spindle diameter (mean± s.e.m.) (D). a,b indicate significant difference (unpaired t-test, $P < 0.05$).

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7. Characterization of Progesterone Receptor Membrane Component 1 localization and putative function in the nucleolus of bovine granulosa cells and oocytes

Foreword

This chapter is a paper that has been accepted pending revision in Reproduction. In the meantime, additional experiments have been performed and the final accepted paper is attached in the appendix.

***Terzaghi L**., Luciano AM., Chediek Dall'Acqua P., Modina SC., Peluso JJ., Lodde V. Characterization of Progesterone Receptor Membrane Component 1 localization and putative function in the nucleolus of bovine granulosa cells and oocytes. Reproduction*

In this paper, we evaluated the role of PGRMC1 in a specific nuclear compartment, the nucleolus, both in somatic cells and in the oocyte to unravel one of its possible mechanisms of action. The localization of PGRMC1 has already been described in both nucleolus of somatic cells and in the zygote, while we evaluated through immunofluorescence its localization in a primary cell line of bovine granulosa cells and in growing and fully grown oocytes and its colocalization with the major nucleolar protein, nucleolin. Moreover, in somatic cells we assessed their functional relationship using RNAi technique to downregulate PGRMC1 expression. Indeed, besides its well-known role in ribosome subunits production, the nucleolus is also involved in cell cycle regulation determining protein modifications such as sumoylation and phosphorylation, sequestrating specific proteins or stabilizing p53 to arrest cell cycle after stress stimuli to the cell. While in the oocyte it completely disassembles during meiosis but still have an important role during previous oocyte growth and reassembles in early embryogenesis.

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7.1 Abstract

Progesterone Receptor Membrane Component-1 (PGRMC1) is a highly conserved multifunctional protein that is found in numerous systems, including the reproductive system. Interestingly, PGRMC1 is expressed at several intracellular locations, including the nucleolus. The aim of this study is to investigate the functional relationship between PGRMC1 and the nucleolus. Immunofluorescence experiments confirmed PGRMC1's nucleolar localization in bovine granulosa cells (bGC) and oocytes. Additional experiments conducted on bGC revealed that PGRMC1 co-localizes with nucleolin, a major nucleolar protein. Furthermore, small interfering RNA (RNAi) mediated gene-silencing experiments showed that when PGRMC1 expression was depleted, nucleolin translocated from the nucleolus to the nucleoplasm. Although PGRMC1 influenced the localization of nucleolin, a direct interaction between these two proteins was not detected using in situ proximity ligation assay. This suggests the involvement of additional molecules in mediating the co-localizaton of PGRMC1 and nucleolin. Since nucleolin translocates into the nucleoplasm in response to various cellular stressors, PGRMC1's ability to regulate its localization within the nucleolus is likely an important component of mechanism by which cells response to stress. This concept is consistent with PGRMC1's well-described ability to promote ovarian cell survival and provides a rationale for future studies on PGRMC1, nucleolin and the molecular mechanism by which these two proteins protect against the adverse effect of various cellular stressors.

7.2 Introduction

Progesterone Receptor Membrane Component 1 (PGRMC1) is a widespread and multifunctional protein that is highly conserved in eukaryotes. It belongs to the membrane associated progesterone receptor (MAPR) family and is expressed in several mammalian organs and tissues (Runko *et al.* 1999, Raza *et al.* 2001, Sakamoto *et al.* 2004, Bali *et al.* 2013a, Bali *et al.* 2013b), including those of the reproductive system (Zhang *et al.* 2008, Luciano *et al.* 2010, Aparicio *et al.* 2011, Luciano *et al.* 2011, Keator *et al.* 2012, Saint-Dizier *et al.* 2012, Tahir *et al.* 2013, Kowalik *et al.* 2016). Specifically, granulosa and luteal cells of human, rodent, bovine and canine ovaries (Engmann *et al.* 2006, Peluso 2006, Aparicio *et al.* 2011, Luciano *et al.* 2011, Tahir *et al.* 2013, Griffin *et al.* 2014, Terzaghi *et al.* 2016) as well as oocytes express PGRMC1 (Luciano *et al.* 2010, Luciano *et al.* 2013, Terzaghi *et al.* 2016).

Multiple functions are attributed to PGRMC1 (reviewed in (Cahill 2007, Brinton *et al.* 2008, Neubauer *et al.* 2013, Peluso & Pru 2014, Cahill *et al.* 2016, Ryu *et al.* 2017)) as reflected by it being localized to numerous sub-cellular compartments. As predicted by the presence of a transmembrane domain, PGRMC1 localizes in several membranous compartments, such as the endoplasmic reticulum, the Golgi apparatus, the nuclear and plasma membranes, the endosomes, and the secretory vesicles (Meyer *et al.* 1996, Raza *et al.* 2001, Bramley *et al.* 2002, Hand & Craven 2003, Shin *et al.* 2003, Sakamoto *et al.* 2004, Min *et al.* 2005, Peluso *et al.* 2006, Zhang *et al.* 2008, Neubauer *et al.* 2009, Ahmed *et al.* 2010, Roy *et al.* 2010, Wu *et al.* 2011, Xu *et al.* 2011, Mir *et al.* 2012, Mir *et al.* 2013, Thomas *et al.* 2014). Interestingly, PGRMC1 is also detected in the nucleus (Beausoleil *et al.* 2004, Peluso *et al.* 2008, Zhang *et al.* 2008, Ahmad *et al.* 2009, Peluso *et al.* 2009, Luciano *et al.* 2010, Peluso *et al.* 2010a, Peluso *et al.* 2012), specifically to the nucleolus (Ahmad *et al.* 2009, Luciano *et al.* 2010, Boisvert *et al.* 2012, Thul *et al.* 2017)] http://www.proteinatlas.org]. This high compartmentalization suggests that at each site PGRMC1 participates in the control of precise cellular processes.

In order to shed light into the intricate story of PGRMC1's biological significance, it is important to dissect the function of PGRMC1 at each sub-cellular compartment. To this end, we have started to address whether PGRMC1 has a role in regulating nucleolar function. Although the nucleolus' main function involves ribosome subunits production, recent advances describe it as a multifunctional subnuclear compartment.

It appears that the nucleolus is a dynamic structure, which disassembles during mitosis and responds to signaling events during interphase. As such, it is involved in cell cycle control, especially regulating protein modifications such as sumoylation and phosphorylation or sequestrating specific proteins (Boisvert *et al.* 2007). Furthermore, it acts as a stress sensor mediating p53 stabilization in order to arrest cell cycle progression (Boisvert *et al.* 2007, Boulon *et al.* 2010). Thus, the overall goal of the present study is to examine the role of PGRMC1 on nucleolar function, particularly its relationship with nucleolin (NCL), a well-characterized nucleolar protein (Boisvert *et al.* 2007, Tajrishi *et al.* 2011).

7.3 Material and methods

7.3.1 Reagents

All the chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO) except for those specifically mentioned. Gene silencing was performed by using the Stealth RNAi™ siRNA technology from Life Technologies as previously described (Terzaghi *et al.* 2016) using PGRMC1 Stealth RNAi (PGRMC1 RNAi: (RNA)-GAG UUG UAG UCA AGU GUC UUG GUC U) within the coding region of the bovine PGRMC1 sequence (RefSeq: NM_001075133). Negative control (cat n. 12935-200) was chosen among the Stealth RNAi negative control (CTRL RNAi) duplexes available from Life Technologies, designed to minimize sequence homology to any known vertebrate transcript. Primary antibodies used in this study are listed in **Table 7.1**.

7.3.2 Sample collection

Bovine samples:

Ovaries from Holstein dairy cows were recovered at the abattoir (INALCA S.p.A., Ospedaletto Lodigiano, LO, IT 2270M CE, Italy) from pubertal females subjected to routine veterinary inspection and in accordance to the specific health requirements stated in Council Directive 89/556/ECC and subsequent modifications. Ovaries were transported to the laboratory within 2 hours in sterile saline (NaCl, 9 g/l) maintained at 26°C and all subsequent procedures, unless differently specified, were performed at 35-38°C. Bovine granulosa cells (bGC) were collected as previously described

(Terzaghi *et al.* 2016). Briefly the content of 2-8 mm ovarian follicles, which typically contain fully-grown oocytes, was aspirated and cumulus-oocyte complexes (COCs) were collected and processed for further immunofluorescence analysis (see below). Remaining follicular cells were washed in M199 supplemented with HEPES 20 mM, 1,790 units/L Heparin and 0.4% of bovine serum albumin (M199-D). The cell pellet was re-suspended in 1ml of Dulbecco's modified growth medium supplemented with 10% of bovine calf serum, 100 U/ml penicillin G, 100 µg/ml, Streptomycin and Glutamax 100U/ml (Gibco). The cell suspension was plated in a 25 cm^2 flask with 6 ml of growth medium and incubated in humidified air at 37°C with 5% CO_2. After 24 hours cells were gently washed with PBS and the growth medium was changed. Cells were incubated until confluence, then collected after trypsinization and re-plated according to the experimental design (see below).

Oocytes in their growing phase, characterized by a diffuse filamentous pattern of chromatin in the nuclear area and the presence of an active nucleolus were collected as previously described from 0.5 to <2 mm early antral follicles by rupturing the follicle wall under the stereomicroscope (Lodde *et al.* 2008); Both COCs collected from 0.5 to <2 mm and 2-8 antral follicles were mechanically denuded using the vortex and fixed for further immunofluorescence analysis.

7.3.3 RNAi treatment

RNA interference (RNAi) experiments on bGC were conducted as previously described (Terzaghi *et al.* 2016). Cells were plated in a total number of 2 X 10^5 bGC cells in 2 ml of medium on cover glasses in 35-mm culture dishes and incubated in humidified air at 37°C with 5% CO_2. After 24 h, cells at 50-70% of confluence were transfected with 6 µl of 20 µM PGRMC1 Stealth RNAi or CTRL RNAi combined with 10 µl of Lipofectamine RNAi MAX (Life Technologies) in a final volume of 2 ml OPTIMEM (Life Technologies), according to the manufacturer protocol, and cultured for 48 h.

7.3.4 Western blot analysis

The levels of PGRMC1 protein expression in CTRL and PGRMC1 RNAi treated bGC were assessed by Western blotting assay as previously described (Terzaghi *et*

al. 2016). PGRMC1 or CTRL RNAi treated bGC were lysed in radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40 (NP-40), and 0.25% sodium deoxycolate], supplemented with protease inhibitors and phosphatase inhibitors. All procedures were conducted on ice. Total amount of protein was determined using the Qubit® Protein Assay Kit and Qubit® fluorometer (Thermo Fisher Scientific). 20 µg of total protein/lane were used for Western blottings. After the run, samples were transferred to nitrocellulose membrane (Bio-Rad), which was then incubated with 5% dry milk powder in TBS containing 0.1% tween (TBS/T) for 2 hours at room temperature. PGRMC1 immunodetection was conducted using the rabbit polyclonal antibodies (see **table 7.1**) in 5% dry milk TBS/T. PGRMC1 was revealed using a stabilized goat anti rabbit IgG peroxidase conjugated antibody and detected using the Super Signal West Dura Extended Duration Substrate (Thermo Fisher Scientific). The nitrocellulose membrane was stripped in stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl [pH 6.7]) at 50°C for 30 min and re-probed with the anti beta tubulin antibody at dilution 1:1000, which was revealed using a stabilized goat anti mouse IgG peroxidase conjugated antibody as loading control.

7.3.5 Immunofluorescence

Immunofluorescence staining was performed on bGC as previously described (Lodde & Peluso 2011, Terzaghi *et al.* 2016). Briefly, cells were fixed in 4% paraformaldehyde in PBS for 7 minutes and permeabilized with 0.1% triton-X in PBS for 7 minutes. Samples were blocked with 20% normal donkey serum in PBS and incubated overnight at 4°C with the rabbit anti PGRMC1 antibody (see **table 7.1**). Double immunostaining was performed on bGC by incubating the samples with the rabbit anti PGRMC1 or the mouse anti NCL antibodies or a combination of the two. After incubation with secondary antibodies for 1 h at room temperature, samples were washed and finally mounted on slides in the antifade medium Vecta Shield (Vector Laboratories) supplemented with 1 µg/ml 40,6-diamidino-2-phenylindole (DAPI). Immunofluorescent analysis on bovine oocytes were performed as previously described (Luciano *et al.* 2010) on 4% paraformaldehyde fixed oocytes. Immunofluorescent staining was performed as described for bGC with the exception

that oocytes were fixed for 30 min at room temperature followed by 30 min and permeabilized with 0.3% Triton-X 100 for 10 minutes.

bGC and oocytes were analyzed on an epifluorescence microscope (Eclipse E600; Nikon) equipped with a 40 X and a 60X objective, a digital camera (Nikon digital sight, DS-U3) and software (NIS elements Imaging Software; Nikon). Immunofluorescence negative controls, which were performed by omitting one or both the primary antibodies, did not show any staining under the same exposure settings. Images that were used for image quantification analysis were captured under the same settings.

7.3.6 In situ Proximity Ligation Assay (PLA)

In situ Proximity Ligation Assay (PLA; Duolink® SIGMA) was used to assess the interaction between PGRMC1 and nucleolin in bGC following following the manufacturer protocol. Primary antibodies for PGRMC1 and nucleolin were the same used for immunofluorescence, while anti-rabbit PLUS and anti-mouse MINUS PLA probes were used as secondary antibodies. Negative controls were performed omitting one of the two primary antibodies. Cells were mounted with Duolink mounting medium.

7.3.7 Image analysis

Quantification of Fluorescent Intensity (FI) signal was performed using the ImageJ software (https://imagej.net). The nucleolar signal of PGRMC1 in bGC was quantified calculating the integrated density of PGRMC1 signal selecting the whole PGRMC1 positive areas in the nucleus of a total of 50 cells for each treatment (CTRL RNAi and PGRMC1 RNAi treated cells) at 48 h after RNAi treatment. Data were polled and the mean FI of the CTRL RNAi treated group was set at 100%. FI values of the CTRL RNAi and PGRMC1 RNAi treatments were expressed as a percentage of the mean CTRL RNAi value. For image quantification of the NCL nucleolar and nucleoplasmic signals, threshold was selected by choosing a cutoff value such that all the nucleolar areas with an intense NCL signal within each cell. Then, the NCL total nuclear FI was assessed by selecting the whole nuclear area and calculating the integrated density of the corresponding regions of interests (ROI) of a total of 50 randomly selected nuclei in CTRL RNAi and PGRMC1 RNAi treated cells. The NCL

nucleolar signal was calculated by analyzing the integrated density of the threshold area in each nucleus, while the NCL nucleoplasmic signal was calculated by subtracting the total nucleolar FI to the total nuclear FI of each nucleus. Data were polled and the mean nucleolar and nucleoplasmic NCL FI of the CTRL RNAi treated group were set at 100%. FI values of the CTRL RNAi and PGRMC1 RNAi treatments were expressed as a percentage of the mean CTRL RNAi value. Background signals did not change significantly among treatments

7.3.8 Statistical analysis

Experiments were run in triplicate, unless otherwise specified. All statistical analysis was done using Prism software (GraphPad Prism v. 6.0e, La Jolla, CA, USA). Data from the replicate experiments were pooled and the data expressed as a mean ± SEM. Student's t test was used to determine differences between two groups.

7.4 Results

7.4.1 PGRMC1 localization

Immunofluorescence analysis indicated that PGRMC1 localized to areas of the interphase nucleus that were not stained by DAPI. PGRMC1's nuclear localization in bGC was the same regardless of which PGRMC1 antibody was used (**Figure 7.1**). However, nuclear staining for PGRMC1 with the Sigma Prestige antibody displayed a diffuse signal with the staining associated with DAPI-negative areas and only slightly more intense than that observed for the overall nucleus. In contrast, the non-DAPI stained areas within the nucleus were more intensely stained using the PGRMC1 antibody provided by Proteintech (**Figure 7.1**). These non-DAPI stained areas typically correspond to areas of the interphase nucleus where the nucleoli resided.

7.4.2 PGRMC1 co-localization with nucleolin

To further characterize PGRMC1 localization in the nucleus, we evaluated its co-localization with the nucleolar marker, nucleolin (NCL), in both bGC and bovine

growing and fully-grown oocytes. Immunofluorescence data indicated that the two proteins co-localized in the nucleolus of bGC as shown in **Figure 7.2**; PGRMC1 signal appeared as a dotted pattern in the area corresponding to the nucleolus compared to NCL signal, which fully covered the nucleolus space (i.e. nuclear areas not stained by DAPI). Although co-localized, in situ proximity ligation assay did not detect an interaction between PGRMC1 and NCL, indicating the absence of a direct interaction between the two proteins in bGC (Data not shown).

In growing bovine oocytes, which are characterized by the presence of an active nucleolus (Fair *et al.* 1996, Lodde *et al.* 2008), NCL marked the nucleolus and showed a light diffuse staining pattern in the nucleoplasm as previously described (Fair *et al.* 2001, Baran *et al.* 2004, Maddox-Hyttel *et al.* 2005). In particular, NCL nucleolar signal was intense and slightly more concentrated at the periphery of the nucleolus. In these oocytes PGRMC1 localized in the nucleolus showing a dotted staining pattern, similar to that observed in bGC nucleoli (**Figure 7.2**). In fully-grown oocytes (**Figure 7.2**), which typically displayed inactive nucleolar remnants (Fair *et al.* 1996, Lodde *et al.* 2008), NCL was mainly dispersed in the nucleoplasm with a faint staining in the nucleolar remnants as previously described (Fair *et al.* 2001, Baran *et al.* 2004, Maddox-Hyttel *et al.* 2005). In these oocytes PGRMC1 concentrated in one or multiple dots where it co-localized with NCL.

7.4.3 Assessment of PGRMC1 and nucleolin functional interaction

In order to establish the possible functional relationship between PGRMC1 and NCL, we silenced PGRMC1 expression in bGC by using RNAi. The RNAi protocol was previously validated by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) showing a significant reduction of PGRMC1 mRNA levels compared to CTRL RNAi treated group (Terzaghi *et al.* 2016). The decline in PGRMC1 expression after 48h PGRMC1-RNAi treatment was confirmed by Western blot analysis, regardless of which PGRMC1 antibody was used. As shown in **Figure 7.3A**, PGRMC1 was present in multiple bands, whose intensity decreased after 48h of PGRMC1 RNAi treatment. Moreover, quantification of PGRMC1 nucleolar immunofluorescent signal in PGRMC1 and CTRL-RNAi treated bGC revealed an approximate 40% decrease in PGRMC1 abundance in the nucleolus, which also gives confirmation of the specificity of PGRMC1's nucleolar localization (**Figure 7.3B** and **7.3C**).

In order to assess the putative functional relationship between PGRMC1 and NCL, the effect of depleting PGRMC1 on the localization of NCL was evaluated. As shown in **Figure 7.4**, when PGRMC1 was depleted, a significantly higher quantity of nucleolin was present in the nucleoplasm when compared to the CTRL-RNAi treated group.

7.5 Discussion

The present findings demonstrate that PGRMC1 localizes to the nucleolus of both bovine granulosa cells and oocytes, suggesting that PGRMC1 has a role in regulating the function of the nucleolus of these two cell types. The prominent nucleolar localization of PGRMC1 as revealed using the Protein Tech antibody is consistent with investigations of non-ovarian cells that detect PGRMC1 within the nucleolus by either immunohistochemistry (http://www.proteinatlas.org) or mass spectrometric analysis (Ahmad *et al.* 2009, Luciano *et al.* 2010, Boisvert *et al.* 2012, Thul *et al.* 2017). However, the rabbit polyclonal antibody to PGRMC1 provided by Sigma Prestige detects PGRMC1 not only within the nucleolus but also in other interchromatin regions that resemble the nuclear speckles (Spector & Lamond 2011). The reason for this discord likely relates to the two antibodies detecting different molecular weight forms of PGRMC1. Western blots using either the Proteintech or the Sigma Prestige antibody detect PGRMC1 as bands at ≈ 25 and ≈ 55 kDa, while the Sigma antibody also detects an additional band at 37 kDA and two bands greater than 55 kDA. All the bands detected by either antibody are specific since their intensity is decreased in PGRMC1 RNAi-treated cells. The different size forms of PGRMC1 are due to dimerization and post-translational modifications such as phosphorylation and sumoylation (Neubauer *et al.* 2008, Peluso *et al.* 2010b, Peluso *et al.* 2012, Kabe *et al.* 2016). Therefore, it is not surprising that polyclonal antibodies obtained using different immunogens may preferentially recognize one or multiple forms of PGRMC1, which in turn might preferentially localize in different subcellular compartment.

Because the Proteintech antibody precisely localizes PGRMC1 to the nucleolus, it was used to determine whether PGRMC1 co-localizes with the nucleolar protein, NCL. This approach reveals that PGRMC1 and NCL co-localize to the nucleolus in bGC. Moreover, depletion of PGRMC1 results in NCL within the nucleolus redistributing to nucleoplasm in these cells. Thus, localization of NCL is likely dependent in part on PGRMC1. This observation is biologically relevant since NCL mobilization from the nucleolus into the nucleoplasm is induced by different types of cellular stress. For example, heat shock, ionizing radiation, and hypoxia all promote the translocation of nucleolin into the nucleoplasm (Daniely & Borowiec 2000, Daniely *et al.* 2002). In particular, NCL redistribution is induced by heat stress in HeLa cells and accompanied by an increase in the formation of a complex between NCL and

Replication Protein A (RPA) (Daniely & Borowiec 2000), which exerts important functions during DNA replication (Iftode *et al.* 1999). NCL-RPA interaction in turn strongly inhibits DNA replication, likely by sequestering RPA away from sites of ongoing DNA synthesis (Daniely & Borowiec 2000). Other studies in U2-OS and U2-OS p53-depleted cells demonstrate that NCL redistribution occurs when stress is induced by γ-irradiation and treatment with the radiomimetic agent, camptothecin. Under these stress conditions, NCL binds p53, which facilitates its transit into the nucleoplasm (Daniely *et al.* 2002). These stress-induced changes in NCL's localization suggest that various stressors change PGRMC1 function, altering its ability to retain NCL, which allows NCL to transit to the nucleoplasm. This concept merits further investigation.

Although PGRMC1 and NCL often co-localize to the same sub-region of the nucleolus of bGC, they do not seem to directly interact, since we were not able to detect a positive signal by means of PLA assay. This might suggest that their functional interaction could involve the participation of another yet to be identified protein or proteins. Interestingly a known PGRMC1 binding protein, Plasminogen Activator Inhibitor 1 RNA-Binding Protein (PAIRBP1) (Peluso *et al.* 2006, Peluso *et al.* 2008, Peluso *et al.* 2013) (also known as SERPINE1 mRNA Binding Protein 1), which is typically found in the cytoplasm, localizes to the nucleolus under specific experimental stress induced conditions in Hela cells, such as treatment with arsenite and the methylation inhibitor adenosine periodate (Lee *et al.* 2014). Therefore, it is possible that PAIRBP1 competes with this putative intermediary protein for binding to PGRMC1. The stress-induced formation of the PGRMC1: PAIRBP1 complex could potentially interfere with PGRMC1' ability to retain NCL within the nucleolus and account for the translocation of nucleolin from the nucleolus into the nucleoplasm under stress condition.

Finally, the present study reveals the relationship between PGRMC1 and NCL in bovine oocytes. PGRMC1 is present in the nucleolus of growing oocytes and the signal is retained to some extent in the nucleolar remnants of fully-grown bovine oocytes. During growth, the oocyte's nucleus is characterized by the presence of a diffuse filamentous transcriptionally active chromatin and by a functional fibrillogranular nucleolus, which is gradually disassembled forming the so called 'nucleolar remnants', along with the progressive inactivation of rRNA synthesis that occurs at the end of oocyte growth (Fair *et al.* 1996, Lodde *et al.* 2008). Ultrastructurally, the nucleolar

remnants appear as electron dense spheres often showing a semilunar fibrillar center-like structures attached (Fair *et al.* 1996, Lodde *et al.* 2008). In bovine oocytes, proteins such as RNA polymerase I and UBF remain associated to the inactive nucleolar remnants, while others, such as NCL and nucleophosmin mostly disperse in the nucleoplasm (Fair *et al.* 2001, Baran *et al.* 2004, Maddox-Hyttel *et al.* 2005). Upon meiotic resumption and during oocyte maturation the nucleolar remnant further disassembles and nucleolar proteins are probably dispersed in the cytoplasm. After fertilization the so called 'nucleolar precursor bodies' (NPBs) appear as electron dense compact spheres in the male and female pronuclei reviewed in (Maddox-Hyttel *et al.* 2005). The NPBs serve for the re-establishment of a functional fibrillogranular nucleolus, which in bovine occurs at the time of major embryonic genome activation (at the 8-16 cell stage). It has been proposed that proteins engaged in late rRNA processing of maternal origin, including NCL, are to some extent re-used for nucleologenesis in the embryo while others need to be de-novo transcribed before being incorporated in the nucleolus (reviewed in (Maddox-Hyttel *et al.* 2005)). In this scenario, PGRMC1 localization in growing and fully-grown oocytes and in the NPBs of bovine zygotes (Luciano *et al.* 2010) suggests a role in both the disassembly and the reassembly of the nucleolus during meiosis and early embryogenesis. Interestingly, in growing oocytes (as in bGC) PGRMC1 and NCL showed a different localization pattern, with PGRMC1 showing a dotted localization. A similar pattern in growing bovine oocytes has been reported for the RNA polymerase I-specific transcription initiation factor, Upstream Binding Factor (UBF) (Baran *et al.* 2004). In future studies, it will be important to assess whether a specific functional interaction between NCL or other nucleolar proteins and PGRMC1 exists during early embryonic development and thereby influences the embryogenesis.

7.6 Tables and Figures

7.6.1 Tables

Table 7.1: list of antibodies used

Cell type	Technique	Primary antibody	Secondary antibody
Bovine Granulosa Cells (bGC)	Immunofluorescence	- Rabbit polyclonal anti-**PGRMC1** (1:50; Protein tech, 12990-1-AP) - Rabbit polyclonal anti-**PGRMC1** (1:50; Sigma, HPA002877) - Mouse monoclonal anti-**nucleolin** (1:2000; Thermo scientific, MA1-20800)	- TRITC-labeled donkey anti-rabbit (1:100; Vector Laboratories, Inc.) - Alexa Fluor 488-labeled donkey anti-mouse (1:1000;Life Technologies)
Oocytes	Immunofluorescence	- Rabbit polyclonal anti-**PGRMC1** (1:200; Protein tech, 12990-1-AP) - Mouse monoclonal anti-**nucleolin** (1:2000; Thermo scientific, MA1-20800)	- TRITC-labeled donkey anti-rabbit (1:100; Vector Laboratories, Inc.) - Alexa Fluor 488-labeled donkey anti-mouse (1:1000;Life Technologies)
Bovine Granulosa Cells (bGC)	In situ proximity ligation assay (PLA)	- Rabbit polyclonal anti-**PGRMC1** (Protein tech, 12990-1-AP -1:50) - Mouse monoclonal anti-**nucleolin** (1:2000, Thermo scientific, MA1-20800)	Anti-rabbit PLUS and anti-mouse MINUS PLA probes (Duolink® In Situ PLA®)
Bovine Granulosa Cells (bGC)	Western blot	- Rabbit polyclonal anti-**PGRMC1** (1:200; Protein tech, 12990-1-AP) - Rabbit polyclonal anti-**PGRMC1** (1:50; Sigma, HPA002877) - Mouse monoclonal anti-**beta tubulin** (1:1000; Sigma, T8328)	- Goat anti rabbit IgG peroxidase conjugated (1:1000; Thermo scientific) - Goat anti mouse IgG peroxidase conjugated (1:1000; Thermo scientific)

7.6.2 Figures

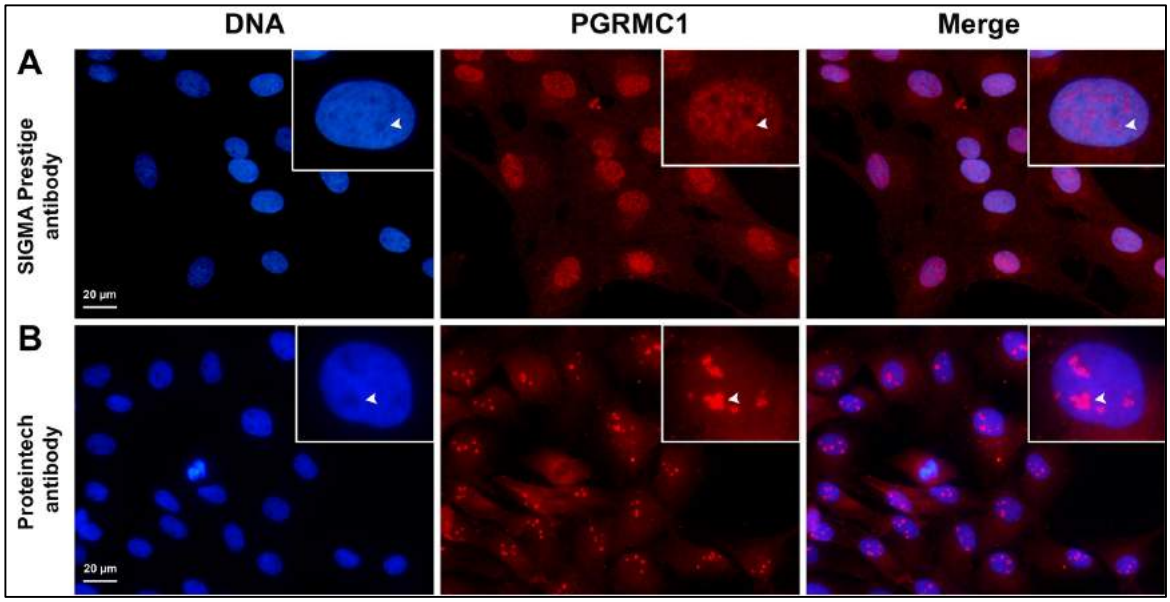

Figure 7.1: PGRMC1 immunofluorescent localization (red) in bGC obtained using SIGMA Prestige (A) and the Proteintech (B) rabbit polyclonal antibodies. DNA is stained with DAPI (blue). Insets show a single magnified nucleus. Note that both antibodies show intense staining in DAPI negative areas (arrows).

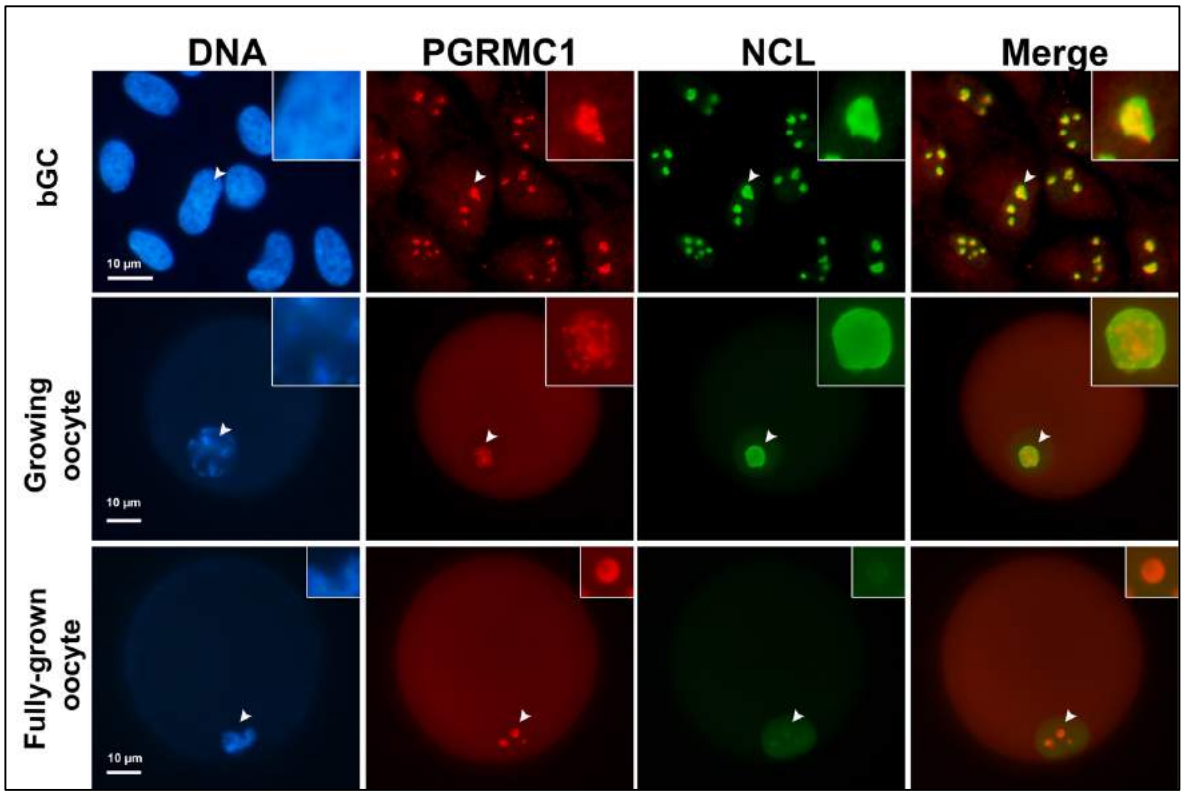

Figure 7.2: PGRMC1 (red) and NCL (green) immunofluorescence localization in bGC, growing oocytes and fully-grown oocytes. DNA is stained with DAPI (blue). Merged images shows partial PGRMC1-nucleolin co-localization (yellow). Insets represent 3X magnification.

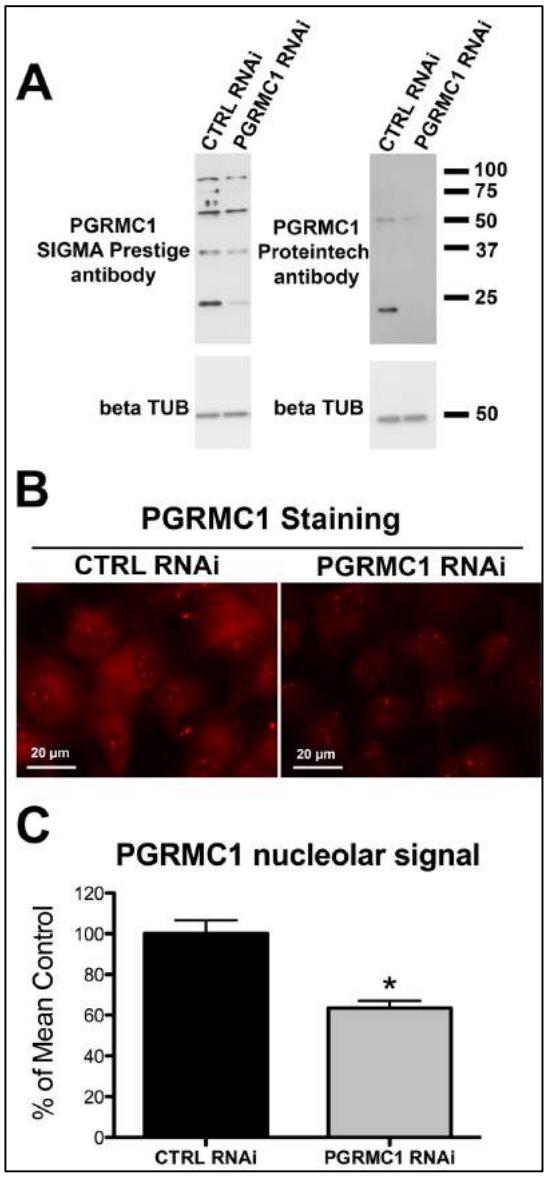

Figure 7.3: Effect of PGRMC1 RNAi mediated gene silencing on PGRMC1 expression. (A) Representative Western blotting analysis showing PGRMC1 protein levels in PGRMC1 and CTRL-RNAi treated bGC after 48 h of treatment using the SIGMA Prestige and the Proteintech rabbit polyclonal antibodies. Beta tubulin was used as loading control. (B) Representative images showing PGRMC1 immunofluorescent staining in PGRMC1 and CTRL-RNAi treated bGC. (C) Graph showing analysis of PGRMC1 immunofluorescence intensity in the nucleolus of PGRMC1 and CTRL-RNAi treated bGC after 48h of treatment; * indicates significant difference (t-test, $p<0.05$).

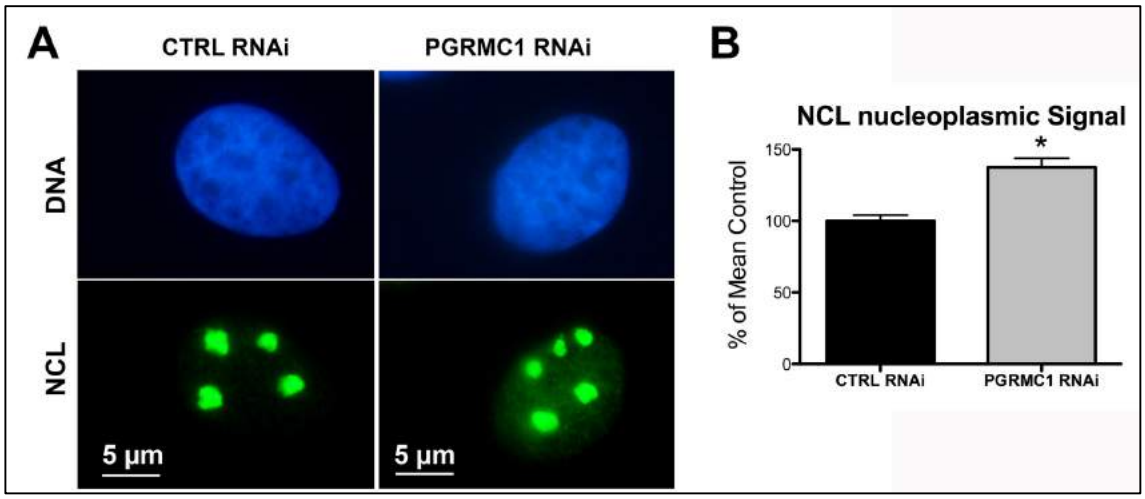

Figure 7.4: Effect of PGRMC1 RNAi mediated gene silencing on NCL localization. (A) Representative images showing NCL immunofluorescent staining in PGRMC1 and CTRL-RNAi treated bGC; note the increased nucleoplasmic signal in PGRMC1 RNAi treated cell. (C) Graph showing analysis of PGRMC1 immunofluorescence intensity in the nucleoplasm of PGRMC1 and CTRL-RNAi treated bGC after 48h of treatment; * indicates significant difference (t-test, $p<0.05$).

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8. PGRMC1 expression in canine mammary tumors: a preliminary study

Foreword

The preliminary data of this project were presented at the 37th National Congress of the Italian Society of Histochemistry - Taormina 22-23 Settembre 2017.

Terzaghi *L., Banco B., Groppetti D., Modina SC., Dall'Acqua PC., Giudice C., Pecile A., Grieco V., Luciano AM, Lodde V. Progesterone Receptor Membrane Component 1 (PGRMC1) expression in canine mammary tumors. European Journal of Histochemistry, 2017; Vol. 61, No 2s, 11-12.*

8.1 PGRMC1 and cancer: an overview

PGRMC1 is reported to be overexpressed in a wide range of tumors, compared to corresponding normal tissues. It was first discovered as a protein induced during dioxin-stimulated tumorigenesis [1] and is also part of a six-gene signature associated with nongenotoxic carcinogens [2]. Then it was found as upregulated, detected as protein or mRNA levels, in different tumors in humans including lung, thyroid, colon, sebaceous glands, oral, head and neck, bladder and reproductive system, such as ovary, cervix and breast cancers [3-10]. PGRMC1 was detected also in plasma [11] and serum [12] of patients with lung and renal cancer, respectively, in which its level was higher compared to healthy patients. Moreover, PGRMC1 levels correlate with tumor stage in ovarian cancer [13] and estrogen receptor status in human breast cancer [14, 15].

Knockdown experiments showed that PGRMC1 promotes tumor cell proliferation, resistance to apoptosis caused by chemotherapy, invasion and metastasis in different tumor cell lines [3, 13, 16]. Also in *in vivo,* mouse xenograft studies showed the role of this protein in increasing tumor growth, angiogenesis and metastasis [16-18].

PGRMC1 also regulates autophagy [19] and could be involved in a conserved hypoxic/anoxic response in human tumors, possibly involved in Warburg effect, since it is part of an expression profile associated with hypoxia in tumors [20] and its expression was induced in a hypoxic zone of comedo-type ductal carcinoma in situ (DCIS) [15]. This is likely a conserved function since also the yeast homologue, DAP1, is inducible by mevalonate pathway activation, leading to steroid synthesis, especially under hypoxic conditions [21].

The precise mechanism of action of PGRMC1 in tumor biology is still not clearly understood, and it could involve interaction with different proteins and diverse pathways. Recently, Kabe et al. demonstrated that heme-dependent PGRMC1 dimerization is essential for its link to EGFR that highly supports its role in cancer. Indeed, EGFR is a tyrosin kinase receptor that is fundamental in activating oncogenic signaling pathways in cancer cells. In particular, phosphorylation of EGFR in response to its ligand (EGF) and its downstream targets AKT and extracellular signal-regulated kinases (ERK) were reduced when PGRMC1 expression was depleted. What's more, their study showed PGRMC1 heme-mediated dimerization was essential in promoting

tumor proliferation of HCT116 colon cancer cells *in vitro* and their metastasis to liver *in vivo* [22].

Finally, Kabe group also demonstrated that in HCT116 colon cancer cell line PGRMC1 dimer promotes chemoresistance to erlotinib and doxorubicin, facilitating the degradation of the latter via its ability to bind CyP450s, in particular the CYP2D6 or CYP3A4, which facilitate doxorubicin metabolism [22]. This suggests that a similar mechanism of action could be involved also in PGRMC1 mediated chemoresistance to cisplatin previously described in triple negative breast cancer cell line, MDA-MB-231 [17] and ovarian cancer cells [13].

Some studies also suggested PGRMC1 as a possible marker of breast cancer risk in women whose breast epithelium is over-expressing this protein, which is important for mediating the strong effect on cell proliferation of certain synthetic progestogens combined with estrogens in this cancer therapy [9, 23] (**Figure 8.1**).

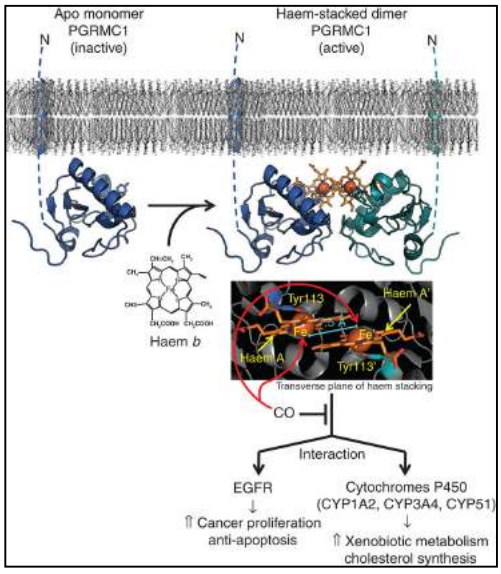

Figure 8.1 *Schematic diagram showing PGRMC1 heme-dependent dimer formation and its role for its interaction with EGFR and cytochromes P450 leading to cell proliferation and chemoresistance in cancer cells. From [22].*

Still there is a lot to investigate on PGRMC1 involvement in cancer to confirm its role and its potential usefulness as diagnostic marker and/or target for therapy.

8.2 Introduction and aim of the study

Given recent studies on PGRMC1 role in cancer as a tumor biomarker and its role in cellular division as main theme of this thesis, we are interested to study its putative role in veterinary oncology. To our knowledge, in veterinary oncology PGRMC1 is only described in cattle bladder cancer, in which the protein is overexpressed in neoplastic tissue and in blood of tumor-bearing animals compared to healthy ones [10].

Therefore, the aim of this preliminary study is to investigate on PGRMC1 expression in canine normal mammary gland and in mammary tumors. The study includes an immunohistochemical analysis of PGRMC1 expression and localization and a further evaluation of different PGRMC1 isoforms using Western blot in healthy and tumoral canine mammary tissue.

Canine mammary tumors (CMT) are the most frequent neoplasm in female dogs [24, 25]. Risk factors include genetics, obesity, exposure to hormones and age (middle aged and older animals) [24].

Histopathological examination is the gold standard for CMT diagnosis and no molecular biomarkers are currently routinely in use. Canine mammary tumors are characterized by a varied morphology, forming simple (only one cell type resembling epithelial luminal or myoepithelial cells), complex (malignant epithelial and benign myoepithelial components), mixed (malignant epithelial component and myoepithelial component together with benign mesenchymal component such as osseous/cartilaginous/adipose tissue), and mesenchymal tumors. Carcinomas are the most common malignant CMTs, together with benign mixed tumors [26, 27].

Clinical prognostic factors found to be most consistently associated with outcome include tumor size, lymph node status, and clinical stage, together with histological invasion and grade (of carcinomas) [24]. Therapy is mainly surgical through mastectomy [24].

Hence finding a good biomarker could be useful for better tumor typing and enhancing precise classification of CMT for a more accurate subsequent prognosis and therapy.

8.3 Material and methods

8.3.1 Sample selection and collection

No animals were killed for this study. All samples were originally submitted for diagnostic purpose to the Diagnostic Service of Veterinary Anatomical Pathology, Department of Veterinary Medicine, University of Milan.

For the immunohistochemical analysis, samples of CMT were retrospectively selected from the department archive. The tumor subtypes and number of samples selected are summarized in **Table 8.1**. The normal/hyperplastic tissue surrounding the tumor was considered as normal tissue for every sample submitted to immunohistochemistry.

For the western blot analysis, fresh tissue biopsies were obtained from dogs undergoing mastectomy for the presence of CMT at the Department of Obstetrics and Gynecology - University of Milan. Tissue biopsies were taken before placing the mammary glands in formalin with a punch (3mm X 7mm, kai medical) and immediately processed (see paragraph 8.3.3). Since surgical therapy consists of unilateral mastectomy with the removal of the entire mammary chain, for these cases we obtained also biopsies of normal tissue from the first or second thoracic mammary glands, free from neoplastic nodules. These further cases were also included for the immunohistochemical evaluation of PGRMC1 expression.

A complete clinical record is available for all cases including histological diagnosis established by board-certified pathologists.

8.3.2 Immunohistochemistry analysis

Immunohistochemical analysis was performed on formalin-fixed and paraffin-embedded samples. Endogenous peroxidase activity was eliminated by incubation with 3% H2O2 in methanol for 45 min. Then sections were incubated with 10% normal goat serum, 0.3% Triton X-100 and 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 min to block non-specific binding of secondary antibody. Samples were then incubated overnight at 4°C with rabbit polyclonal anti-PGRMC1 primary antibody (SIGMA, cat. HPA002877, dilution 1:200).

Primary antibodies were detected by using a biotinylated anti-rabbit IgG (Vector Laboratories cat. BA-1000) diluted 1:400 in PBS with 1% of BSA and detected with the Vectastain Elite ABC kit (Vectastain Elite ABC kit, Vector Laboratories). Finally, samples were incubated for 2 minutes with DAB substrate (DAB substrate kit for peroxidase, Vector Laboratories) for color development and then counterstained with Mayer's haematoxylin for nuclear staining. Bovine ovarian tissue was included as internal positive control, while negative controls were performed omitting the primary antibody.

Protein labeling was evaluated independently by 3 veterinarians and classified by a scoring including the percentage area of the tumor expressing PGRMC1 and the intensity of the staining as weak (A), moderate (B) and strong (C).

8.3.3 Western blot analysis

Western blot analysis was performed as previously described [28]. Briefly, punch biopsies of tumor tissue and normal/hyperplastic tissue, taken from the thoracic mammary glands not affected by neoplasia, were obtained after surgery. Tissue was immediately homogenized and lysed in radioimmunoprecipitation assay (RIPA) buffer. Extracted proteins were quantified with Qubit™ Protein Assay Kit and Qubit™ fluorometer (Thermo Fisher Scientific). A total of 50 µg protein/lane for each sample were loaded and equal protein loading was verified by Ponceau staining. PGRMC1 immunodetection was performed using rabbit polyclonal anti-PGRMC1 antibody (SIGMA, cat. HPA002877, dilution 1:500) or mouse anti-pan Cytokeratin AE1/AE3 antibody (Novusbio, cat. NBP2-29429, dilution 1: 400) overnight at 4°C. PGRMC1 and cytokeratins were revealed using a goat anti rabbit or mouse IgG peroxidase conjugated antibody antibodies (Thermo scientific, cat.32460-32430, dilution 1:1000) and detected using Clarity Western ECL substrate (BIORAD, cat. 170-5060). Negative controls were included omitting primary antibodies.

Relative amount of protein was quantified on scanned films using Image J software. Since PGRMC1 was expressed only in epithelial cells of mammary tissues we evaluated its levels relatively to the quantity of epithelial tissue in each sample. Hence, the intensity of all PGRMC1 bands was normalized for the intensity of the corresponding cytokeratins bands.

8.3 Results

8.3.1 PGRMC1 expression and localization in canine mammary tissue and tumors

Canine normal mammary gland and hyperplastic areas, both surrounding the tumors and from tumor-free mammary glands, were consistently positive to PGRMC1. The protein was expressed in all epithelial cells of ducts and alveoli with a very strong (C) intensity of the staining (**Figure 8.2 A**).

In simple adenomas 80-100% of the tumor expressed PGRMC1 in the epithelial cells with a moderate-high intensity (B-C) (**Figure 8.2 B**). The same expression pattern was observed in complex adenomas and in some samples also mioepithelial cells were positive to PGRMC1 with a moderate intensity (B) (**Figure 8.2 E**)

In simple carcinomas, the expression pattern was the most heterogeneous; up to 60% of the tubulopapillary part of the tumor expressed PGRMC1 with a moderate to weak intensity (B-A). In solid parts of the tumor PGRMC1 was expressed only in 20-30% of the area and the signal is weak (A), while other areas were negative for the protein (**Figure 8.2 C-D**). The same pattern was observed in the complex/mixed carcinomas samples (**Figure 8.2 E**).

The PGRMC1 expression patterns in canine mammary gland and CMTs are summarized in **Table 8.2.**

8.3.2 PGRMC1 isoforms expression levels

Western blot analysis confirmed PGRMC1 presence both in normal and in neoplastic tissue. PGRMC1 monomer (25 kDa) was present in all samples, while its dimer was mainly expressed in normal/hyperplastic samples. An additional 37 kD band appears in only two tumoral tissues. The relative quantification of PGRMC1 presence relatively to epithelial cells, marked with a pan-cytokeratins antibody, revealed 3/5 samples PGRMC1 expression as higher in normal tissue compared to neoplastic tissue, while 2/5 PGRMC1 expression was higher in the tumor (**Figure 8.3**). This could be explained, at least for tumoral sample n. 4, for the presence of epitheliosis foci. Indeed, epitheliosis is an intraductal hyperplasia, considered as a pre-neoplastic

lesion, in which PGRMC1 was expressed with very high intensity (C), so this could have determined the discordant result (**Figure 8.4**).

8.4 Discussion

To the best of our knowledge our data showed for the first time PGRMC1 expression pattern in canine normal mammary glands and in neoplastic mammary tissue. PGRMC1 expression is high in normal mammary gland and in adenoma, while it decreases in carcinoma neoplastic tissue, being less expressed in less differentiated parts of the tumor. This pattern mirrors the oestrogen α and progesterone receptors expression in CMT, which decrease with tumor malignancy [29].

PGRMC1 presence and levels of expression were in part confirmed by western blot analysis. Recently, Kabe et al. described the importance of PGRMC1 dimer for PGRMC1 role in cancer, in particular in cell proliferation and chemoresistance, so the presence of the dimer band in canine normal tissue is controversial [22]. Nonetheless, normal/hyperplastic tissue could be actively proliferating hence this could explain the predominant presence of PGRMC1 dimer here rather than in tumoral tissue.

This preliminary study prompted us to continue investigate on PGRMC1 role in CMT including more tumor subtypes and increasing the number of cases. Moreover, it would be interesting to evaluate PGRMC1 specific subcellular localization with different techniques since, according to current hypothesis, PGRMC1 function could vary according to its localization in different systems. We should assess if there is any difference in PGRMC1 expression pattern consequent to differences in the hormonal mammary background in intact or ovariectomised dogs.

Finally, canine spontaneously occurring tumors are used as a model for different types of human tumors concerning study on tumor biology, diagnosis and development of new therapies [30, 31]. In particular, in human breast cancer (BC) there is limited literature on PGRMC1 immunohistochemical pattern of expression. In studies using the same antibody of ours [32], PGRMC1 is expressed by epithelial cells and in normal mammary gland it was moderately expressed, while in ductal carcinoma (the only subtype considered) its expression is moderate to strong (*http://www.proteinatlas.org)*. This apparent difference between BC and CMT could be due to several factors; for example, different tumor subtypes considered, a complete different endocrinological background and single patient differences.

Hence, the importance to study precise tumor subtypes that could be correctly compared is essential to draw any conclusion together with considering the wide individual variations.

8.5 Figures and tables

8.5.1 Figures

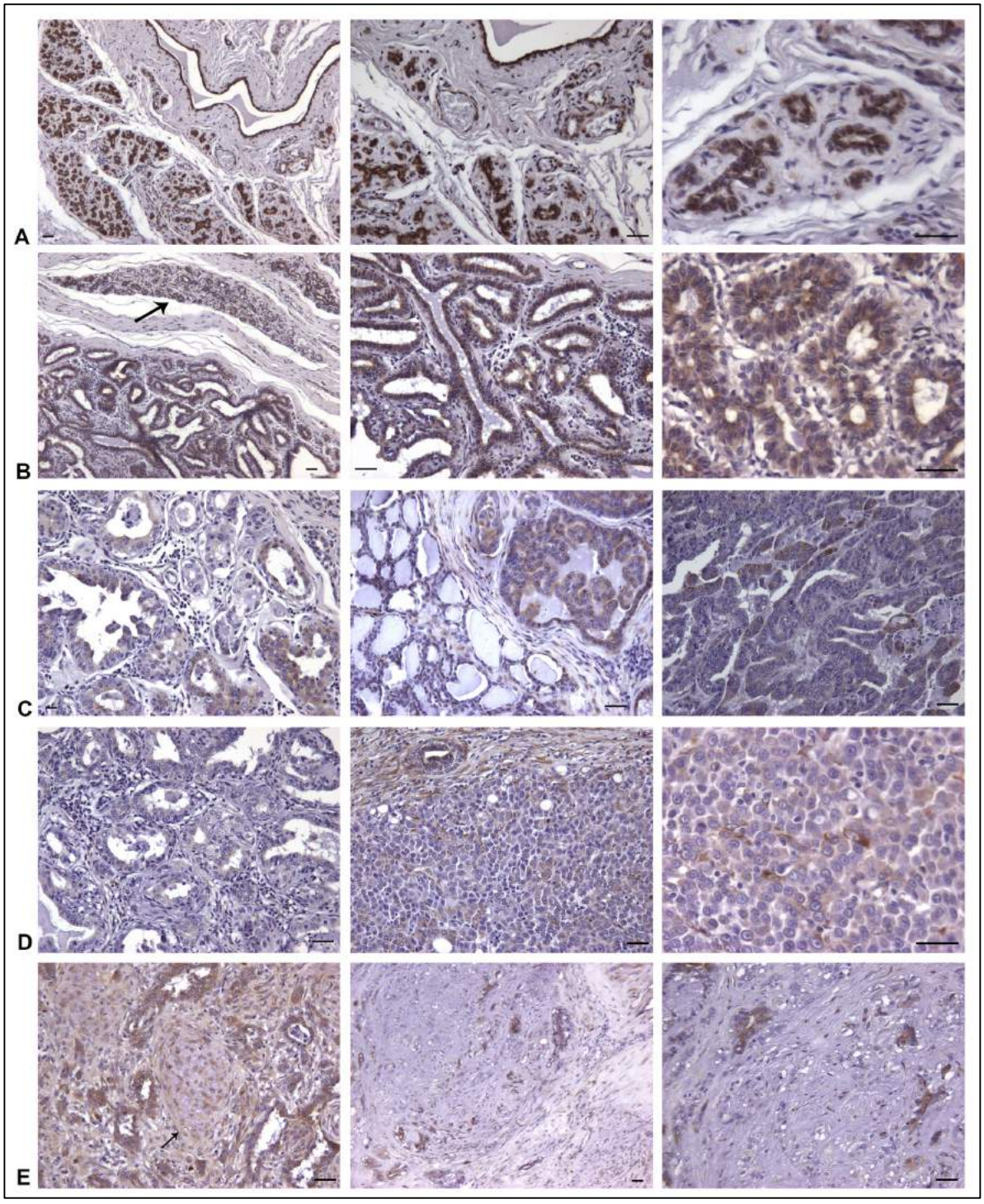

Figure 8.2 Representative images of PGRMC1 expression in canine mammary tumors. Scale bars are 50 µm.
A. Normal mammary gland tissue. B. Simple Adenoma and normal surrounding tissue (arrow)
C. Simple carcinoma, tubulopapillary part D. Simple carcinoma, solid part
E. First image shows a complex adenoma, with also mioepithelial cells positive to PGRMC1 (arrow). The other two images represent a complex carcinoma.

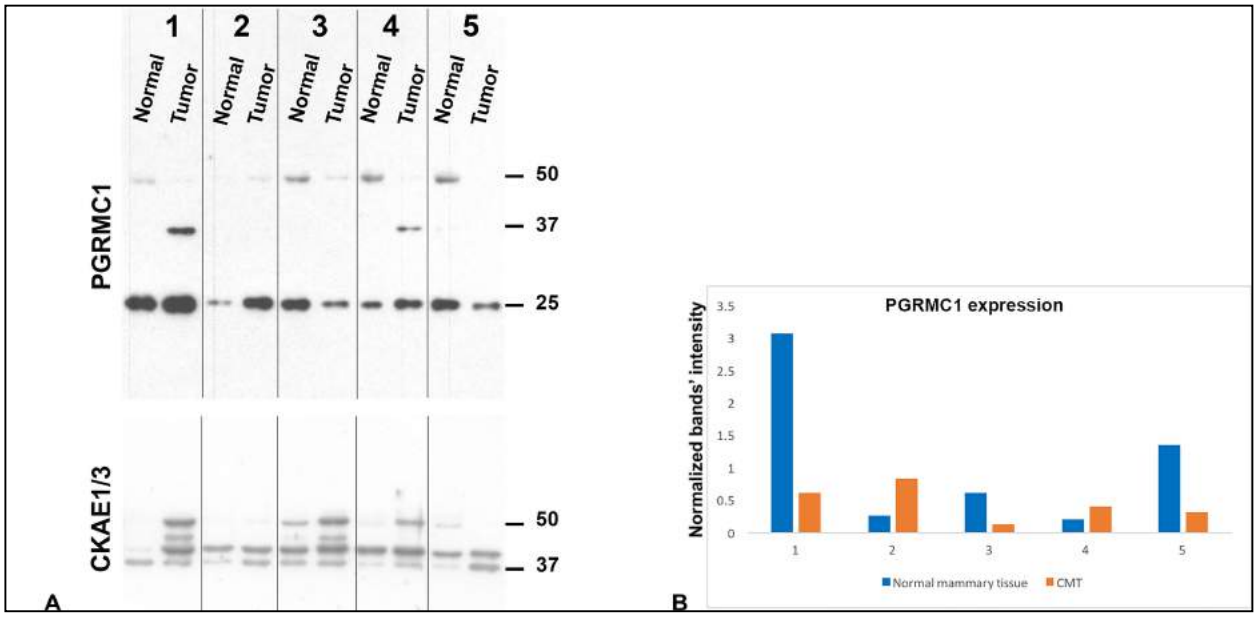

Figure 8.3
A. Western blot analysis showing levels of PGRMC1 and CKAE1/AE3 in biopsies of normal and relative tumoral tissue.
B. Graph showing PGRMC1 levels normalized to CKAE1/AE3 expression for each case. Tumor types details:
Case 1. Complex carcinoma in mixed tumor.
Case 2. Simple cribriform carcinoma
Case 3. Carcinoma in mixed tumor
Case 4. Simple solid carcinoma, cribriform, with epitheliosis
Case 5. Tubular carcinoma, in part complex

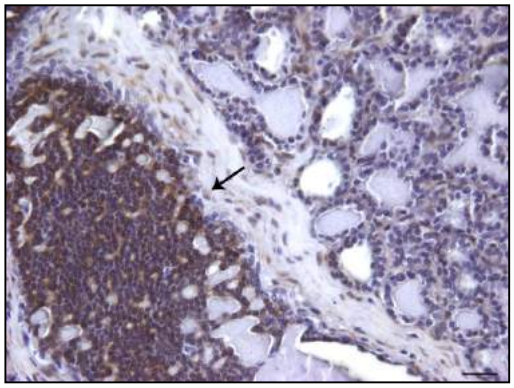

Figure 8.4 Example of epitheliosis foci (arrow) in sample 4 tumor. Scale bar is 50 µm.

8.5.2 Tables

Table 8.1 *Canine Mammary Tumor cases selected for immunohistochemical analysis for PGRMC1 expression.*

Tumor subtypes	Number of samples
Simple Adenoma	7
Complex Adenoma	5
Simple Carcinoma	9
Complex Carcinoma	5

Table 8.2 *Summary of PGRMC1 expression and corresponding scoring attributed for each tumor subtypes evaluated in the immunohistochemical study.*

Tumor subtype	Percentage of PGRMC1 expression	Intensity of PGRMC1 staining
Normal/hyperplastic mammary tissue	100%	C
Simple adenoma/Complex adenoma	80-100%	B-C
Simple carcinoma/Complex carcinoma	Tubulopapillary: up to 60% Solid: 20-30%	Tubulopapillary: A-B Solid: A to Negative staining

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9. General discussion and perspectives

During the course of this 3 years PhD program we have deepened previous studies conducted at the Reproductive and Developmental Biology Laboratory that consider PGRMC1 as one of the key factors involved in oocyte quality and follicular development, which are main systems involved in a successful reproductive outcome.

Among the multiple steps needed to reach the production of a viable embryo and consequent offspring, folliculogenesis and the production of a good quality female gamete is one of the crucial point, during which several defects that might occur could consequently affect reproductive efficiency. As described in the introduction chapter, nowadays infertility is a major issue in dairy cows that needs to be quickly solved as milk yield and production demand is constantly increasing and it is a major cause of economical loss besides impacting also on animal welfare.

Therefore, studying PGRMC1 role in bovine follicular system, according to its different subcellular localization, would deepen our knowledge on mechanisms that could impact mammalian follicle development and, subsequently, mammalian fertility.

Our studies give new insights into some aspects of the versatile PGRMC1 protein in reproductive field.

Disrupting PGRMC1 function through gene silencing by RNAi or pharmacological inhibition lead to failure in both somatic cells and oocyte division, indicating PGRMC1 is required for this process given its localization at the mid-zone and mid-body of meiotic and mitotic spindles. Especially, our results showed the impairment of later stages of cell division impeding the correct completion of cytokinesis in somatic cells and polar body extrusion and metaphase II plate proper formation in the oocyte. These findings indicate for the first time the precise stage of cell division on which PGRMC1 might exerts its role. Moreover, our findings suggest a possible mechanism of action involving PGRMC1 interaction with AURKB in both systems, paving the way to perform more studies on the precise action of this protein in this particular subcellular site.

Moreover, we also addressed the hypothesis of PGRMC1 as a mediator of P4 action comparing PGRMC1 and nPGR role in meiotic division. We demonstrated that inhibiting nPGR through Aglepristone and RU486 altered the progression of meiotic division affecting the proper spindle formation. Yet, the effects on the spindle are not

dramatic as what happens disrupting PGRMC1 function. This suggests that the two receptors might be involved in different stages of maturation and developmental competence of bovine oocyte mediating different P4 actions.

Since PGRMC1 function might depend on the specific subcellular domain in which it localizes, we gave our contribution showing its localization and possible role in the nucleolus of bovine granulosa cells and in the oocyte, which has been poorly investigated up to now. In granulosa cells PGRMC1 might have a functional relationship with nucleolin since our preliminary experiments showed their nucleolar colocalization and depleting PGRMC1 determines nucleolin shift into the nucleoplasm. This function is probably mediated by other proteins since the two proteins seem not to directly interact. While in the oocyte our localization studies suggested PGRMC1 putative role in disassembly and reassembly of the nucleolus during meiosis and early embriogenesis, respectively.

Overall, our studies on bovine granulosa cells and oocyte could be relevant for further understanding PGRMC1 role as a key molecule in folliculogenesis and reproductive function.

Practically, the results of our studies could improve assisted reproductive technologies widely used in livestock; for example, through the identification of markers suitable to predict oocyte competence and its ability to give rise to healthy offspring or more basically to achieve a deeper insight in reproductive physiology. Moreover, PGRMC1 is expressed also in human oocyte and ovarian cells and changes in its expression reflect impaired ovarian function. Indeed, in some women with polycystic ovarian syndrome [232] or premature ovarian failure [232, 233] reduced PGRMC1 levels are present, while PGRMC1 overexpression was found in infertile patients undergoing gonadotropin-induced ovulation and in vitro fertilization [234]. Hence, the knowledge deriving from our studies could set the stage for further investigations also in human infertility and contribute to improve assisted reproductive techniques as well, helping in choosing the best gametes and conditions to gain the highest fertility rate with the lowest discomfort for the patient.

Finally, PGRMC1 role in granulosa cells division could be applied in a wider view of PGRMC1 function in somatic cells proliferation of other systems, in particular in the presence of diseases such as cancer. Indeed, oncology is another field that might benefit from these studies. Given its overexpression in many types of cancer and its emerging role in tumor proliferation and chemoresistance, PGRMC1 is more and

more studied as a putative biomarker and therapy target. So, besides PGRMC1 role in cell division in granulosa cell line, starting from our additional preliminary studies on PGRMC1 showing its presence and putative role in canine mammary tumors might unravel intriguing information on this very wide and contemporary topic.

We can conclude that our studies contribute to widening the notions about the complex understanding of the puzzling and intriguing PGRMC1 protein.

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11. Appendix: published full papers, list of publications and communications

Full papers

Terzaghi L., Luciano AM, Chediek Dall'Acqua P, Modina SC, Peluso JJ & Lodde V. PGRMC1 localization and putative function in the nucleolus of bovine granulosa cells and oocytes *Reproduction* 2018;**155 :** 1–10

Tessaro I., Modina S.C, Lodde V, Sivelli G., Franciosi F., **Terzaghi L.**, Luchini P., Rumio C., Luciano AM. Ultra-low doses of follicle stimulating hormone and progesterone attenuate the severity of polycystic ovary syndrome features in a hyperandrogenized mouse model. J Reprod Infertil. 2017;18(3):288-297

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Terzaghi L., Banco B., Groppetti D., Dall'Acqua PC., Giudice C., Pecile A., Grieco V., Luciano AM. & Lodde V.
Progesterone Receptor Membrane Component 1 (PGRMC1) expression in canine mammary tumors: a preliminary study.
(Manuscript in preparation)

Communications/Abstracts

2015

January 16th 2015 - Seminar in a welcome meeting in Milan for Prof. José Buratini Junior, visiting Professor from University of Sao Paulo UNESP (Botucatu Campus), Brazil – **Oral communication**: "Role of Progesterone Receptor Membrane Component 1 (PGRMC1) in mitosis".

Terzaghi L., Luciano AM., Zuccotti M., Merico V., Garagna S., Modina SC., Lodde V. Progesterone Receptor Membrane Component-1 (PGRMC1) plays a role in bovine granulosa cells mitosis. In: Proceedings of 36°congresso della Società Italiana di Istochimica - 61° convegno Gruppo Embriologico Italiano (Pisa), 7-10 June 2015. European Journal of Histochemistry Eur J Histochem; 2015: 21-21

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