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MOLECULAR DETERMINANTS OF MAMMALIAN FERTILITY: ROLE OF PROGESTERONE RECEPTOR MEMBRANE COMPONENT 1 (PGRMC1)

(SSDVET01)

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1. OUTLINE

Declining fertility is a major obstacle in maintaining profitability of dairy farms [1-3]. One likely cause of the reduced fertility is that over the past 30 years breeding schemes have almost solely been based on selecting traits for high milk production. This has likely resulted in alterations in a large number of genes that adversely affect fertility [2, 3]. For this reason there is an urgent need to identify the genes that are responsible for the reduced fertility in diary cattle. This would immediately lead to advancement in the management practices in breeding programs and could improve the reproductive performance of high milk-producing dairy cows.

Progesterone receptor membrane component 1 (PGRMC1) belongs to the membrane-associated progesterone receptor (MAPR) proteins family involved in regulating numerous biological functions [4]. PGRMC1 is mainly localized in intracellular membranes and colocalizes with the endoplasmic reticulum and Golgi apparatus in rat liver cells and hippocampal Pukinje cells. However, several studies described a nuclear or chromosomal localization. Several reports suggest the participation PGRMC1 in progesterone signaling in the reproductive system in different mammalian species. PGRMC1 has been detected in nuclear extracts of HeLa cells among the protein of the human mitotic spindles, in oocyte nucleus and recently has been proposed in regulating microtubule stability in human and rat ovarian cell mitosis [5]. Interestingly, deletion and point mutations of PGRMC1 have been associated with human premature ovarian failure (POF) [6]. Moreover, defects in spindle formation and/or function can generate chromosome instability and aneuploidy, a condition that is the major cause of defective early embryonic development, miscarriages and birth defects [7]. In humans, the frequency of aneuploidy

increases with female age, with the decline of ovarian function, the gradual depletion of ovarian follicle reserve and the reduced ability to produce oocytes competent for fertilization and embryo development [8]. However, this occurs also in young infertile and subfertile women affected by POF [9, 10].

Because of the possible relevance in reproductive function, the present study was undertaken in order to clarify the role of PGRMC1 in bovine reproductive system and ovarian function. Finally, since we recently demonstrated that about 5% of individuals in the current population of culled dairy cows 4-8 years old is affected by a significant reduction of follicles population [11] this study analyzed the possible involvement of PGRMC1 in cow infertility and in reproduction in mammals.

2. INTRODUCTION AND HISTORY OF PGRMC1 AND SUB-CELLULAR LOCALIZATION

Progesterone receptor membrane component 1 (PGRMC1) belongs to the so-called membrane-associated progesterone receptor (MAPR) protein family that is widespread in eukaryotes [4]. The protein now termed PGRMC1 was cloned in 1996 as a membrane progesterone receptor from a porcine cDNA library by Falkenstein et al. after the N-terminal amino acid sequence had been determined [12]. In the same year, Selmin et al. reported the DNA sequence of a gene whose expression was induced in rat liver upon treatment with 2,3,7,8-tetrachloro dibenzodioxine (TCDD) and termed *25-Dx* [13]. The human homolog (Hpr6) of porcine membrane progesterone receptor was identified in 1998, and the gene was localized to the X chromosome [14]. PGRMC1 was recognized as an immunologically defined antigen that had been named inner zone antigen (IZA), localised in the zona fasciculata and reticularis of the adrenal cortex [15]. PGRMC1 expression had been observed also during embryogenesis of the mouse spinal cord and optic chiasm, suggesting an important role in regulating axon guidance at the midline of the developing embryonic central nervous system [16]. In 2004 the same authors identified a lower eukaryotic PGRMC1 homolog VEM-1 in Caenorhabditis elegans nematode. By a transgenic null mutation it was demonstrated that VEM-1 was involved in neuron guidance and axon formation in the nematode ventral midline, which was the context that had led to the identification of mammalian previous study [17]. In unicellular eukaryotes as Saccharomices cerevisae yeast, PGRMC1 homolog, named Dap1 (damage-associated protein), has been found to interact with cytochrome P450 proteins to synthesize sterols and protect cells from DNA damage [18, 19]. In conclusion phylogenetic tree construction based upon similarity of sequences of PGRMC1-related members of the MAPR family shows a wide eukaryotic distribution [4].

PGRMC1 was first purified from rat liver (microsomal) membrane fractions [20], and was

shown to cofractionate with the endoplasmic reticulum in liver extracts [21]. Perinuclear localization, consistent with endoplasmic reticulum, was observed in MCF-7 cells [22]. Two more reports suggest a nuclear or chromosomal localisation for PGRMC1. Beausoleil et al. detected phosphopeptides from PGRMC1 in nuclear extracts of HeLa cells [23], and Sauer et al. detected phosphorylated PGRMC1 in extracts of human mitotic spindles [24].

Raza et al. [15] observed a 28 kDa isoform in microsomal and mitochondrial fractions, whereas the 58 kDa form was observed in the cytosolic fraction of rat adrenal inner zones. In 2005 Min et al. observed that rIZA1 was distributed diffusely in the cell, forming vesicular structures suggesting its association with the membrane compartments, and colocalising with cytochrome *b*5 of the endoplasmic reticulum in HeLa cells [25]. Immune histochemistry studies on neonatal hippocampal Pukinje cells also suggested an endoplasmic reticulum and Golgi apparatus distribution [26].

At least some fractions of the PGRMC1 in spontaneously immortalized granulosa cells (SIGCs) were available for biotinylation on the surface of non-permeabilised cells [27, 28]. PGRMC1 was also one of the proteins that was detected after the biotinylation of extracellular proteins in various cancer cell lines by Shin et al. in 2003, in a study that revealed the presence of various topologically ‘cytoplasmic’ proteins in the extracellular compartment [29]. It is worthy of mention here that the distantly related Neudesin MAPR family member was identified as an extracellular mediator of neurotropic (anti neuro-apoptotic) responses, including the regulation of proliferation and differentiation decisions in the central nervous system [30, 31], suggesting that the cytochrome *b*5 domain of PGRMC1 could also induce specific extracellular responses if externalized, perhaps by mediating intercellular steroid transport. Sperm seem also to possess PGRMC1 on the cell surface because an antibody to the N-terminal region could specifically block the progesterone-driven acrosome reaction that accompanies sperm fusion with the egg upon fertilisation [32, 33]. However, it is unclear from the above studies whether this

extracellular PGRMC1 component constitutes a major or minor fraction of the total PGRMC1 localised to those respective cells.

Progesterone treatment of spinal chord-injured rats led to increased PGRMC1 expression, and the protein was immunolocalized on the cell membranes of dorsal horn and central canal neurons [34]. Bramley et al. reported co-precipitation of PGRMC1 with an antisera to caveolin [35], and this was dependent upon the use of the cholesterol-chelating reagent digitonin (see section on Potential Ligands). Failure to detect PGRMC1 immunofluorescence except in permeabilized cells in the developing CNS of rodent embryos [16] suggested that: “PGRMC1 is associated exclusively with intracellular membranes or that it only transiently resides at the cell surface before being cleared, possibly, via endocytosis” [36]. Munton et al. observed a fifteen fold higher relative abundance of PGRMC1 in a murine brain subcellular post-synaptic density fraction than from synaptic membranes. Intriguingly, tyrosine-phosphorylated PGRMC1 was only detected in the synaptosomal preparation rather than in preparations of synaptic vesicles or postsynaptic density [37].

The HC5 protein, corresponding essentially to the truncated N-terminus of PGRMC1 including the transmembrane domain, nevertheless, does not seem to be located at the membrane level because an antibody generated to this region, detected a 10 kDa protein in liver cytosolic but not microsomal fractions, as mentioned above. The anti HC5 antibody and IZA monoclonal antibody, recognized 28 and 25 kDa proteins in microsomal fractions, yet only a 25 kDa protein in cytosolic fractions, suggesting that PGRMC1 may alternate between membrane-bound and cytosolic forms [38].

In their 2004 review, Min et al. suggest that PGRMC1 “might form multimers via a reducing reagent resistant bond, and the change from monomer to multimer might result in changes in intracellular distribution of these molecules and also of their functions” [39]. Therefore it is probably prudent to assume that PGRMC1 can alter its sub-cellular location

under certain circumstances, which are as yet poorly understood, and that this redistribution could be important for PGRMC1-directed biological processes. Indeed it is difficult to avoid the conclusion that the key to unraveling the role of PGRMC1 lies in understanding the regulation of its function in various sub-and extra-cellular compartments. It also remains unclear whether only the N-terminus up to the transmembrane domain can be extracellular and available for biotinylation, or whether the entire protein may be translocated to the extracellular space.

3. PGRMC1: FUNCTIONS AND POTENTIAL LIGANDS

PGRMC1 has been implicated in the binding of several ligands, all of which could yet prove to be of biological relevance.

3.1 PGRMC1 and Progesterone/steroids

PGRMC1 was initially cloned in search of membrane receptors for progesterone that were distinct from the classical hormone activated PR transcription factor. Meyer et al. [20] showed that swine liver microsomes have a high affinity (k_d = 11 nM) and a low affinity (k_d= 286 nM) binding site for progesterone. In a 2006 paper these authors referred to this as the approximate k_d of PGRMC1 for progesterone, citing the data from the 1996 paper: "Interestingly the k_d for PGRMC1 is in the 0.2 to 0.3 μM range" [28], however the actual molecular identity of the progesterone receptor in microsomes was not formally proven. The major binding activity was purified, and the fraction with most activity contained the 28 and 56 kDa proteins that have already been described above, and which led to the cloning of porcine and later human PGRMC1. However there were undoubtedly many other additional proteins in this fraction. An antibody against the N-terminal peptide recognized

28 and 56 kDa bands that could be competed by that peptide, in a protein complex that was approximately 200 kDa as estimated by size exclusion chromatography [40]. Falkenstein et al., from the Mannheim group, showed in 1999 [33] and in 2001 [41] that pig liver microsomes bind progesterone as a radio-ligand, and that the binding form consisted of a higher order complex containing at least two molecules of the 28 kDa PGRMC1 monomer. When excess PGRMC1 was introduced by transfecting an expression plasmid, the progesterone binding activity of the microsomal fractions increased [33]. Yet it is possible that PGRMC1 caused the induction or permissive modification of another progesterone binding protein. Using selective chemical modification of amino acids, they concluded that progesterone binding required carboxyl, methionine and tryptophan groups [41]. However possible allosteric effects on protein structure by these modifications were not adequately controlled, which could mean that none of those amino acids actually contacts a bound progesterone ligand. Furthermore, those authors only demonstrated a correlation between chemical treatment of microsomes and reduced levels of progesterone binding of microsomes, a result that might be expected regardless of the identity of the receptor. They did not actually demonstrate that the receptor was PGRMC1, which they correctly acknowledged in their discussion.

Ovarian surface epithelial cells express PGRMC1 and respond to progesterone at doses consistent with a low affinity receptor characterized in liver microsomes [28]. Yet overexpression of PGRMC1 increased progesterone binding and the responsiveness of SIGCs to progesterone's anti-apoptotic actions. Although an antibody directed against the extracellular domain of PGRMC1 blocked progesterone's capacity to inhibit apoptosis, it remains to be demonstrated that the actual receptor of progesterone is not another protein such as PAIRBP1, which is co-expressed with PGRMC1 in those cells, and which has been observed in a protein complex with PGRMC1. Indeed, this is likely.

In 2006 Viero et al. suggested that PGRMC1 may mediate the response to neurosteroids in murine embryonic sensory neurons, however the only evidence was the demonstration

by PCR that PGRMC1 was expressed in the responsive cells [42]. This was consistent with the prior work of Sakamoto et al., who observed in 2004 that PGRMC1 is expressed in neonatal rat Purkinje cells at precisely the time when they synthesise progesterone from cholesterol, and when they respond to that steroid. A variety of previous evidence suggested that progesterone acts on Purkinje cells via membrane-associated progesterone-binding proteins to promote neonatal dendritic growth, spinogenesis and synaptogenesis. Those authors furthermore conclude that: “mifepristone (RU486) protects Purkinje cells from developmental cell death (...). Because this protective effect of RU486 is considered to be independent on the activation of nuclear PRs [i.e., transcription factor PR] (...), 25-Dx-mediated [PGRMC1] mechanisms might be involved in the protection of Purkinje cells.” [26].

It must be noted that none of the studies cited here actually demonstrated progesterone binding to PGRMC1, but rather to microsome membranes containing PGRMC1 and multiple other proteins, or to progesterone-responsive tissues expressing PGRMC1. Biologically relevant binding of progesterone to PGRMC1 still remains to be demonstrated under conditions where PGRMC1 might not be complexed with or otherwise influence PAIRBP1, leaving open the possibility that PAIRBP1 or another protein(s) provides the actual binding activity. Indeed, in 2005 Ghosh et al. [43] could not detect specific progesterone binding to purified recombinant PGRMC1, however they did detect heme-binding under the same conditions. Similarly, Min et al. [25] also failed to observe specific progesterone binding to a bacterially expressed purified GST-PGRMC1 (GST-hIZA1) fusion protein that reversibly bound heme with modest affinity. Thus, the situation has essentially remained unchanged since the original cloning of Hpr6 in 1998, where Gerdes et al. stated that: “These correlations provide evidence that the progesterone binding sites and the Hpr6.6 proteins are identical or at least localised within the same complex” [14].

3.2 PGRMC1 and cholesterol synthesis

The cholesterol synthetic pathway is an important target for treating cardiovascular disease and inhibiting fungal infections. PGRMC1 likely regulates cholesterol synthesis in two ways. First, PGRMC1 binds and activates the P450 protein Cyp51/lanosterol demethylase [19], which catalyzes an essential reaction in the sterol synthetic pathway [44]. This finding was derived directly from research in the fungi Saccharomyces cerevisiae and Schizosaccharomyces pombe, where the PGRMC1 homologue, called Dap1, activates Cyp51 [18, 19, 45, 46]. However, Dap1–Cyp51 binding has not been demonstrated in S. cerevisiae, and the two proteins only partially overlap in their localization [45].

Cyp51 is the target for the azole class of antifungal drugs, which are widely used to treat yeast infections, and Dap1 mediates resistance to azole drugs by activating Cyp51 [18, 19, 46]. Dap1 also regulates a second P450 protein, called Erg5, in the cholesterol synthetic pathway [18, 19, 46] suggesting a general role for Dap1 in P450 activation. As in yeast, human PGRMC1 binds to Cyp51, and human embryonic kidney cells depleted for PGRMC1 have a partial arrest in cholesterol synthesis [19].

In addition to activating Cyp51, PGRMC1 binds to the proteins Insig (insulin-induced gene) and Scap [SREBP cleavage activating protein [47]], which span the endoplasmic reticulum and sense cholesterol levels. SREBP (sterol regulatory element binding protein) is a transcription factor which exists in a precursor form in the endoplasmic reticulum [19, 48]. Insig inhibits the processing and activation of SREBP by binding to Scap [48, 49]. When Insig is itself inhibited, Scap is released to escort SREBP to the Golgi for processing [48]. The biological role of PGRMC1 in regulating Insig and Scap is unclear, and whether Insig and Scap are part of the P450 or progesterone-binding complexes associated with PGRMC1 is not known.

3.3 Interactions between PGRMC1 and P450 proteins

In yeast and humans, PGRMC1 binds directly to P450 proteins. Dap1 binds to Cyp51/lanosterol demethylase in yeast, and PGRMC1 binds to Cyp51, Cyp3A4, Cyp7A1/cholesterol 7α-hydroxylase and Cyp21A2/21-hydroxylase in humans [19]. This implies a role for PGRMC1 in cholesterol synthesis, drug and hormone metabolism and bile acid synthesis. Indeed, monoclonal antibodies to PGRMC1 block the 21-hydroxylation of progesterone in rat adrenal tissue [50], and PGRMC1 activates Cyp21 when the two proteins are coexpressed [25, 39, 46], indicating that PGRMC1 promotes progesterone turnover. As in yeast, human PGRMC1 is required for the Cyp51-catalyzed step in cholesterol synthesis [19]. PGRMC1 and its homologues have been likened to “helping hands for P450 proteins” [51], and PGRMC1 is highly expressed in the rodent and human liver [13-15, 52], suggesting that PGRMC1 may contribute to multiple P450-mediated pathways.

P450 proteins require a reductase partner, and Dap1 (the yeast PGRMC1 homologue) has reducing activity [53], suggesting that PGRMC1 class proteins may be more than “helping hands”. PGRMC1 is also related to cytochrome b5, an important coactivator of numerous P450 reactions [54]. However, Dap1 binds to heme through a penta-coordinate mechanism [53], which is distinct from that of cytochrome b5, and hyper-expression of cytochrome b5 could not complement loss of the DAP1 gene in yeast [46], suggesting that their functions do not overlap. Furthermore, Dap1 has a much higher affinity for ferric heme than ferrous heme [53], suggesting that Dap1 would release heme during a redox cycle. Thus, the precise mechanism through which Dap1 activates P450 proteins is unclear.

Like Dap1, PGRMC1 binds to heme, although its relative affinity for ferric and ferrous heme and its reducing activity have not been determined. The putative structure of PGRMC1 from the protein database predicts a prominent groove at the center of the protein (Figure 1). The Asp120 residue, which is required for heme binding [55], is at the

center of the groove, and a proposed phosphorylation site for the Abl tyrosine kinase [4] is located near the opening of the groove (Figure 1, right, the tyrosine residue of the GPY). The Tyr164 residue, which corresponds to the yeast Tyr138 site, is required for heme binding in S. cerevisiae Dap1 [53] and S. pombe Dap1 [19]. The Tyr107 and Tyr113 residues (indicated by arrows in the GPY site and adjacent to it) are required for heme binding in rat PGRMC1/IZA1 [39], and all of these residues localize to the same central groove of Pgrmc1 as Asp120 (Figure 1). Dap1 and PGRMC1 have similar roles in cholesterol synthesis and damage resistance, and this is reflected in the conserved residues that surround the central groove (Figure 1, conserved residues are indicated by asterisks). In this depiction, residues that are different in Dap1 and PGRMC1 cluster at one end of the protein and are indicated by pound signs in Figure 1.

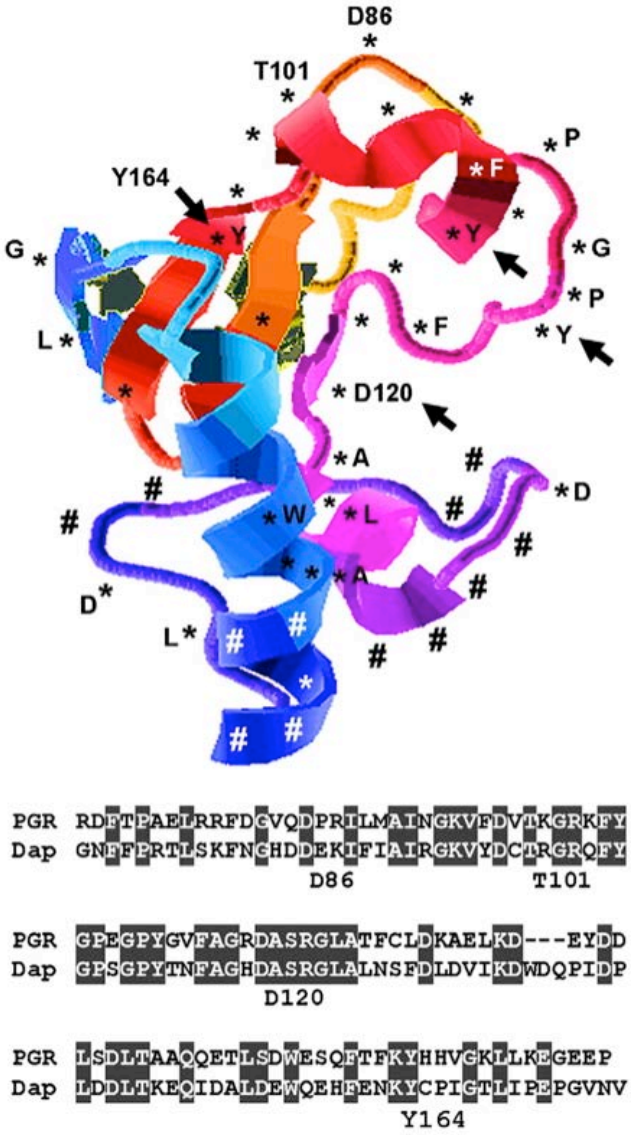


Figure 1. A model for the structure of residues 71–171 of PGRMC1. Asterisks indicate identical residues between PGRMC1 and its yeast homologue, Dap1, while pound signs indicate key non-conserved residues. The sequences of the two proteins are shown below with the identical sequences shaded in gray. The positions of four residues are indicated as a reference point. PGRMC1 residues Asp120, Tyr107, Tyr113 and Tyr164 are required for heme binding in PGRMC1, and the residues that are analogous to Asp120 and Tyr164 are required for heme binding in Dap1 [56].

3.4 PGRMC1 AND CANCER

3.4.1 PGRMC1 expression in clinical tumor samples

PGRMC1 is over-expressed in breast tumors and in cancer cell lines from the colon, thyroid, lung, and cervix [57]. In ovarian cancer, PGRMC1 expression increased in advanced stage tumors, and PGRMC1 was homogeneously expressed within the tumors [58]. Microarray analyses have also detected PGRMC1 expression in colon, lung, ovarian and breast tumors [59-62]. In breast cancer, PGRMC1 is part of a protein signature that predicts hypoxia [60], which is reminiscent of its induction during hypoxia in S. pombe [19]. Conversely, the PGRMC1 promoter is hypermethylated in ovarian cancer [63].
One P450-mediated pathway to which PGRMC1 could contribute is the synthesis and metabolism of estrogen and progesterone. Both hormones are synthesized from cholesterol via steps requiring multiple P450 proteins, including P450scc (Cyp11A1), Cyp17 and Cyp19 (aromatase). Estrogen (E2 and E1) is hydroxylated by Cyp1A1, Cyp1A2, Cyp3A4 and Cyp1B1 to form active metabolites 2-hydroxyestradiol (2-OH-E2) and 4-hydroxyestradiol (4-OH-E2). Both 2-OH-E2 and 4-OH-E2 are further oxidized into carcinogenic compounds estrogen-2,3-quinone and estrogen-3,4-quinone [64, 65]. While it is intriguing to speculate whether PGRMC1 could contribute to hormone synthesis, signaling and turnover, its biological role in these pathways remains to be elucidated.

3.4.2 A six gene carcinogenicity signature includes PGRMC1

PGRMC1 is also implicated as a biomarker in other steps in cancer progression. Nie, et al. identified a carcinogenicity signature in rats, which included PGRMC1 and five other genes, in response to 52 known carcinogenic compounds [66]. Hokaiwado, et al. performed a similar analysis and identified PGRMC1, among others [67]. PGRMC1 was originally cloned as 25-Dx based on its induction by the non-genotoxic carcinogen 2,3,7,8-tetrachlorodibenzop-dioxin in the rat liver [13], which also induces P450 proteins such as

Cyp1A1 [68]. It is unclear how P450 proteins like Cyp1A1 trigger tumor formation, although P450-mediated oxidative damage is a candidate mechanism [69]. It is notable that human PGRMC1 promotes cell death in cancer cells after oxidative damage [22], possibly due to its activation of P450 proteins, activates the pro-survival protein kinase Akt and inactivates the cell death-associated protein IκB [22], suggesting multiple avenues for regulating cell survival.

Interestingly, PGRMC1/25-Dx/mPR induction by TCDD is genderspecific, so that PGRMC1 expression is induced in male rats but repressed in females [70]. TCDD induces liver tumors in females but not in males [71], suggesting that gender-specific expression patterns may contribute to TCDD hepatocarcinogenicity. PGRMC1/25-Dx expression is repressed by progesterone and estrogen in murine neurons [52], and it is possible that these hormones repress PGRMC1 in the liver following dioxin treatment. Thus, it is tempting to speculate that PGRMC1 could have a signaling function in male liver tissue that contributes to the early stages of cancer progression.

3.4.3 Chemotherapy sensitivity in human tumor cells

Because of the role of Dap1 in damage resistance, Rohe et al. [56] tested whether PGRMC1 has an analogous function in cancer cells. PGRMC1 was inhibited by expression of a dominant-negative, heme binding deficient mutant or by siRNA, and either treatment sensitized breast cancer cells to the chemotherapeutic drugs doxorubicin and camptothecin [55]. These drugs are inhibitors of topoisomerase II and topoisomerase I, respectively. Peluso et al. reported similar results in ovarian cancer cells treated with cisplatin [58]. PGRMC1 expression is induced by chemotherapy [55, 72] and in mouse cells with short telomeres [73], which suffer chromosomal damage during senescence and crisis. Together these results suggest that PGRMC1 induction is a consequence of DNA damage, and PGRMC1 plays a role in suppressing damage-induced cell death in cancer cells. In theory, PGRMC1 could be targeted by antagonists of the PGRMC1 complex, in

combination with chemotherapy, to improve its tumoricidal activity, and targeting the larger PGRMC1 complex may have the same effect.

3.5 PGRMC1 expression in the brain

PGRMC1/25-Dx is expressed in various areas of the brain (hypothalamic area, circumventricular organs, ependymal cells of the lateral ventricles, meninges), particularly in structures involved in CSF production and in osmoregulation [74]. PGRMC1/25-Dx is expressed at a basal level in the cerebellum when rats are born, and the mRNA and protein level of PGRMC1 are increased neonatally and decreased thereafter in both male and female rats, without significant difference between the genders [26]. PGRMC1 is expressed mainly at Purkinje and external granule cells in the cerebellum of neonate rats, whereas in the adult cerebellum, PGRMC1 is expressed in Purkinje cells [26]. Cerebellar Purkinje cells are the sites of de novo synthesis of progesterone from cholesterol, where the steroids promote dendritic growth, synaptogenesis and spinogenesis. Progesterone is neuroprotective in a number of different model systems for brain lesions [75], and PGRMC1 is induced by progesterone after traumatic brain injury [34]. In addition, pseudopregnant rats have increased expression of 25-Dx [74], which also corresponds with neuroprotection. However, it is unclear whether PGRMC1 directly regulates neuroprotection and what its mechanism might be. One intriguing clue is the neurotrophic activity of the PGRMC1 family member neudesin, which is secreted [76]. Neudesin binds to heme and directly stimulates MAP kinase and Akt signaling in cultured neurons [76]. However, the receptor system for neudesin remains to be characterized, and it is unclear whether a portion of PGRMC1 might be secreted in a similar manner.

3.6 Other phenotypes associated with PGRMC1

Some phenotypes that are readily detectable in yeast have not been analyzed in detail in mammals. In S. cerevisiae, dap1 mutants are deficient in endocytosis [18]. The phenotype

was detected by measuring dye uptake, and the majority of sterol pathway mutants have increased dye uptake [77], probably due to membrane defects that allow the dye to leach into the cells. In contrast, dap1Δ mutants have decreased dye uptake, suggesting that Dap1 contributes to endocytosis. This phenotype suggests a role for Dap1 in membrane uptake and/or intracellular trafficking. Indeed, Dap1 localizes to an endosomal fraction [45] and contributes to the transport or storage of iron in yeast [45]. It is unclear whether this endocytosis phenotype is conservedin humans, but the localization of PGRMC1 to the endoplasmic reticulum or to punctuate cytoplasmic sites overlaps with that of the yeast homologue [21, 55, 58, 78].

In addition to regulating endocytosis, PGRMC1 has a role in regulating protein kinase-associated signaling [22] in which PGRMC1 increases Akt activation. Akt is phosphorylated by the PDK1 protein kinase, and there is a putative PDK1 binding region on PGRMC1 [4]. The exact mechanism through which PGRMC1 activates Akt is unknown, but a recent work suggests a role for PGRMC1 in membrane-associated signaling in cancer cells [79]. The ability of PGRMC1 to activate signaling resembles that of yeast dap1 mutants, which have a defect in membrane-associated signaling from a G-protein coupled receptor cascade for the yeast α-factor mating pheromone [18]. Interestingly, there is some indirect evidence that PGRMC1 binds to caveolin [35], which controls signaling through a number of receptor complexes [80-82]. Thus, one potential model through which PGRMC1 regulates signaling is that a PGRMC1-caveolin complex contributes to the secretion, stability or activation of signaling proteins at the cell membrane.

Another area of research that began in model organisms involves the transcriptional regulation of Dap1 and PGRMC1. In S. pombe, the DAP1 gene is under the control of the SREBP (sterol regulatory element binding protein) homologue SRE1, particularly under hypoxic growth conditions [19], raising the possibility that PGRMC1 is similarly regulated by SREBP. Promoter mapping software predicts a sterol regulatory element in the

PGRMC1 promoter, but it is unclear whether this site is active. Because PGRMC1 binds to Insig and Scap [47], one intriguing model is that PGRMC1 could be part of a feedback mechanism in regulating SREBP processing.

4. STRUCTURE AND FUNCTIONAL MOTIFS

4.1. Cytochrome b5 domain fold

Mifsud and Bateman recognised in 2002 that the MAPR family of which PGRMC1 is a member contains a cytochrome *b*5 domain fold [83]. In the same year, the NMR solution structure of the cytochrome *b*5-domain putative ligand binding domain of the *Arabidopsis* protein "Putative Steroid-Binding Protein" (Genbank Accession GI:40889041) was submitted by Suzuki and colleagues from the RIKEN Genomic Sciences Center, Yokohama, to the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Base (PDB) with PDB Accession 1J03. The structure was published in December 2003, and appeared in the scientific literature under the name of the corresponding *At2g24940* gene in April 2005 [84]. Another essentially identical determination of the core structure of the same gene product was published in 2004 by Song et al. from the University of Wisconsin under PDB accession 1T0G, which was determined on a slightly longer version of the protein [85]. This review refers to the initial 1J03 structure, which corresponds to the region that is highly homologous to the core mammalian cytochrome *b*5 domains of the PGRMC1 and PGRMC2 proteins.

4.2. PGRMC1 is a structural homologue of Arabidopsis 1J03

To consider potential heme-binding ligands, Min et al. [25] modelled the PGRMC1 cytochrome *b*5 domain on the distantly related PDB structure 1CXY, the *Ectothiorhodospira vacuolata* cytochrome *b*558, which is a prokaryotic homologue of

cytochrome *b*5 [86]. Alignment of the cytochrome *b*5 domain of the *Arabidopsis* protein "Putative Steroid-Binding Protein" (1J03) with PGRMC1 confirms that the major structural elements and spacing between them are better conserved between these proteins, as seen in Figure 2 which shows the helices and beta strands of the cytochrome *b*5 domain according to the nomenclature of Mifsud and Bateman [83], with the exception that H-2◦ has been added because it was not depicted in the Mifsud and Bateman alignment, yet is present in the MAPR family. Many of the non-identical amino acids between 1J03 and PGRMC1 within the beta strands or alpha helices furthermore represent conservative substitutions. This demonstrates that these proteins are structurally homologous over this cytochrome *b*5 domain, and that the NMR structure of 1J03 can validly be superposed with the amino acid backbone of PGRMC1 and related proteins to help understand their structure/function relationships. Although similar theoretical structures based on the 1J03 fold have been calculated for many of the sequences in the MODBASE data base [87], that is linked from SwissProt, MODBASE was not used for Figure 3.

Figure 2. PGRMC1 is a structural homologue of *Arabidopsis* 'putative steroid receptor' (1J03). The cytochrome *b*5 domains of both proteins are aligned. Nomenclature for helices (H1–H4) and beta sheets (β1–β4) follow Mifsud and Batemen [83], with the exception of the helix labelled H2◦ which is present in PGRMC1 and other MAPR family members but absent from their nomenclature [4].

4.3 ScanSite predicted motifs

Potential protein motifs were predicted by the ScanSite "MotifScan" module function [88] for the PGRMC1 amino acid sequence. For a protein of just 194 amino acids (21.5 kDa), in addition to the cytochrome *b*5 domain a plurality of short motifs putatively concerned with protein interactions and signal transduction molecules were predicted, including two SH2 target sequences, an SH3 target sequence, a tyrosine kinase site, two acidophilic kinase sites (nominally referred to as CK2 in this review), and consensus binding sites for ERK1 and PDK1. Figure 3 shows the position of these predicted motifs in the PGRMC1 sequence. In 2006 Peluso et al. also noted the presence of the predicted SH2 and SH3 motifs using Scansite [28], as did workers from ProteoSys AG in Mainz and the University Women's Hospital of Tuebingen (International Patent Application PCT/EP2006/009351, submitted 26 September, 2005). Note that these are not SH2 or SH3 domains, but rather peptide sequences that may be targeted by those domains. Notably, these motifs are all on the accessible surface of the folded protein (see below). In the original paper describing the 25-Dx clone, Selmin et al. noted that the transmembrane domain and the N-terminal cytoplasmic flanking region were similar to conserved sequences found in the cytokine/growth factor/prolactin receptor superfamily. This region, corresponding to the proposed SH3 target sequence, is particularly similar to an IL-6R sequence involving a series of prolines that is conserved in cytokine receptors where it is called box-1, which is necessary for the interaction with the tyrosine kinase JAK2 [13]. The truncated HC5 protein mentioned above does not include this putative SH3 target site. Although not all of these sites may be biologically relevant, this, nevertheless, strongly suggests that PGRMC1 may be able to function as an adaptor molecule in signalling processes. Although only the N-terminal putative CK2 site (S56) was detected under high stringency Motif Scan parameters, the predicted SH2 target sequence (centred on Y179) that was detected under high stringency contains the predicted medium stringency C-terminal CK2 site (S180).

4.4 Phosphorylation

Both acceptor serines of the predicted CK2 sites have been detected as phosphoserine peptides in PGRMC1 from HeLa cell nuclear extracts [23]. Phosphoserine 180 was also detected in PGRMC1 from human mitotic spindle preparations 41], and from a trypsin digest of total mouse liver protein extract [89]. Because phosphorylated peptides are notoriously difficult to detect, it is possible that serine 56 was phosphorylated in all those samples. Working in mouse brain samples after subcellular fractionation, Munton et al. observed phosphorylation of murine tyrosine 180 only from synaptosomal and not from synaptic vesicle or post-synaptic density preparations. This amino acid is homologous to the human Y179 tyrosine phosphate acceptor of the predicted C-terminal SH2 target sequence, suggesting that PGRMC1 may be relocalised by tyrosine phosphorylation. This result was notable in this context because PGRMC1 was almost 15-fold more abundant in the post-synaptic density fraction where no phosphorylated species were detected [37]. Selmin et al. [70] also noted the presence of slightly higher molecular weight species of PGRMC1 in MCF7 breast cancer cell line which they speculated may have represented differentially phosphorylated forms induced by 10nM dioxin treatment. They concluded: “Our results indicate that the mPR protein (PGRMC1) may exist in different states of phosphorylation, which would cause changes in the apparent MW. This latter explanation would be in line with the fact that RT-PCR experiments always detect one single transcript, and previous Northern assays which showed a single mPR RNA size, corresponding to a protein of ≈25 kDa”.

4.5 ITAM motifs and vesicular trafficking

Runko and Kaprielian [17] noticed the presence of several conserved YXX(Φ) consensus motifs (where represents an aliphatic amino acid) in PGRMC1 and related proteins from several organisms. These tyrosine-based motifs are thought to be involved in vesicle transport, and particularly so when they occur in a so-called tight turn on the protein

surface, in the endocytic internalisation of proteins from the cell surface at clathrin-coated pits that contain caveolin [90]. YXX(Φ) motifs are also known in the literature as immunoreceptor tyrosine-based activation motifs (ITAMs), which are characterized by canonical YXX(L/I/V) sequences [91-94]. The observed colocalisation of PGRMC1 (called NGPR by those authors) with caveolin and the effects of digitonin treatment on ligand binding [35, 95] are consistent for such a proposed role in endocytic processes and membrane trafficking, although this remains undemonstrated.

4.6 Structural model

A model for PGRMC1 is shown as Figure 3, based upon homology to *Arabidopsis* 1J03 (Figure 2), and showing the structural motif labeling nomenclature discussed. Using the aligned domain topology of these two protein structures, note that the N-terminal and C-terminal regions of the cytochrome *b*5 domain would be on the same side of the structure, which would be the opposite side of the protein to the ligand-binding pocket. Therefore the predicted PGRMC1 N-terminal SH3 target recognition site and CK2 site centred on residues P62 and S62, are most probably adjacent and functionally overlapping to one another on the surface of the related PGRMC1 protein directed away from the ligand binding pocket. Similarly, the predicted SH2 domain and CK2 site centred on Y179 and S180, respectively, are also probably located on the same surface on the opposite side of the protein from the ligand-binding site. This suggests that ligand-binding of PGRMC1 could occur simultaneously with protein interactions through these sites. It is worthwhile to recall that ligand-binding to nuclear hormone receptors induces large structural alterations in those proteins, to the extent that the ligands are often totally shielded from aqueous solution by the protein ligand-binding pockets [96]. So it is easily conceivable that ligand-binding could affect the conformations and specificities of any putative protein interaction motifs in PGRMC1.

The predicted Shc consensus-like SH2 target sequence as evolutionarily inserted as a

loop between conserved cytochrome *b*5 domain helices 3 and 4 ('H3-H4 SH2' in Figure 3) in the MAPR family that is absent in the ancestral cytochrome *b*5 domain fold. In the crystal structure of bovine cytochrome *b*5, these two helices are joined by a short linker of just a few amino acids at the rim of the ligand-binding pocket. In the plant 1J03 structure the amino acids between helices 3 and 4, which exhibit marked amino acid homology to the predicted SH2 target motif of PGRMC1, fold in the direction away from the ligand pocket leaving the ligand-binding region accessible. If these amino acids do function as the target for an SH2 domain in vertebrates, we are faced with an extremely interesting situation. The SH2 domain is thought to have evolved along with multicellularity in eukaryotes, and examples have been identified on the basis of consensus SH2 signatures in plants [97]. The vertebrate PGRMC1 and PGRMC2 exhibit absolute conservation of the putative Y138 phosphate acceptor amino acid of PGRMC1 [17]. The plant protein 1J03 also exhibits strong homology across this region, with several amino acids in the putative SH2 target loop exhibiting absolute conservation, and several others having undergone conservative substitutions. However the position homologous to Y138 of PGRMC1 is occupied by a serine (also a phosphate-accepting amino acid) in the plant protein (see Figure 2), which merits the initiation of experiments aimed at functional classification of this stretch of amino acids in plants and mammals if the SH2 target sequence status is verified.

Interestingly, structural alignment of PGRMC1 with the plant protein also revealed that P108 at the centre of the putative ERK1 binding site was exposed at the surface of the protein immediately N-terminal to helix 2◦ (Figure 3), while T160 at the centre of the putative PDK1 kinase binding site was also exposed on the surface of helix 4. In fact, the P108-centred putative ERK1 binding site is situated on the lip of the binding pocket adjacent to the position where a cytochrome *b*5 histidine interacts with the heme ligand, and this proline is conserved in related proteins from yeast to mammals [83]. Therefore the putative ERK1 binding site could conceivably be inhibited by recruitment of the ligand

to the binding pocket, or vice versa. Similarly, the predicted phosphorylation of Y112 by Abl or Lck kinase could also potentially influence ligand binding. However in the absence of experimental analyses, these alluring speculations remain rather circumspect. Notably, several of the YXX (Φ)/ITAM potential motifs described by Runko and Kaprielian (particularly Y165 and perhaps Y112: Figure 3) are actually accessible at the protein surface on sharp turns following a helix in the modeled PGRMC1 structure, which is the structural prerequisite for involvement of ITAMs in vesicle trafficking [90]. Y42 immediately follows the transmembrane helical domain, and could also be involved in a sharp backbone turn. This situation is highly unlikely to have arisen by chance, and supports the prediction of Runko and Kaprielian that PGRMC1 is involved in membrane trafficking. The functional relevance of these putative structural motifs certainly merits further scrutiny.

4.7 Functional model

These combined observations lead to a proposed model for the cellular function of PGRMC1. As stated above, the preponderance of protein motifs putatively associated with signal transduction in the predicted 21.5 kDa protein is highly suggestive that the protein indeed functions as a signalling adaptor molecule involved in membrane trafficking, and that the activity may be kinase- and/or ligand-regulated. Strikingly, the spatial juxtaposition of the SH2 and SH3 target motifs in Figure 3 would induce intimate local concentration of putative interacting proteins relative to each other, such as possibly kinases and their substrates, or proteins located on different sub-cellular membranes, potentially greatly facilitating biological activities. This provides a highly plausible scenario for the mode of action of PGRMC1. The protein is schematically represented in Figure 4 as a ligand-binding signalling adaptor protein. In the CK2-phosphorylated state of this hypothetical model, one or more of the N-terminal SH3 target motif and C-terminal SH2 target motif are phosphorylated by an acidophilic kinase such as CK2, and do not interact with other proteins. Since the C-terminal CK2 site is more highly evolutionarily conserved,

and overlaps exactly with the corresponding predicted SH2 target, it is the more likely of these motifs to be functionally regulated by CK2. However in the non-CK2-phosphorylated state the CK2 site/s is/are dephosphorylated in the model, possibly leading to relocation of PGRMC1 to specific subcellular compartments as perhaps observed in various literature reports. This would entail accompanying tyrosine phosphorylation if an SH2 target sequence was involved. The predicted SH2 target between helices 3 and 4 of the cytochrome *b*5 domain may interact with other proteins. It is tempting to speculate that interactions through these putative motifs are regulated by ligand-binding, tyrosine phosphorylation of the putative Abl/Lcklike site at Y112, and/or binding of either ERK1 and/or PDK1 to their respective predicted sites on the edge of the ligand pocket.

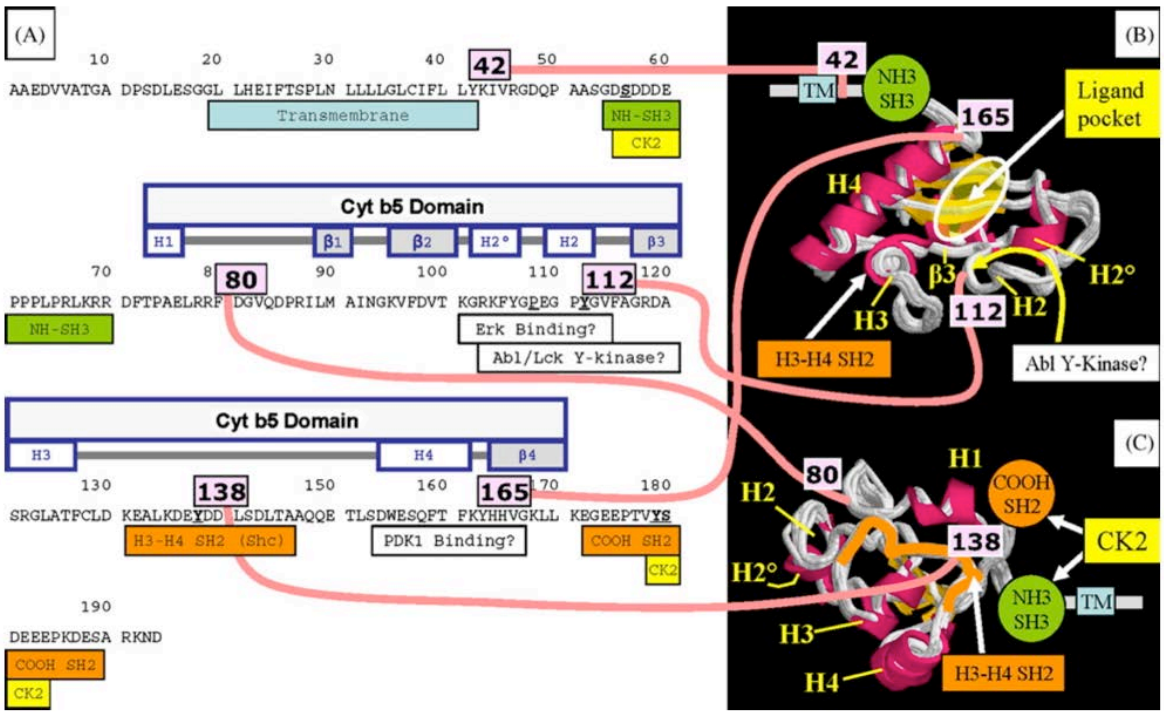


Figure 3. PGRMC1 modelled using the *Arabidopsis* 1J03 NMR structure. (A) The amino acid sequence of PGRMC1 (SwissProt O00264), showing predicted functional motifs from Table 1 below the sequence. The cytochrome *b*5 domain is indicated above the sequence, with the positions of helices and beta sheets after Mifsud and Bateman [83] and Figure 2. The position of tyrosines of the putative ITAM/YXX(Φ) motifs according to Runko Kaprielian [17] are shown in pink boxes above the amino acid sequence. (B and C) The structure of PGRMC1 as modelled with the *Arabidopsis* 1J03 coordinates (which were depicted with RasMol), showing the positions of the colour-coded features from (A). (B) View from the side of the ligand binding pocket. (C) View from the side opposite to that containing the ligand-binding pocket. The positions of PGRMC1 features, which are not present in the 1J03 structure, are schematically portrayed by circles, which are colour-coded to correspond with (A). H2 was not shaded red as a helix by the RasMol software, although the helical arrangement of the peptide backbone is obvious [4].

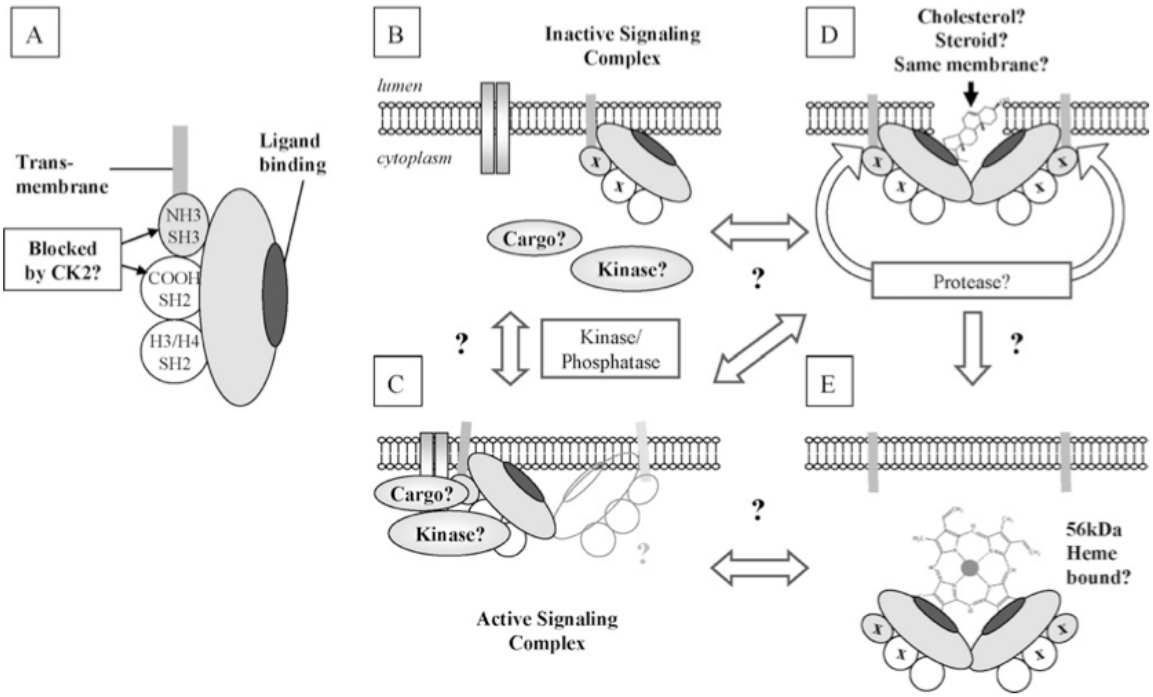


Figure 4. Hypothetical model for PGRMC1 function as a ligand-binding adaptor molecule. (A) Schematic model of PGRMC1, based upon the model structure of Figure 3. (B) According to the hypothetical model, when PGRMC1 is phosphorylated by CK2 at S56 and S180 the putative SH3 and SH2 target sequences are unavailable to interact with SH3- and SH2-domain-containing proteins. (C) In the absence of S56 and/or S180 phosphorylation of these interaction motifs PGRMC1 possibly forms a functional protein complex. (D) Cholesterol and/or steroid binding may require dimerisation of PGRMC1 from a '28 kDa' monomer to a '56 kDa' dimer. It is conceivable that the subunits bind to membranes on different subcellular organelles. (E) Protease action, such as by the S1P or S2P proteases which cleave the cholesterol sensitive SREBP [98], may release a '56 kDa' dimer to the cytoplasm, where it can bind heme. These speculative scenarios are intended to be mechanistically illustrative, and neither mutually exclusive nor functionally comprehensive. Translocation(s) between sub-cellular locations may be involved in change between putative functional scenarios (arrows) [4].

5. POSSIBLE DISEASE RELEVANCE

PGRMC1 was cloned as 25-DX as a dioxin-inducible gene, which suggests a possible function in stress-alleviation or stress response, an interpretation which seems to be supported by subsequent data. Progesterone has a neuroprotective effect. We have already seen that progesterone treatment of spinal chord-injured rats led to increased PGRMC1 expression in the rat spinal cord [34], and PGRMC1 is also expressed in brain regions involved in water homeostasis, where it is up-regulated after traumatic brain injury [74]. Although there is still no evidence of direct steroid binding to PGRMC1, as reviewed above, there is reasonable evidence for a role in steroid binding in complex with other proteins, and for influencing steroid synthesis via the cholesterol regulatory system, and/or metabolism. Another potential avenue of disease relevance lies in the immune system. PGRMC1 has been identified in intraepithelial CD8-positive and estrogen receptor beta-positive T lymphocytes from human oviduct epithelium, where a role in the hormonal responsiveness of that tissue was suggested [99]. However no diseases associated with PGRMC1 have yet been reported in these systems.

In a recent review, Kampa and Castanas argue that membrane steroid receptors may constitute major future targets for therapy in neoplastic cells [100], and the strongest indication of a disease-related function for PGRMC1 so far is in cancer. Nie et al., from Johnson and Johnson Pharmaceuticals, identified PGRMC1 in a 2006 toxicogenomics report as one of six genes whose expression profiles could predict the action of nongenotoxic carcinogens with over 80% accuracy. Yet typically for this protein, they were unable to classify PGRMC1 into one of the functional networks predicted by an Ingenuity Systems Pathways Knowledge Base because PGRMC1 was not functionally annotated in that database [66]. This further highlights the enigmatic level at which the biology of PGRMC1 is presently understood against a backdrop suggesting that its role is, nevertheless, probably quite important.

PGRMC1 is more abundant in several tumours and/or tumour cell lines than in control healthy tissues. In 2003 Difilippantonio and colleagues from the Charit´e Hospital in Berlin and the National Cancer Institute in Bethesda, Maryland, found by subtractive hybridisation cloning that PGRMC1 was consistently about 2–4 fold more abundant in cell lines from lung cancers than from healthy lung cell lines, however PGRMC1 was only one of multiple identified genes, and the finding was not extended to primary clinical tissues [59]. In 2005 Crudden et al., from the Craven and Mannheim groups, showed by Western blot that PGRMC1 is more abundant in breast cancer than in healthy tissue and it is variously overexpressed in some colon and thyroid tumours [57]. The same year, Craven and coauthors further proposed that PGRMC1 belonged to a group of genes that regulate susceptibility of cancer cells to chemotherapy [72]. Kurek, Clare, Neubauer, and colleagues from the University Women's Hospital of Tuebingen and ProteoSys AG in Mainz identified three isoforms of PGRMC1 in breast cancer tumors by 2D-PAGE, two of which were differentially more abundant in tumours lacking the estrogen receptor (ER), a phenotype which correlates with both an activated wound response and negative clinical outcome. Immune histochemistry studies suggested a more micro-heterogenous subcellular distribution than the diffuse cytoplasmic staining observed in tumors that were ER-positive (international patent applications WO2006029836, submitted 14th September 2004; PCT/EP2006/009351, submitted 26 September 2005).

It has been proposed that PGRMC1 centrally regulates susceptibility to chemotherapy by heme-binding [55], although we have already noted that no involvement of a cytochrome P450 interaction or accessory redox function has yet been demonstrated. Peluso, Wehling, Losel and colleagues [28, 101] argue that the protein complex of PGRMC1 and PAIRBP1 induces the anti-apoptotic effect of progesterone on granulosa cells, an effect which could also be relevant for cancer and other pathogenic cells.

However it is possible that PGRMC1 exerts functions as an adapter protein independently of its ligand-binding, or perhaps regardless of the bound ligand. In addition to PAIRBP1,

we have seen that PGRMC1 is implicated in the UNC-40/DCC receptor for Notch on the surface of migrating neurons, in a signalling system which does not harbor an obvious or reported requirement for ligand binding. In *C. elegans*, the UNC-6/Netrin response is associated with presence of two receptors: UNC-40/DCC and UNC-5. These belong to the class of dependence receptors which induce one of two responses, such as survival or apoptosis, in the presence or absence of ligand. It has emerged that the Netrin, UNC-40/DCC, UNC-5 pathway is crucially important in mammalian tumorigenesis. In the absence of Netrin its receptors induce p53-dependent apoptosis, and the Netrin gene is transcriptionally regulated by p53. Hence the UNC-5 family and UNC-40/DCC receptors have been identified as tumor-suppressors whose deletion can lead to cancer, which explains why Netrin is a survival factor [102-105]. Therefore any ability of PGRMC1 to interact with DCC and influence its pro-apoptotic activity in the absence of Netrin could be of paramount importance for tumour cells. It is unknown whether these molecules are involved in the apparent upregulation of PGRMC1 after tissue injury that was observed by Velardo et al. from the University of Florida [106], who performed systematic gene expression profiling in a model based upon physically induced mouse spinal chord injury. Those authors identified waves of co-regulated genes that were temporally induced after tissue damage. PGRMC1 mRNA was up-regulated relatively late (many days to weeks after damage) along with a cluster of genes involved in tissue repair.

In relation to cholesterol regulation, it was mentioned above that HMG-CoAR is transcriptionally activated by SREBPs after they are escorted from the endoplasmic reticulum to the Golgi by the PGRMC1-interacting protein SCAP, thereby activating cholesterol synthesis under conditions of low cholesterol. This could conceivably contribute substantially to neoplastic status. Signalling requires the presence of proteins accumulated in cholesterol-enriched, detergent-insoluble and caveolin-associated microdomains which have come to be called lipid rafts [107]. In fact it has been demonstrated that cellular cholesterol levels determine the properties of rafts, and thereby

regulate the biological response of cells [108, 109]. However any putative neoplasminfluencing effects of PGRMC1 need not necessarily involve cholesterol or rafts. HMG-CoAR catalyses the reduction of acetyl CoA to l-mevalonate, which is the precursor for the so-called mevalonate pathway, which leads not only to the synthesis of the carbon-skeleton of cholesterol and steroids, but also to the synthesis of isoprenoids that are necessary for the prenylation and attendant membrane localisation of proteins involved in signal transduction such as the G-protein RhoA. The mevalonate pathway has therefore emerged as one of the recently most investigated systems for anti-cancer treatment, particularly involving statins, which inhibit HMGCoAR activity [110-112].

6. PGRMC1 AND REPRODUCTION

Recently PGRMC1 has been demonstrated in female reproductive tissues of different mammalian species, suggesting a role for it in reproduction.

6.1. Uterus and pregnancy

Chen et al. [113] compared the protein profiles differentially expressed in the human endometrium between the proliferative and secretory phases of normal menstrual cycles by 2D differential in-gel electrophoresis. Mass spectrometry identified 76 proteins representing 41 different gene products and immunohistochemistry confirmed the observed changes in 3 representative proteins (Rho-GDIalpha, CLIC1, PGRMC1). PGRMC1 was more highly produced in the proliferative than the secretory phase and immunostaining studies revealed that it was confined to the stromal component of proliferative phase endometrium. Importantly PGRMC1 expression was observed predominately during the midproliferative phase when circulating progesterone levels are very low, suggesting that progesterone may not be its biologically relevant ligand. Given that progesterone resistance and dysregulation across the endometrial cycle is an

important feature of endometriosis and infertility [114] it may be that PGRMC1 dysregulation contributes to these disorders.

These findings were confirmed in uterine tissues in mice [115] where endometrial expression of PGRMC1 was up-regulated in the stromal compartment from estrus to metestrus and reduced during diestrus. PGRMC1 expression was studied also during early gestation. Immunohistochemical analyses demonstrated that PGRMC1 was either diffusely expressed throughout stromal cells cytoplasm or highly abundant in the nucleus. The protein continued to show dynamic localization during the decidualization process. Stromal cells at the outer margins of the secondary decidualization zone showed only nuclear expression of PGRMC1, while stromal cells having undergone terminal differentiation expressed PGRMC1 throughout the cytoplasm. PGRMC1 was also detected in the myometrium during pregnancy and was found dually expressed in decidualized stromal cells at both the antimesometrial and mesometrial poles of the implantation site. Interestingly, PGRMC1 was expressed in the microvasculature at the mesometrial aspect of the implantation site. These findings suggest that PGRMC1 promotes differentiation, since the protein expression increases in endometrial cells undergoing steroid-depended terminal [39]. In addition, the same report described PGRMC1 in the human placenta and associated membranes with the most abundant expression in smooth muscle of the placental vasculature, villous capillaries and the syncytiotrophoblast suggesting the hypothesis that this protein functions in events important in early pregnancy including cellular differentiation, modulation of apoptosis and steroidogenesis [116, 117].

The expression of PGRMC1 has been described also in early equine conceptuses [118]. Horse conceptuses recovered on days 7, 10 and 14 after ovulation were examined for PGRMC1 mRNA expression using quantitative rtPCR. mRNA for PGRMC1 was detected at all stages examined and PGRMC1 expression increased during the day 7-14 period.

The study demonstrates that equine conceptuses express PGRMC1 during the period encompassing rapid conceptus growth, differentiation and maternal pregnancy recognition.

6.2. Ovary and oocyte

Other reports investigated the localization and function of PGRMC1 in the ovary. In particular immunohistochemical studies, performed in rat, localized PGRMC1 in ovarian germinal epithelium cells, granulosa, theca and luteal cells [28] and verified that PGRMC1 expression in granulosa and luteal cells was developmentally regulated [28]. In preantral and antral follicles before eCG treatment in fact, PGRMC1 was detected in a limited number of granulosa cells, apparently localized to the nuclei of many PGRMC1-expressing granulosa cells. In a few granulosa cells before eCG treatment, PGRMC1 appeared to be concentrated at or near the plasma membrane, with its expression restricted to a limited section of the plasma membrane. After eCG treatment the localization dramatically changed such that PGRMC1 expression was almost exclusively at or near select regions of the plasma membrane. With hCG induction of ovulation and luteinization, PGRMC1 expression increased. Unlike granulosa cells, 100% of the luteal cells expressed high levels of PGRMC1, with PGRMC1 being distributed throughout the cell.

Lately, the same authors proved that PGRMC1 regulates granulosa cell viability verifying its role as mediator of Progesterone's antiapoptotic action [78, 119]. In an attempt to identify some of the genes whose expression might be part of the putative genomic action of PGRMC1, mRNA levels of numerous genes involved in apoptosis were assessed using real-time PCR microarrays. Treatment with PGRMC1 siRNA in the absence of P4 significantly altered that pattern of gene expression compared to treatment with scrambled

(control) siRNA. The overall effect was to increase the mRNA levels of many genes that are involved in promoting apoptosis. This suggests that PGRMC1, which is not bound to P4, regulates gene expression in a manner that would make the cells more susceptible to undergoing apoptosis. This finding fits well with PGRMC1's cell survival function [4, 120] and is consistent with the observations that depletion of PGRMC1 makes ovarian [58, 78], endometrial [121] and breast cancer [55, 121] cells more sensitive to the apoptotic effects of various chemotherapeutic agents.

Expression of PGRMC1 mRNA was found in granulosa or luteal cells in humans [101], mice [122], pigs [123] and cows [124].

Fertility in mammals is dependant on females having an adequate primordial follicle pool to supply oocytes for fertilization and its number is one determinant of the reproductive lifespan [125]. Primordial follicle assembly occurs during fetal development before birth [126]. Microarray analysis and RT-PCR indicated that PGRMC1 is continuously and highly expressed throughout all the development of bovine fetal ovary [127]. This result raises the possibility that PGRMC1 may support the primordial follicle assembly.

PGRMC1 is also expressed in bovine, rat [28] and human oocytes [128]. Recently PGRMC1 was detected among the proteins of the mitotic spindle [129], suggesting a role for it in mitosis. Moreover, the mRNA that encodes PGRMC1 is present in bovine oocytes at a very high level similar to that of actin [130]. In spite of its high level of expression, little is known about PGRMC1 and oocyte function. Since PGRMC1 is present in mitotic spindle [24, 129], it could be directly involved in regulating oocyte maturation (meiosis).

7. AIM OF THE STUDY

Because of the possible relevance in reproductive function, the present study was undertaken in order to characterize the role of PGRMC1 in bovine infertility. In particular the study has been developed in three parts. In the first part the expression and the localization of PGRMC1 in the bovine reproductive system during the different phase of the estrous cycle were evaluated. In the second part the role of PGRMC1 in oocyte function and specifically in the control of meiotic division and its partecipation in the process of chromosomal congression was analyzed. In the third part of the study we assessed the specific role of PGRMC1 in the decreased developmental capability of oocytes from dairy cows of reproductive age with a reduced ovarian reserve, as a proposed model of premature ovarian failure.

8. Part I

Expression of Progesterone Receptor Membrane Component-1 in bovine reproductive system during estrous cycle

SUMMARY

Several reports suggest the participation of progesterone receptor membrane component 1 (PGRMC1) in progesterone signaling in the reproductive system. This study aimed to investigate the presence and localization of PGRMC1 in bovine ovary, oviduct and uterus, during the follicular and luteal phases of the estrous cycle.

In the ovary, PGRMC1 has been detected in surface germinal epithelium, granulosa cells, theca cells and in the germinal vesicle of the oocytes at all stages of folliculogenesis. In the corpus luteum the expression of PGRMC1 was influenced by the stage of the estrous cycle. In the oviducts and in the uterus horns, PGRMC1 was immunolocalized in the luminal epithelium, in the muscle layer cells and in the endothelial cells. In the uterus, PGRMC1 was intensely localized also in the glandular endometrium. However, in the oviducts and in the uterus horns, the localization of PGRMC1 was independent of the stage of the estrous cycle and of whether evaluating the ipsilateral or the contralateral organ.

In conclusion, the present immunohistochemical study showed that PGRMC1 is located in various compartments of the bovine female reproductive organs and, with the exception of the corpora lutea, PGRMC1 localization showed similar pattern during different stage of the estrus cycle.

INTRODUCTION

Progesterone receptor membrane component 1 (PGRMC1) belongs to the membrane-associated progesterone receptor (MAPR) proteins family that is widespread in eukaryotes and involved in regulating numerous biological functions [4]. PGRMC1 is predominantly located in intracellular membranes and colocalizes with the endoplasmic reticulum and Golgi apparatus in rat liver cells and hippocampal Pukinje cells [21, 26]. Several studies described a nuclear or chromosomal localization. PGRMC1 was detected in nuclear extracts of HeLa cells [23], among the protein of the human mitotic spindles [24, 129], and recently has been proposed in regulating microtubule stability in human and rat ovarian cell mitosis [5]. Various ovarian cell types including granulosa cells and ovarian surface epithelial cells as well as ovarian tumors express PGRMC1 where it plays an essential role in promoting the survival of both normal and cancerous ovarian cell in vitro [131].

Recently PGRMC1 expression has been demonstrated in several female reproductive tissues in different mammalian species. PGRMC1 has been detected in uterine and placental tissues of the mouse and human [115] and it is among the proteins that were differentially expressed in the human endometrium during menstrual cycle [113].

Other reports investigated the localization and function of PGRMC1 in the ovary. In particular immunohistochemical studies localized PGRMC1 in granulosa cells, theca cells and ovarian surface epithelial cells (germinal epithelium) in rat [28]. Moreover the expression of PGRMC1 mRNA was found in granulosa or luteal cells in humans [101], mice [122], pigs [123] and cows [124]. Several works suggest that progesterone mediates its antiapoptotic action through PGRMC1 [78, 119].

PGRMC1 is also expressed in bovine [132], rat [28] and human oocytes [128]. Recently, PGRMC1 was associated to the meiotic spindle suggesting a role in oocyte

maturation, which may be specifically related to the mechanism by which chromosomes segregate [132]. However, with regard to localization of the protein, a recent work did not detect PGRMC1 in the bovine oocyte [133].

To date there is little information on the localization of PGRMC1 in the reproductive organs of the cow. Thus, the aim of the present study was to examine the presence and localization of PGRMC1 in a variety of cow reproductive organs, including ovary, oviduct and uterus during the follicular and luteal phases of the estrous cycle using immunohistochemistry.

MATERIAL AND METHODS

Tissue collection

All the chemicals used in this study were purchased from Sigma Chemical Company except for those specifically mentioned. Bovine ovary, oviduct and uterus were recovered at a local abattoir (INALCA JBS S.p.A., Ospedaletto Lodigiano, LO, IT 2270M CE, Italy) from non-pregnant dairy cows, 4 to 8 yrs old, subjected to routine veterinary inspection and in accordance to the specific health requirements stated in Council Directive 89/556/ECC and subsequent modifications, and were transported to the laboratory on ice. The stage of the estrous cycle was assessed by morphological observations of the ovary [134]. In particular, animals in which one of the two ovaries presented an active corpus luteum were classified as belonging to luteal phases of estrous cycle while animals in which one of the two ovaries presented the ovulatory follicle and a regressed corpus luteum were classified as belonging to follicular phases of estrous cycle [135, 136].

For each animal, both ovaries were transversely cut and 2-4 fragments of corpus luteum and 2-4 fragments of ovarian cortex were collected; from both oviducts samples of isthmus, ampulla and infundibulum were separated. Finally the apical third of each uterine horns were isolated dissecting at about 5 cm from the uterus-isthmic junction [137]. All samples were fixed in B5 (Formalin-Mercury II Chloride) fixative (Bio-Optica, Milan, Italy) for 12-24 hours, dehydrated by a graded series of ethanol, cleared with xylene, paraffin embedded and sectioned at 5 μm on glass slides, previously treated with Vectabond (Vector Laboratories, Burlingame, CA, USA) to enhance the adherence of tissue.

Western blot analysis

Western blot analysis was performed as previously described with minor

modifications [132]. Briefly, aliquots of 50 mg of ovarian cortex and corpus luteum were homogenized in RIPA buffer, which was supplemented with complete protease and phosphatase inhibitor cocktails, incubated for 30 minutes on ice and then centrifuged at 14000 g for 20 min at 4°C. Total amount of protein was determined using the Bio-Rad Protein Assay (Biorad) and 20 µg of total protein was used for western blot analysis. Equal protein loading was verified by Ponceau staining. PGRMC1 immunodetection was conducted using different concentrations (1 µg/ml and 0.3 µg/ml) of a rabbit polyclonal antibody (Sigma Prestige, Cat. No. HPA002877) or a goat polyclonal antibody (AbCam, Cat. No. ab48012) over night at 4°C. PGRMC1 was revealed using an anti-rabbit or anti-goat HRP-labelled antibodies (1:8000) and SuperSignal® West Pico Chemiluminescence Substrate (PIERCE Biotechnologies Inc, Rockford, IL, USA). Negative controls were conducted by omitting the primary antibodies.

Immunohistochemistry

Indirect immunohistochemistry was carried out to evaluate the expression and localization of PGRMC1. Endogenous peroxidase activity was eliminated by incubation with 3% (v/v) H_2O_2 in methanol for 30 minutes. Then sections were incubated with 10% (v/v) normal goat serum or normal rabbit serum, 0.3% (v/v) Triton X-100 and 3% (w/v) bovine serum albumin (BSA) in phosphate buffered saline (PBS) for 30 minutes to block non-specific binding of secondary antibody. In a preliminary study the sections were incubated overnight at 4°C with different concentrations (0.96, 0.48, 0.24 or 0.16 µg/ml) of polyclonal rabbit anti-PGRMC1 (Sigma Prestige) or polyclonal goat anti-PGRMC1 (Abcam) diluted in PBS with 1% (w/v) BSA and 0.3% (v/v) Triton X-100. In all the subsequent experiments the rabbit polyclonal antibody was used at a concentration of 0.48 µg/ml. Primary antibodies were detected by using a biotinylated anti-rabbit or anti goat IgG (Vector Laboratories), diluted 1:400 in PBS with 1% (w/v) of BSA and detected

with the Vectastain Elite ABC kit (Vectastain Elite ABC kit, Vector Laboratories). For color development, all sections were incubated with DAB substrate (DAB substrate kit for peroxidase, Vector Laboratories) for 2 minutes. After staining, the samples were counterstained with hematoxylin QS (Vector Laboratories). Negative controls were performed by omitting the primary antibodies. Samples were analyzed on a Nikon Eclipse microscope (Nikon Corp., Tokyo, Japan) in bright field at a magnification of 200X-400X.

RESULTS

Preliminary studies were conducted in order to compare the specificity and the sensitivity of two antibodies (polyclonal rabbit anti-PGRMC1 and polyclonal goat anti-PGRMC1) by western blot and immunohistochemical analysis with different primary antibodies concentrations.

Western blot analysis confirmed that PGRMC1 was specifically detected in ovarian cortex as a ≈ 26 kDa protein (Figure 5A). However, only the polyclonal rabbit anti-PGRMC1 detected the protein, while under the same experimental conditions the goat polyclonal antibody did not. Conversely, the goat polyclonal antibody only detected the 26 kDa protein when higher amounts of total proteins (100 and 50 µg) were loaded on the gel (Figure 5B). Moreover, antibody titration on immunohistochemistry showed a higher sensitivity of the polyclonal rabbit anti-PGRMC1 since this antibody detected PGRMC1 in the granulosa cells, in the theca layer and in the endothelial cells of blood vessels at all concentration tested, whereas the goat polyclonal antibody detected a much weaker signal at the corresponding concentration (Figure 6A and 6B lower panel). In addition the rabbit polyclonal detected a much higher PGRMC1 signal in cumulus cells and germinal vesicle of the oocyte when compared with the goat polyclonal used at the same concentration (Figure 6B upper panel). Therefore, the polyclonal rabbit anti-PGRMC1 antibody, at a concentration of 0.48 µg/ml was utilized in further experiments to assess PGRMC1 localization in bovine ovary, oviduct and uterine horn.

A total of 8 animals (4 for each estrous cycle) were analyzed for PGRMC1 localization. Data are and shown in Figures 7, 8 and 9 and summarized in Table 1.
In all experiments, negative controls were conducted in the absence of the primary antibody and did not show any staining.

In the ovary, immunohistochemical studies showed that PGRMC1 was expressed

in the follicles during all the stages of folliculogenesis, in the oocyte and in its surrounding cumulus cells, both in the nucleus and inside the cytoplasm (Figure 7). Oocyte showed a positive staining in the germinal vesicle (GV), while the ooplasm displayed only a weak signal (Figure 7, A and B). In the cumulus oophorus, granulosa cells lying closer to the oocyte exhibited a more intense signal than granulosa cells in the outer layers (Figure 6 B, up left). PGRMC1 was also detected in theca layers (Figure 7 C). Furthermore, PGRMC1 was immunolocalized in the germinal epithelium of the ovarian surface (Figure 7 D) and in the endothelial cells of the stromal vessels (arrows in Figure 7 A, B and C). On the contrary, in ovarian stromal tissue cells PGRMC1 was weakly detected.

Immunolocalization of PGRMC1 revealed a similar expression pattern for the follicular and luteal phase of the estrous cycle and differences were not observed between the two ovaries of the same animal.

In contrast, in the corpus luteum PGRMC1 was differently immunolocalized depending on the stage of the estrous cycle (Figure 8). During the luteal phase (Figure 8 A) most of the luteal cells showed a cytoplasmatic staining for PGRMC1, with signal intensity varying from intense to weak, while the signal was completely absent only in a few cells. During the follicular phase (Figure 8 B) PGRMC1 was detected as a weak signal in the cytoplasm of most of the cells. In both phases PGRMC1 was expressed in the endothelial cells and was detected as an intense to weak signal in the nuclei of the luteal cells.

Microscopic examination of PGRMC1 localization in the oviduct revealed a specific nuclear and cytoplasmic staining for PGRMC1 in the luminal epithelium (Figure 9 A and B), in the muscle layers (Figure 9 C) as well as in the endothelial cells. On the contrary PGRMC1 was weakly expressed in the cells of lamina propria and submucosa (stromal cells). No differences were observed between infundibulum (Figure 9 A), ampulla (Figure 9 B), and isthmus (Figure 9 C), neither in follicular nor luteal phase, either between the

two organs of the same animal.

In the uterus, PGRMC1 was observed as an intense signal in the luminal epithelium (Figure 9 E), glandular endometrium (Figure 9 F), as well in the muscle layers (Figure 9 F) and in the vessels while a weak signal was present in the stromal compartment. As in the oviduct, a positive staining was present both in the nucleus and in the cytoplasm of all the cells and any difference was observed between the estrous stages and between the two horns.

Table 1. Immunohistochemical localization of PGRMC1 in the reproductive tracts during estrus cycle. PGRMC1 is indicated as intense (+) weak (±) or negative signal in the different organs.

	Estrous cycle			
	Follicular phase		Luteal phase	
Tissue/cell type	Cytoplasm	Nucleus	Cytoplasm	Nucleus
Ovary follicle				
Oocyte	±	+	±	+
Granulosa cells	+	+	+	+
Theca cells	+	+	+	+
Endothelial cells	+	+	+	+
Stromal tissue				
Stromal cells	±	±	±	±
Endothelial cells	+	+	+	+
Germinal epithelium	+	+	+	+
Corpus luteum				
Luteal cells	±	±	+	±
Endothelial cells	+	+	+	+
Oviduct				
Epithelial cells	+	+	+	+
Stromal cells	±	±	±	±
Muscle layer cells	+	+	+	+
Endothelial cells	+	+	+	+
Uterus				
Luminal epithelial cells	+	+	+	+
Glandular epithielial cells	+	+	+	+
Stromal cells	±	±	±	±
Muscle layer cells	+	+	+	+
Endothelial cells	+	+	+	+

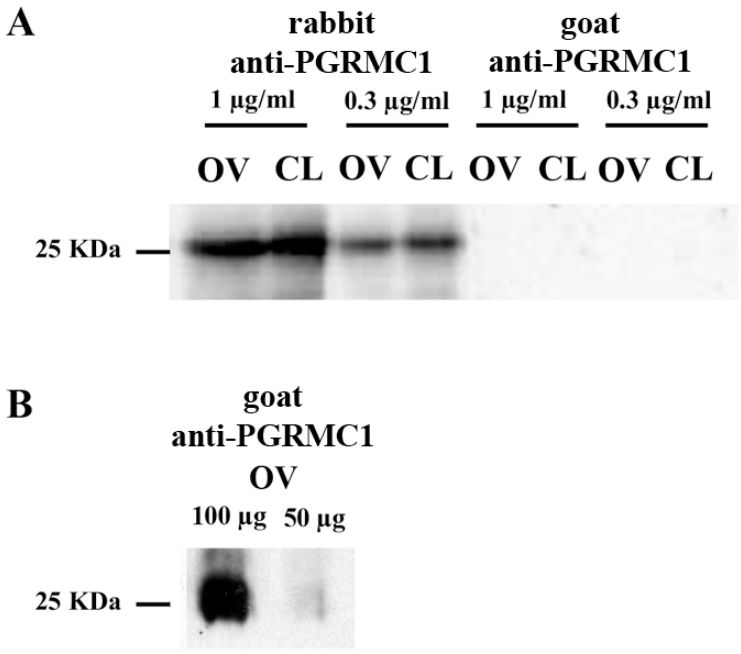


Figure 5. Detection of PGRMC1 in bovine ovarian cortex and corpus luteum by western blot analysis

A) Western blot analysis showing PGRMC1 expression in bovine ovarian cortex and corpus luteum (20µg total protein/lane) using the rabbit polyclonal (Sigma-Prestige) or the goat polyclonal (Abcam) antibodies at different concentrations. Note that only the rabbit polyclonal anti-PGRMC1 antibody detected the protein, while under the same experimental conditions the goat polyclonal antibody did not. **B)** Western blot analysis of PGRMC1 expression in ovarian cortex with the goat polyclonal antibody using increasing amount of total protein (50 and 100µg).

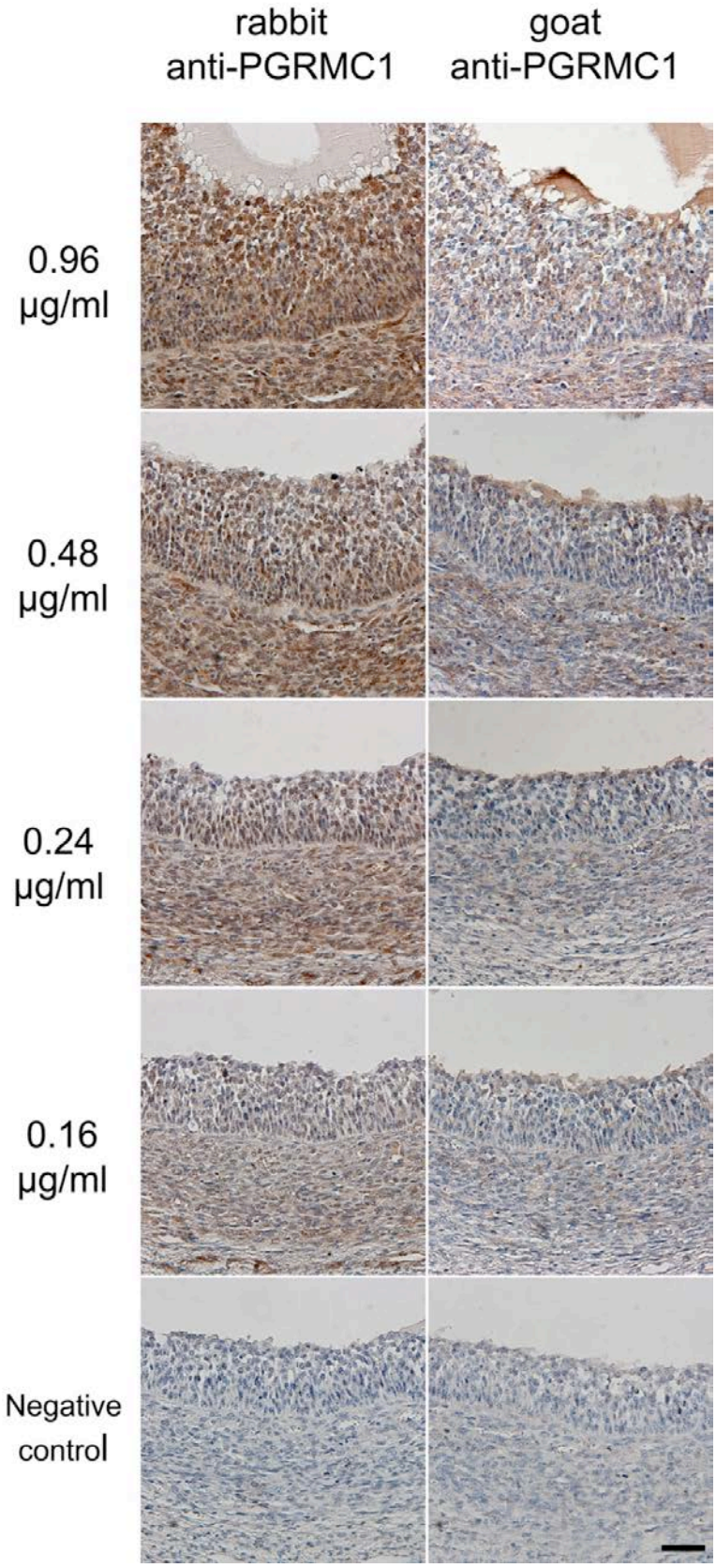


Figure 6A (legend on next page)

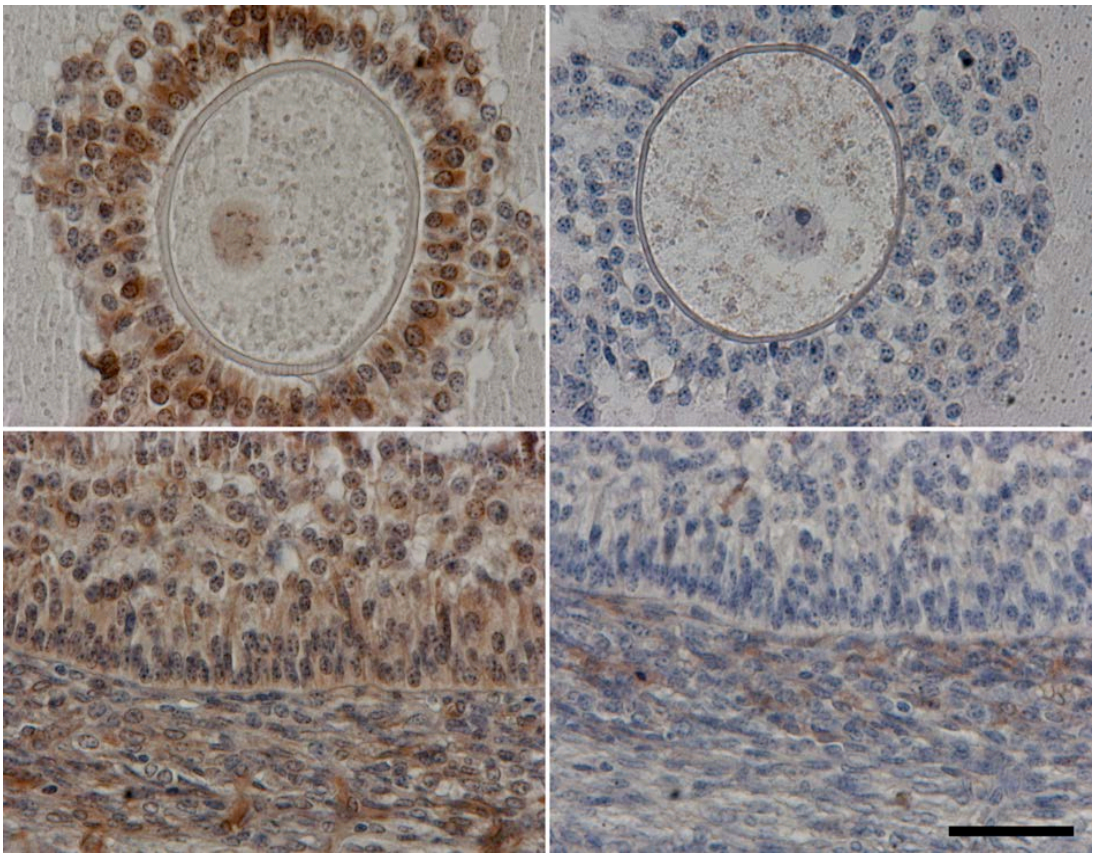


Figure 6B. Titrations of different primary antibodies to detect PGRMC1.

A: Representative images of PGRMC1 localization in a medium antral follicle wall using rabbit polyclonal (Sigma-Prestige) and goat polyclonal (Abcam) primary antibodies. The values on the left refer to the concentrations (µg/ml) of the primary antibodies used. Note that rabbit polyclonal antibody shows a specific staining for PGRMC1 in the granulosa cells, in the theca layer and in the endothelial cells of blood vessels at all concentration used, whereas a weaker signal was detected by the goat polyclonal antibody under the same experimental conditions. No immunoreactivity was seen in negative controls where incubation with the primary antibodies was omitted. Final magnification = 200X, scale bar = 50 µm.

B: Representative images showing details of PGRMC1 localization in oocytes and medium antral follicle wall using the two antibodies under the same experimental conditions at a concentration of 0.48 µg/ml. Note that rabbit polyclonal primary antibody

exhibits a specific staining for PGRMC1 in cumulus cells, germinal vesicle, granulosa cells, theca layer and endothelial cells when compared with the goat polyclonal primary antibody. Final magnification = 400X, scale bar = 50 µm.

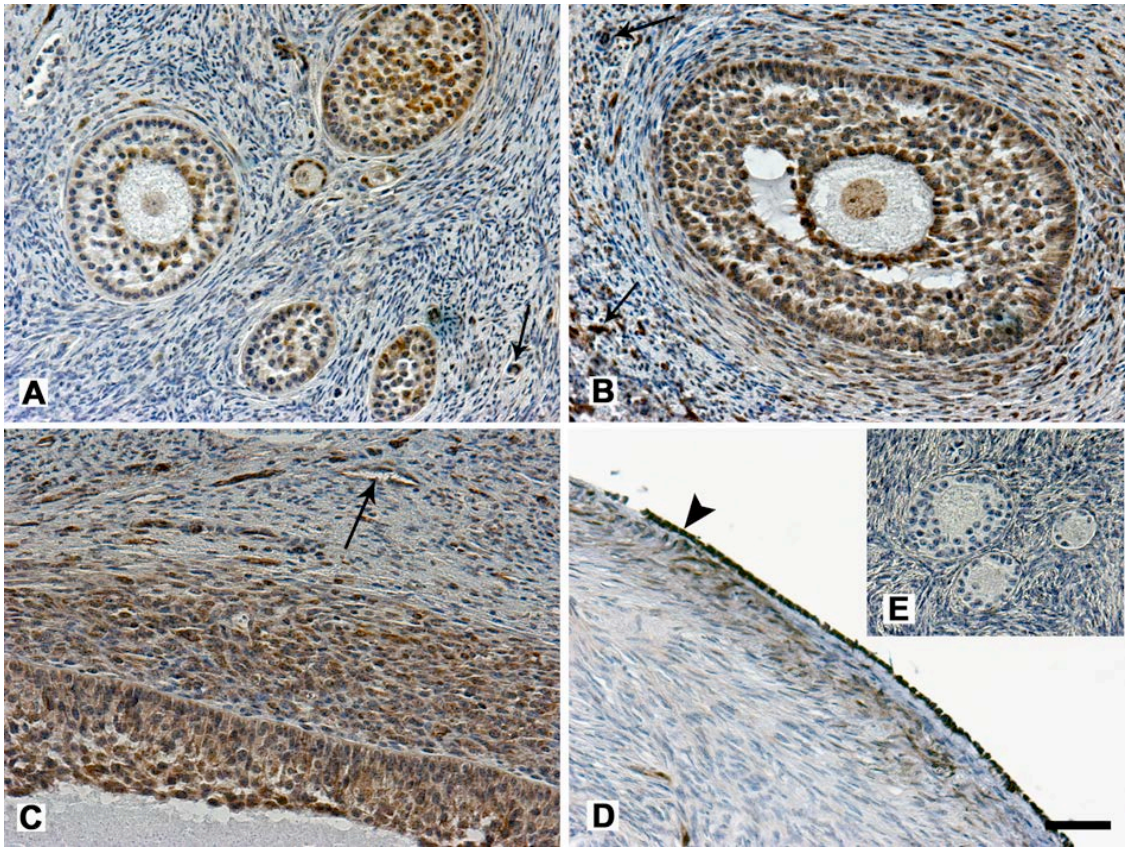


Figure 7. Immunohistochemical localization of PGRMC1 in bovine ovarian cortex.

PGRMC1 is localized in primordial, primary and secondary follicles (A), in early antral follicles (B) and in antral follicles (C). Note that the expression of PGRMC1 is more evident in the granulosa cells closer to the oocyte, in the GV and in the internal theca layer. PGRMC1 was detected in the endothelial cells (Arrows) and in the germinal epithelium at the ovarian surface (D, Arrowhead). No immunoreactivity was seen in negative control where incubation with the primary antibody had been omitted (E). Final magnification = 200X, scale bar = 50 µm.

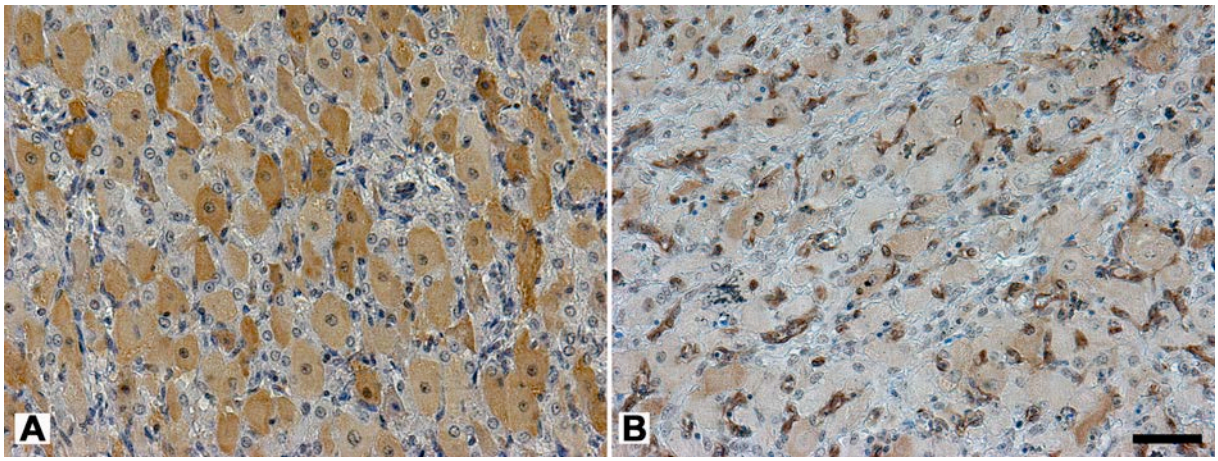


Figure 8. Immunohistochemical localization of PGRMC1 in bovine corpora lutea during the luteal and follicular phase of the estrus cycle.

During the luteal phase (A) PGRMC1 is localized in the cytoplasm of the luteal cells displaying an heterogeneous signal, varying from intense to weak to completely absent. During the follicular phase (B) PGRMC1 is detected as a weak signal in the cytoplasm of most of the luteal cells. In both phases PGRMC1 is expressed in the endothelial cells and is weakly detected in the nucleus of the luteal cells. Final magnification = 200X, scale bar = 50 μm.

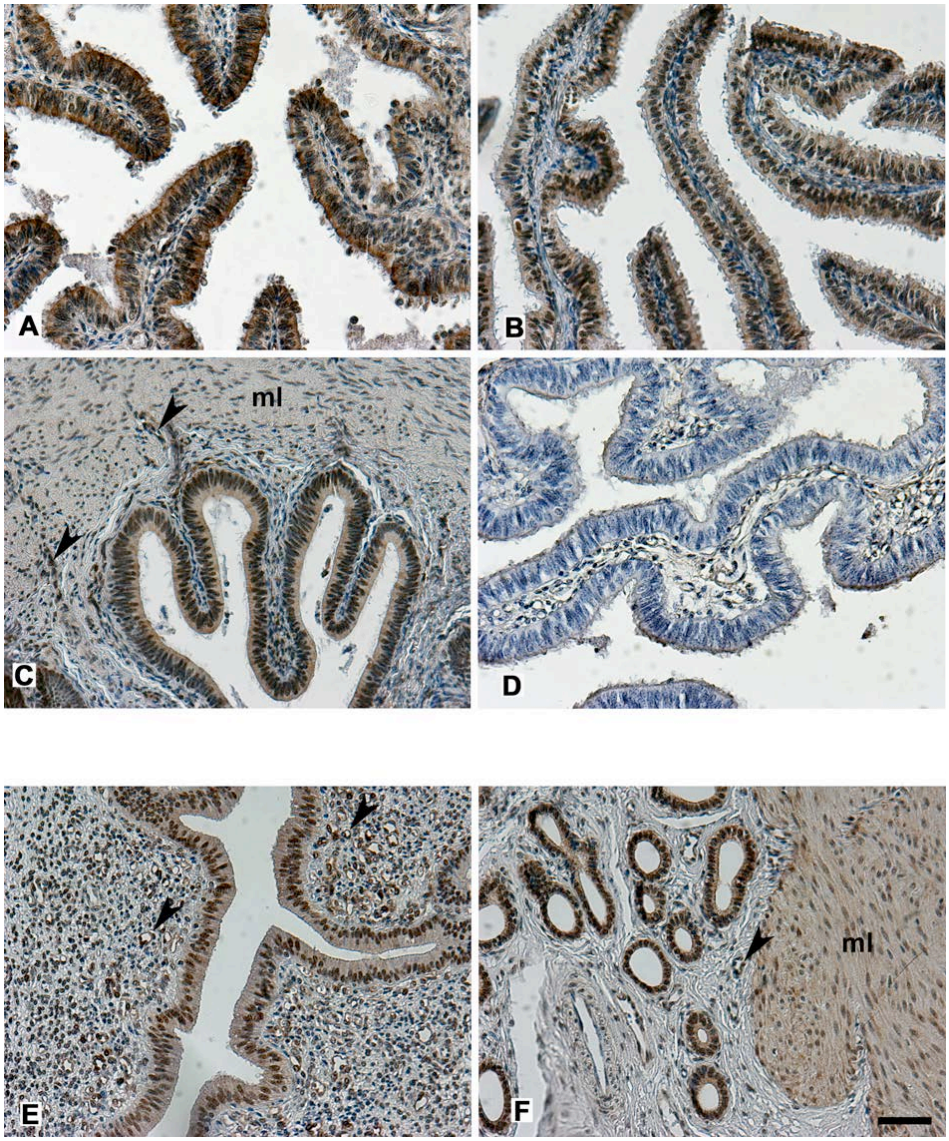


Figure 9. Immunohistochemical localization of PGRMC1 in bovine oviduct and uterus.

PGRMC1 is expressed in the different tracts of the oviduct, infundibulum (A), ampulla (B) and isthmus (C) in luminal epithelium, in the muscle layers (ml) and in the vessels (Arrowheads). In the uterus PGRMC1 is localized in luminal (E) and glandular (F) epithelium, in the muscle layers (ml) and in the endothelial cells (Arrowheads). Note that the nuclear staining of epithelial cells is intense both in oviduct and uterus. No immunoreactivity was seen in negative control where incubation with the primary antibody had been omitted (D). Final, scale bar = 50 µm.

DISCUSSION

To the best of our knowledge, this is the first study that describes PGRMC1 immunolocalization in various parts of bovine reproductive system. In the ovary, PGRMC1 has been detected in germinal epithelium, granulosa cells, theca cells, and oocyte in all stages of folliculogenesis, in agreement with previous studies conducted in rat [28]. Data from the literature indicate that, in the ovary, PGRMC1 is continuously and highly expressed during fetal development in rat and cow and it has been suggested that PGRMC1 participates through a progesterone-dependent mechanism to the process of primordial follicle assembly [127, 138].

In this study, PGRMC1 was intensely localized in the GV of oocytes. This confirms our previous immunofluorescence study in the oocyte where PGRMC1 was restricted in the GV after follicle isolation [132]. In the oocyte, PGRMC1 has a role in oocyte maturation, which may be specifically related to the mechanism of chromosomes segregation. However, other Authors were unable to detect PGRMC1 in the bovine oocyte [133] while it was detected in the surrounding granulosa cells. This difference may be due to the different sensitivity of the antibody used and to the shorter incubation time employed in the previous study (3 h). As indicated in our study, the polyclonal rabbit anti-PGRMC1 antibody showed a much higher sensitivity respect to the polyclonal goat anti-PGRMC1 antibody and the titration assay demonstrated that only high concentrations of the polyclonal goat anti-PGRMC1 antibody were able to detect a specific signal by western blot and immunohistochemistry under our experimental conditions.

Our observations in the ovary and corpus luteum support previous studies where the expression of PGRMC1 was found in granulosa and luteal cells in human [101], mouse [122], rat [28], pig [123] and cow [124, 139]. PGRMC1 positivity in endothelial cells of the corpus luteum reported in previous study on PGRMC1 expression in cow [139] was

also confirmed by our observations.

Furthermore, in the present work, the expression of PGRMC1 in the corpus luteum was found to be influenced by the stage of the estrous cycle. In the luteal phase, PGRMC1 was intensely localized in the cytoplasm while in the follicular phase a weak PGRMC1 signal was detected in the cytoplasm of most of the cells. This stage-specific localization of PGRMC1 could be explained through the functional status of the corpus luteum. Beside its possible involvement in steroid synthesis in the active corpus luteum, the ability of PGRMC1 in promoting luteal cell viability by mediating progesterone anti-apoptotic action was demonstrated [101].

PGRMC1 was detected among the proteins that were differentially expressed in the human endometrium during the menstrual cycle [113]. This study showed that PGRMC1 protein was more highly present in the proliferative (luteal) than the secretory (follicular) phase and immunostaining data revealed that it was confined to the stromal component of proliferative phase endometrium. Analogously, microarray profiling of progesterone-regulated endometrial genes during the rhesus monkey secretory phase showed a down regulation of PGRMC1 [140]. These findings were confirmed also in mice [115] where PGRMC1 expression was up-regulated in the stromal compartment of the uterus from estrus to metestrus and reduced during diestrus. In the bovine, our study showed that PGRMC1 localization in the oviducts and in the uterus horns, was similar in the ipsilateral or the contralateral organs and was irrespective of the stage of the estrous cycle. This difference between species may be explained by the different technique adopted since the immunochemical detection of the protein must be confirmed by a quantitative analysis. Moreover, the level of the transcript must be determined in order to assess whether or not the phase-independent localization of PGRMC1 observed in this study, in bovine oviduct and uterus, is accompanied by a phase-independent PGRMC1 transcript level.

In the present study we did not observe differences in PGRMC1 localization in the uterus between the luteal and follicular phases. However, we do not exclude that these differences could arise during pregnancy. In fact, a decrease in PGRMC1 has been observed in human myometrium during term or preterm labor, where PGRMC1 down-regulation might contribute to the ‘functional withdrawal' of progesterone’s action and shift the balance to a state of increased uterine contractility [141]. Moreover, PGRMC1 was described in the human and mouse placenta and associated membranes with the most abundant expression in smooth muscle of the placental vasculature, villous capillaries and the syncytiotrophoblast suggesting that this protein functions in events important to early pregnancy, including cellular differentiation, modulation of apoptosis and steroidogenesis [115]. Therefore, the study of PGRMC1 localization and expression of PGRMC1 during pregnancy in the cow would be of particular interest since dissimilarities between species could be due to the different involvement of the endometrium in the process of placentation in these species. Rodents and primates are characterized by an haemochorial placenta while cows have a synepitheliochorial placenta. Further studies are needed in order to confirm this hypothesis.

In conclusion, the present immunohistochemical study showed that PGRMC1 is located in various compartments of the bovine female reproductive organs. Despite some difference in PGRMC1 expression have been reported in human and mouse, our data suggest that with the exception of the corpora lutea, PGRMC1 localization does not seem to be differentially regulated during different stage of the estrus cycle. However, a quantitative study must be conducted in order to clarify the phase-independency of PGRMC1 expression in this species.

9. Part II

Progesterone Receptor Membrane Component-1 Expression and Putative Function in Bovine Oocyte Maturation, Fertilization and Early Embryonic Development

Summary

Although mRNA that encodes progesterone receptor membrane component-1 (PGRMC1) is present in mammalian oocytes, nothing is known about either PGRMC1's expression pattern or function in oocytes during maturation, fertilization and subsequent embryonic development. Since in somatic cells PGRMC1 associates with the mitotic spindle, we hypothesized that PGRMC1 is involved in oocyte maturation (meiosis). Western blot analysis confirmed the presence of PGRMC1 in bovine oocytes. The present study also shows that PGRMC1 is present at the GV- and MII-stage oocyte and is associated with male and female pronucleus formation of the zygote and is highly expressed in blastocysts. A more detailed examination of PGRMC1 localization using confocal imaging demonstrated that in germinal vesicle (GV) stage oocytes, PGRMC1 was concentrated throughout the GV but did not localize to the chromatin. With the resumption of meiosis in vitro, PGRMC1 concentrated in the centromeric region of metaphase I chromosomes, while in anaphase I/telophase I stages the majority of PGRMC1 concentrated between the separating chromosomes. At the metaphase II stage, PGRMC1 re-associated with the centromeric region of the chromosomes. A colocalization study demonstrated that PGRMC1 associated with the phosphorylated form of Aurora kinase B, which localizes to the centromeres at metaphase. Finally, PGRMC1 antibody injection significantly lowered the percentage of oocytes that matured and reached the metaphase II stage after 24 h of culture. The majority of the PGRMC1 antibody-injected

oocytes arrested in the prometaphase I stage of meiosis. Furthermore in most of the PGRMC1 antibody-injected oocytes, the chromosomes were disorganized and scattered. Taken together these data demonstrate that PGRMC1 is expressed in bovine oocytes and its localization changes at specific stages of oocyte maturation. These observations suggest an important role for PGRMC1 in oocyte maturation, which may be specifically related to the mechanism by which chromosomes segregate.

INTRODUCTION

Progesterone receptor membrane component-1 (PGRMC1) is an evolutionary conserved protein that is involved in regulating numerous biological functions [4]. Recently PGRMC1 was detected among the proteins of the mitotic spindle [129], suggesting a role for it in mitosis. Interestingly, PGRMC1 is also expressed in bovine [130], rat [28] and human oocytes [128]. Moreover, the mRNA that encodes PGRMC1 is present in bovine oocytes at a very high level similar to that of actin [130]. In spite of its high level of expression, little is known about PGRMC1 and oocyte function. Since PGRMC1 is present in mitotic spindle [129], it could be directly involved in regulating oocyte maturation (meiosis).

In mammalian ovaries, oocytes are arrested in prophase of the first meiotic division (i.e. germinal vesicle stage) until the gonadotropin surge induces meiosis in oocytes of preovulatory follicles. Nuclear meiotic maturation of oocytes includes condensation of chromosomes and the sequential progression through prometaphase I, metaphase I, anaphase I and telophase I. The meiotic progression then arrests at metaphase II [142]. This sequence of meiotic events also occurs in vitro. For example in cows, fully-grown oocytes extracted from follicles larger than 2 mm are capable of resuming meiosis in vitro with approximately 90% of the oocytes reaching metaphase II [143]. Regardless of whether meiosis occurs in vivo or in vitro, this process requires the precise coordination between the chromosomes and the tubulin-based meiotic spindle.

To guarantee the coordinated interaction between the chromosomes and the meiotic spindle, the activity and localization of various spindle-associated proteins is strictly regulated [144]. One important spindle-associated protein is Aurora B (AURKB). AURKB is a protein kinase that is a component of the chromosomal passenger complex (CPC), which also consists of an inner centromere protein, INCENP, which binds AURKB [145]. During mitosis, AURKB plays a critical role in chromosome-microtubule interactions

[146]. This kinase is localized to the kinetochores from prophase to metaphase and relocates to the central spindle and the mid-body in cytokinesis [147]. The altered expression AURKB is implicated in defects of centrosome function, spindle assembly, and chromosomal instability [148, 149].

AURKB is also expressed in mammalian oocytes [150-153]. It gradually becomes phosphorylated on Thr-232 during oocyte maturation. Phosphorylation of AURKB activates this kinase and leads to the phosphorylation of Histone H3 and the gradual condensation of chromatin [150, 152, 154]. Moreover, altered AURKB activity increases the risk in chromosome congression failure, non-disjunction and aneuploidy in mammalian oocytes [155].

Since PGRMC1 is highly expressed in bovine oocytes and zygotes [130] and it is localized on the mitotic spindle [129], we hypothesized that PGRMC1 is involved in meiosis. The present studies were designed to determine the role of PGRMC1 in oocyte meiotic division by monitoring its 1) localization during oocyte in vitro maturation, fertilization and early development, 2) relationship to changes in the activity of AURKB and 3) function by injecting an antibody to PGRMC1 into oocyte prior to in vitro maturation.

MATERIAL AND METHODS

Oocyte Collection and Culture

All the chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO) except for those specifically mentioned. Bovine ovaries were recovered at the abattoir (INALCA JBS 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^{-1}) maintained at 26°C. All subsequent procedures, unless differently specified, were performed at 35-38°C. Cumulus-oocyte complexes (COCs) were retrieved from mid-sized antral follicles (2-6 mm) with a 16-gauge needle mounted on an aspiration pump (COOK-IVF, Brisbane QLD, Australia). COCs were washed in TCM199 supplemented with HEPES buffer (20 mM HEPES, 1790 units/L Heparin and 0.4% of bovine serum albumin (BSA)) and examined under a stereomicroscope. Only COCs medium-brown in color, with five or more complete layers of cumulus cells with oocytes with finely granulated homogenous ooplasm were used.

Groups of 25-30 COCs were in vitro matured in TCM-199 supplemented with 0.68 mM L-glutamine, 25 mM $NaHCO_3$, 0.4% BSA fatty acid free, 0.2 mM sodium pyruvate and 0.1 IU/ml of recombinant human FSH (r-hFSH, Gonal-F, Serono, Rome, Italy) in humidified air under 5% CO_2 at 38.5°C as previously described [156]. Oocytes were mechanically separated from cumulus cells either just after collection from ovaries (immature oocytes at GV stage) or after 4, 8, 12, 16, 20 and 24 h of culture.

In Vitro Fertilization and Embryo Culture

After 24h of in vitro maturation, oocytes were fertilized as previously described [156]. Briefly, the contents of a straw of cryopreserved bull spermatozoa (CIZ, S. Miniato Pisa, Italy) was thawed and cells separated on a 45-90% Percoll gradient. Sperms were counted and diluted to a final concentration of $0.5x10^6$ spermatozoa/ml in fertilization medium that was a modified Tyrode's solution (TALP) supplemented with 0.6% (w/v) BSA fatty acid free, 10 µg/ml heparin, 20 µM penicillamine, 1 µM epinephrine, and 100 µM hypotaurine. COCs and sperms were incubated for 18 hours at 38.5° C under 5% CO_2 in humidified air. After in vitro fertilization, presumptive zygotes were washed and cumulus cells were removed by vortexing for 2 min in 500 µl of a modified synthetic oviduct fluid (SOF, [157]) supplemented with 0.3% (w/v) BSA fraction V, fatty acid free, MEM essential and non-essential aminoacids, 0.72 mM of sodium pyruvate, and buffered with 10 mM of HEPES and 5 mM of $NaHCO_3$. Presumptive zygotes were rinsed and transferred in embryo culture medium, which was SOF buffered with 25 mM of $NaHCO_3$, supplemented with MEM essential and non-essential aminoacids, 0.72 mM of sodium pyruvate, 2.74 mM of myo-inositol, 0.34 mM of sodium citrate and with 5% of calf serum (Gibco, Invitrogen, San Giuliano Milanese, Milan, Italy). Incubation was performed at 38.5° C under 5% CO_2, 5% O_2 and 90% N_2 in humidified air. Zygotes and blastocyst stage embryos were collected after 18 and 196 hours post insemination, respectively [158].

Western Blot Analysis

Freshly isolated COCs were denuded by vortexing in 500 µl of TCM199 supplemented with 20 mM HEPES and 5% calf serum (Gibco, Invitrogen, Life Technologies, Carlsbad, CA, USA) for 2 min at 35 Hz. Oocytes (500-700 per extracts) and aliquots of 50 mg of ovarian cortex were homogenized in lysis buffer [10 mM Tris-Cl, pH 4,

1% w/v NP-40, 150 mM NaCl, imM EDTA, 0.2% Zwitterion], which was supplemented with complete protease inhibitor cocktail and incubated for 1 hour on ice then centrifuged at 14000 g for 15 min. The supernatant was then mixed with 4X SDS-Laemmli loading buffer and extensively boiled for 15 minutes. After centrifugation for 5 min at 14000 g, each cell extract was loaded onto 12% acrylamide gel and transferred to nitrocellulose. The nitrocellulose was then incubated with 5% nonfat dry milk powder overnight at 4° C to saturate nonspecific sites. The nitrocellulose blot was incubated with polyclonal rabbit anti-PGRMC1 (Prestige antibodies, Sigma) for 2 hours at room temperature at a dilution of 1:500. Western blots were processed using a horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000) for 45 minutes at room temperature and developed using HRP Immuno-Blot Assay kit (Bio-Rad Laboratories, Segrate, Milan, Italy).

Immunofluorescence Staining

Indirect immunofluorescence was carried out to evaluate the cellular localization of PGRMC1, Aurora Kinase B (AURKB) and Aurora Kinase B phosphorylated on Threonine 232 (phospho-Thr^{232} AURKB) in oocytes at different stages of meiosis (See Table 2). All the oocytes and the zygotes were freed of cumulus cells by vortexing. Then the zona pellucida was removed from oocyte, zygotes and embryos using 0.5% pronase as previously described [159]. After being washed three times in phosphate buffered saline containing 0.1% polyvinylalchol (PBS-PVA), the oocytes, the zygotes and the embryos were fixed in 4% paraformaldehyde in PBS for 15 min at 37°C, then transferred to 4°C for the next 45 min. The fixed oocytes, zygotes and embryos were washed three times with PBS-PVA and permeabilized with 0.3% Triton-X 100 in PBS for 10 min at room temperature. Non-specific binding was blocked by incubating the samples in 10% donkey serum, 1% BSA in PBS for 30 min at room temperature. The samples were then incubated with the primary antibody overnight at 4°C. PGRMC1 immunolocalization was

carried out using a rabbit polyclonal anti-PGRMC1 antibody (Prestige Antibodies by Sigma). For the co-localization studies, the oocytes were incubated either with a solution of rabbit polyclonal anti-PGRMC1 antibody and mouse monoclonal anti-AURKB antibody (Abcam, Cambridge, UK) or with a solution of mouse monoclonal anti-AURKB antibody and rabbit polyclonal anti-phospho-Thr^{232} AURKB antibody (BioLegend, San Diego, CA, USA). All the primary antibodies were diluted 1:50 in PBS containing 1% BSA. After being washed three times in PBS-PVA for 10 min each, the oocytes were incubated with TRITC labeled donkey anti rabbit antibody (dilution 1:100; Vector Laboratories, Inc., Burlingame, CA, USA) or, for the colocalization experiment, with a solution of Alexa Fluor 488 labeled donkey anti mouse antibody (dilution 1:500; Invitrogen, Life Technologies, Carlsbad, CA, USA) and TRITC labeled donkey anti rabbit antibody for 30 min at room temperature. The samples were washed 3 times in PBS-PVA and mounted on slides in the antifade medium Vecta Shield (Vector Laboratories Inc., Burlingame, CA, USA) supplemented with 1 µg/ml DAPI. Samples were analyzed on a Nikon Diaphot epifluorescence microscope or a Nikon C1si confocal laser-scanning microscope (Nikon Corp. Tokyo, Japan). In each experiment, negative controls were performed by adding an equal concentration of rabbit IgG (Vector Laboratories) or omitting the primary antibodies and did not reveal any staining.

PGRMC1 Blocking Antibody Injection Study

The anti-PGRMC1 antibody (Prestige Antibodies by Sigma) was spin-dialyzed and concentrated using 10kD Millipore Ultrafree 0.5 PBGC filter unit (Millipore Corp., Bedford, MA, USA). GV stage oocytes within their cumulus cell complexes were injected with 15-35 pl of a 1.2 mg/ml stock solution of anti-PGRMC1 antibody in PBS. This resulted in a final concentration of 0.15-0.30 µM of antibody in the oocyte. The injected volume represented approximately 1.5-3% of oocyte volume. As control a group of COCs in each experiment

was injected with an equal volume of rabbit IgG (Vector Laboratories) dissolved in PBS at the same concentration or PBS only. The procedure was carried out as previously described in mouse [160, 161] and adapted to bovine oocyte volume. A microinjection apparatus (Narishige CO LTD, Tokyo, Japan) was used to guide the holding and injecting micropipettes into a 50 µl drop of TCM199-HEPES buffer covered with mineral oil. The injecting pipette was connected to a FemtoJet (Eppendorf Srl, Milano, Italy) that allowed the injection of minute quantities of solution. After being injected, COCs were washed in TCM199-HEPES buffer and matured in vitro for 24 h as above described. After the maturation time, the oocytes were freed of cumulus cells and fixed in 500 µl of 60% Methanol in PBS for 30 min at 4°C. The oocytes were stained with 0.5 mg/ml of Propidium Iodide to assess the stage of meiotic progression by observation at 200-400x under fluorescence microscopy [156].

Statistical Analysis

All experiments were replicated at least three times. Observations from all the experiments were pooled. The images shown in each figure are representative. The actual number of oocytes at each stage of maturation that were analyzed is provided in the legend of each figure. Differences in maturation rate in the PGRMC1 blocking antibody injection study were analyzed by Chi square test. Probabilities of less than 0.05 were considered statistically significant.

RESULTS

PGRMC1 Expression in Oocytes, Zygotes and Embryos

As shown in Figure 10, western blot analysis demonstrated that PGRMC1 was specifically detected in bovine oocytes as a ≈ 26 kDa protein. Having established that this antibody specifically detects bovine PGRMC1, it was utilized to localize PGRMC1 in bovine oocyte undergoing meiosis in vitro. Note that under culture conditions employed in these studies, GV stage oocyte resume meiosis with 93% of the oocytes reaching metaphase II by 24 h of culture (Table 2). The rate of in vitro fertilization and blastocyst development was respectively 89.5% and 37.3%. In a preliminary study, PGRMC1 was localized at the time of collection or after in vitro maturation, in vitro fertilization and embryo culture, and then observed in epifluorescence microscopy. As shown in Figure 11, PGRMC1 was present within GV in immature oocytes and associated with the MII plate of in vitro matured oocytes. After IVF, PGRMC1 was associated with the female and the male pronucleus. At the blastocyst stage, virtually all the blastomeres showed positive staining for PGRMC1.

Having confirmed that PGRMC1 was present in bovine oocytes and embryos, changes in the localization of PGRMC1 during oocyte maturation was examined in more detail utilizing confocal microscopy. This analysis revealed that PGRMC1 associates with the metaphase II chromosomes at distinct focal points (Figure 12), which suggests that PGRMC1 localizes to the centromere.

As shown in Figure 11 PGRMC1 was not detectable in the ooplasm. In fact, the fluorescent staining of the cytoplasm in the IgG control was similar to that observed in the PGRMC1 antibody immunostained group (compare Figure 11 A and B).

Table 2. Sequential meiotic stages during bovine oocyte in vitro maturation.

Time* (h)	*n*	Pro I	Promet I	Met I	Ana I	Telo I	Met II
0	68	**99**	1				
4	76	**64**	36				
8	71	25	**62**	13			
12	72	10	39	**43**	7	1	
16	70		6	30	**37**	17	10
20	76	1	3	12	13	**32**	39
24	72				3	4	**93**

*Time in culture. Values in **bold** indicate the highest percentage of oocytes in specific stages of meiosis for each time in culture. Pro I, prophase I; Promet I, Prometaphase I; Met I, metaphase I; Ana I, anaphase I; Telo I, telophase I; Met II, metaphase II.

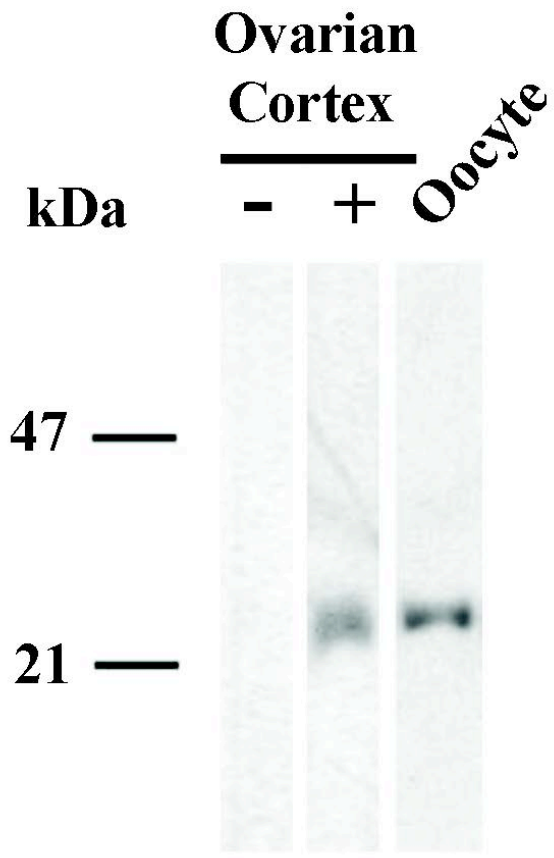


Figure 10. Detection of PGRMC1 in bovine oocytes. Western blot analysis showing PGRMC1 expression in bovine oocytes. A western blot using lysate prepared from bovine ovarian cortex, conducted in the absence of the PGRMC1 antibody, is shown as a negative control (-) while a western blot using this lysate conducted in the presence of the PGRMC1 antibody, is shown as a positive control (+). Western blot using lysate prepared from bovine oocytes is shown in lane 3. Representative blots from three independent experiments are shown.

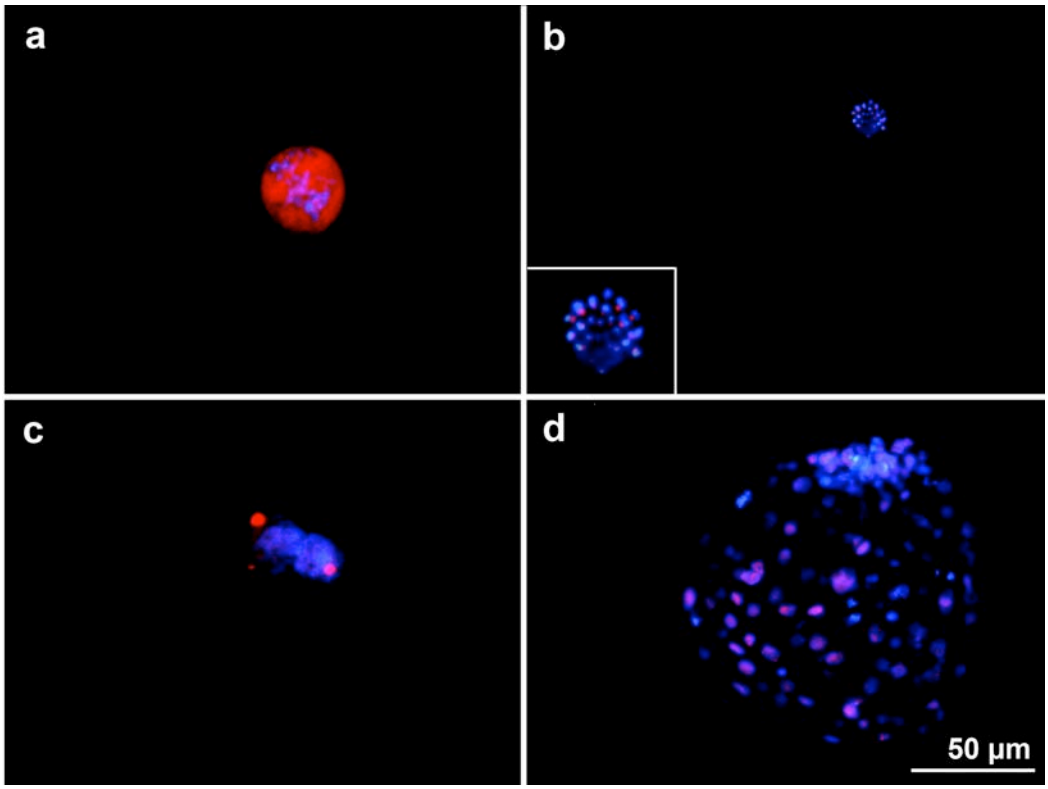


Figure 11. PGRMC1 localization in prophase I (GV stage) (a), metaphase II oocyte (b), pronuclei stage (c) and blastocyst stage embryo (d). Note that PGRMC1 appears to associate with germinal vesicle in immature oocyte and with the metaphase chromosomes in MII oocyte. In (b) the insert shows the magnified metaphase II plate. In zygotes, PGRMC1 is associated with male and female pronucleus and is localized in the blastomeres up to the blastocyst stage of development. Representative images in epifluorescence are shown.

This immunocytochemical analysis was conducted on a total of 98 oocytes at the following stages: 39 in prophase I; 27 in metaphase II I; 17 in pronuclear; 15 in blastocyst.

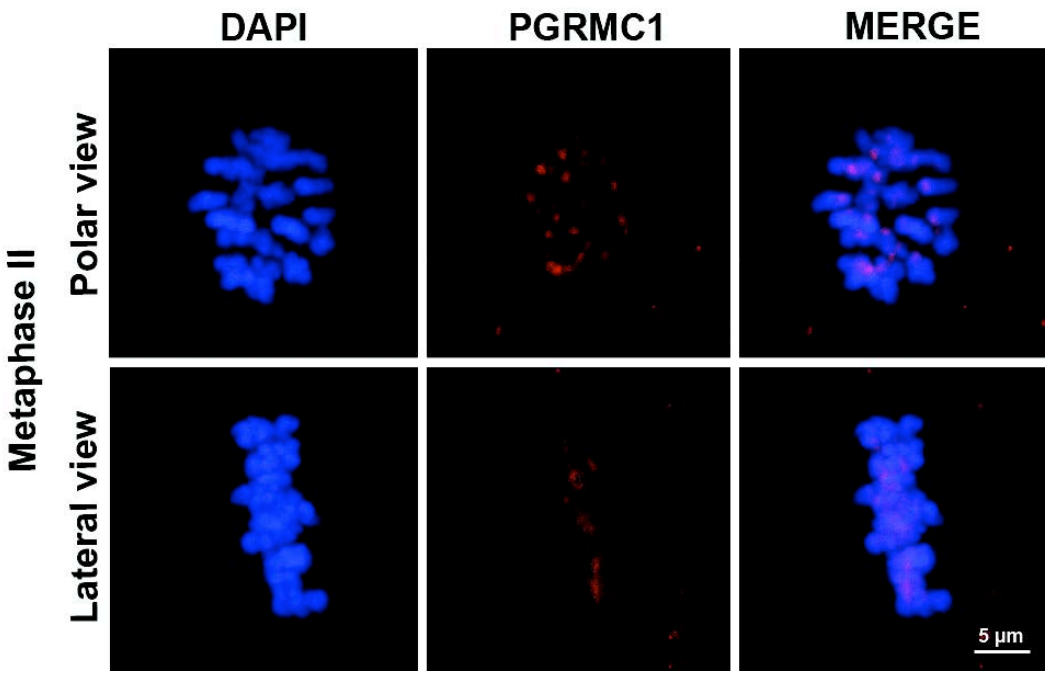


Figure 12. PGRMC1 localization in metaphase II stage oocytes. Polar and lateral views are shown. Images were obtained by scanning the sample on z-axis at 0.2 µm of thickness throughout the plane of focus containing the chromosomal equatorial plane. The z-series were then projected to obtain a three dimensional image using the software provided by Nikon Microscopes Inc. PGRMC1 appears to associate with the metaphase chromosomes at precise focal points, consistent with a centromeric localization. The localization of PGRMC1 in 90 oocytes metaphase II was examined. Representative images are shown.

PGRMC1 Expression in Oocytes During Meiosis in vitro and PGRMC1 and AURKB Colocalization

To test this assumption, PGRMC1 was colocalized with AURKB, which is known to associate with proteins that comprise the centromere at metaphase [145]. This study

demonstrated that in prophase I (GV stage) oocytes, PGRMC1 and AURKB were highly expressed within the GV, but neither protein associated with the chromatin. At prometaphase I (i.e. after GV breakdown), both proteins began to associate with the chromosomes and this association was more evident in metaphase I oocytes. During anaphase I/telophase I stages, both proteins dissociated from the chromosomes and concentrated in the midzone and the midbody of the separating chromosomal plates. At anaphase I, these two proteins colocalized throughout the midzone. During telophase, some AURKB colocalized to PGRMC1 at the center of the midbody between the chromosomal plates. Interestingly some AURKB at the periphery of the midbody was not colocalized with PGRMC1. PGRMC1 and AURKB appeared to localize to the spindle pole in some oocytes. However, this was not consistently observed and its functional significance, if any, remains to be determined in further studies. Finally at the metaphase II stage, both PGRMC1 and AURKB again colocalized to the centromeric region of the chromosomes (Figure 13).

In a third study, the relationship between the AURKB and the Thr-232 phosphorylated (active) form of AURKB, was assessed. This study illustrated that AURKB began to associate with the chromatin in all of the prometaphase I oocytes, but the active form of AURKB was only barely detectable in about half of these oocytes. By metaphase I, staining for phosphorylated AURKB was readily detectable in all the oocytes and remained so up to the metaphase II stage (Figure 14). Importantly after GV breakdown, PGRMC1 was always concentrated to those subcellular compartments where the active, phosphorylated form of AURKB was present. This was particularly evident at the telophase I stage (compare Figure 13 and 14).

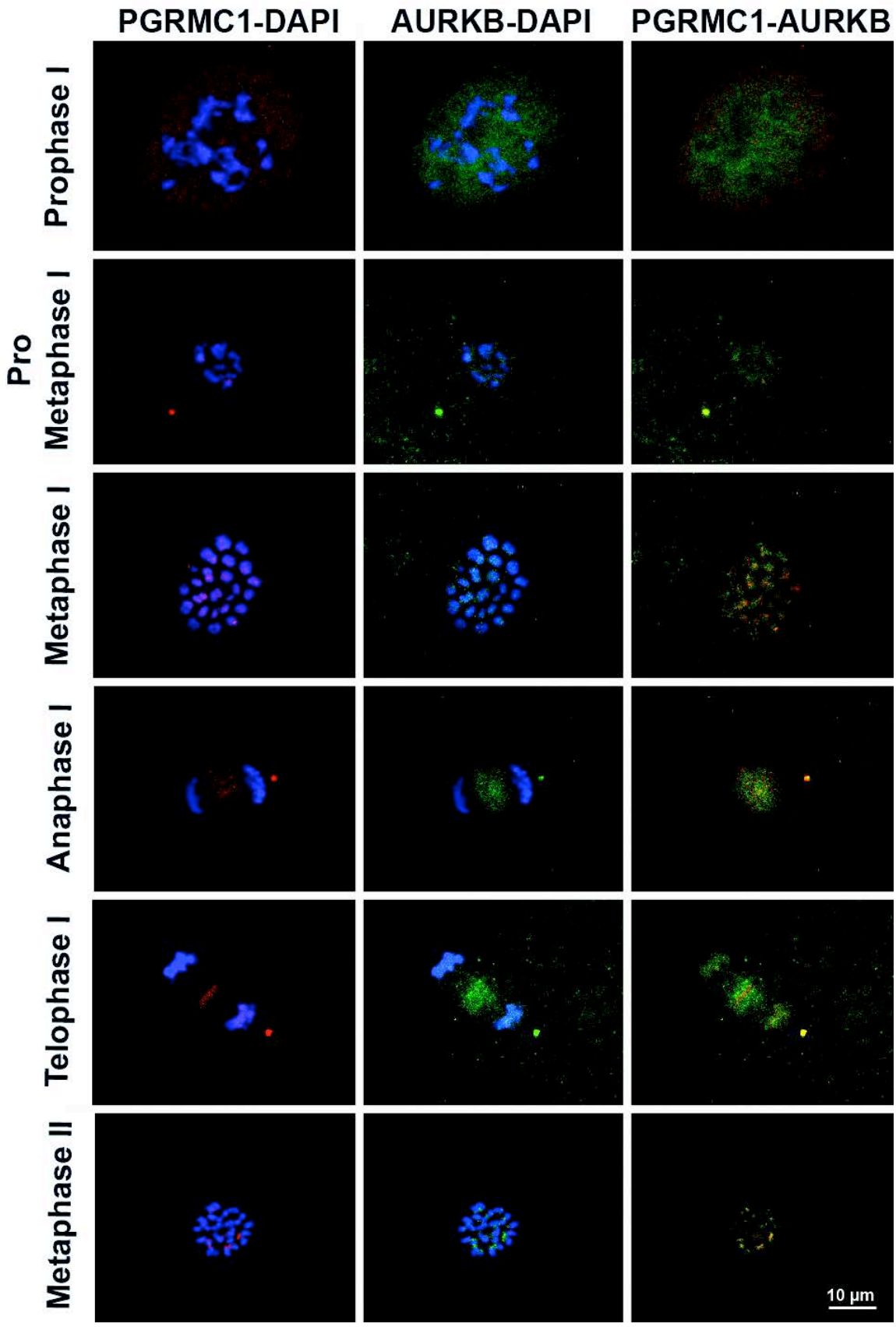


Figure 13. (legend on next page)

Figure 13. Colocalization of PGRMC1 and AURKB during bovine oocyte maturation. Representative confocal images showing PGRMC1 and AURKB localization at the indicated meiotic stages, from the prophase I (GV stage) to the metaphase II. Images were cropped to show the area containing the chromatin. PGRMC1 (red) and AURKB (green) immunostaining are merged with DAPI (DNA; blue) counterstained image to visualize the specific protein-chromosomes associations. In the right column PGRMC1 and AURKB images are merged to reveal sites of their colocalization (orange). Note that the images of anaphase I and telophase I are in lateral view in order to better illustrate the relationship between PGRMC1 and AURKB at the midzone and the midbody. The images of metaphase I and metaphase II are shown in a polar view in order to better illustrate the relationship between PGRMC1, AURKB and the chromosomes. This immunocytochemical analysis was conducted on a total of 199 oocytes at the following stages: 21 in prophase I; 17 in prometaphase I; 39 in metaphase I; 38 in anaphase I; 34 in telophase I and 50 in metaphase II.

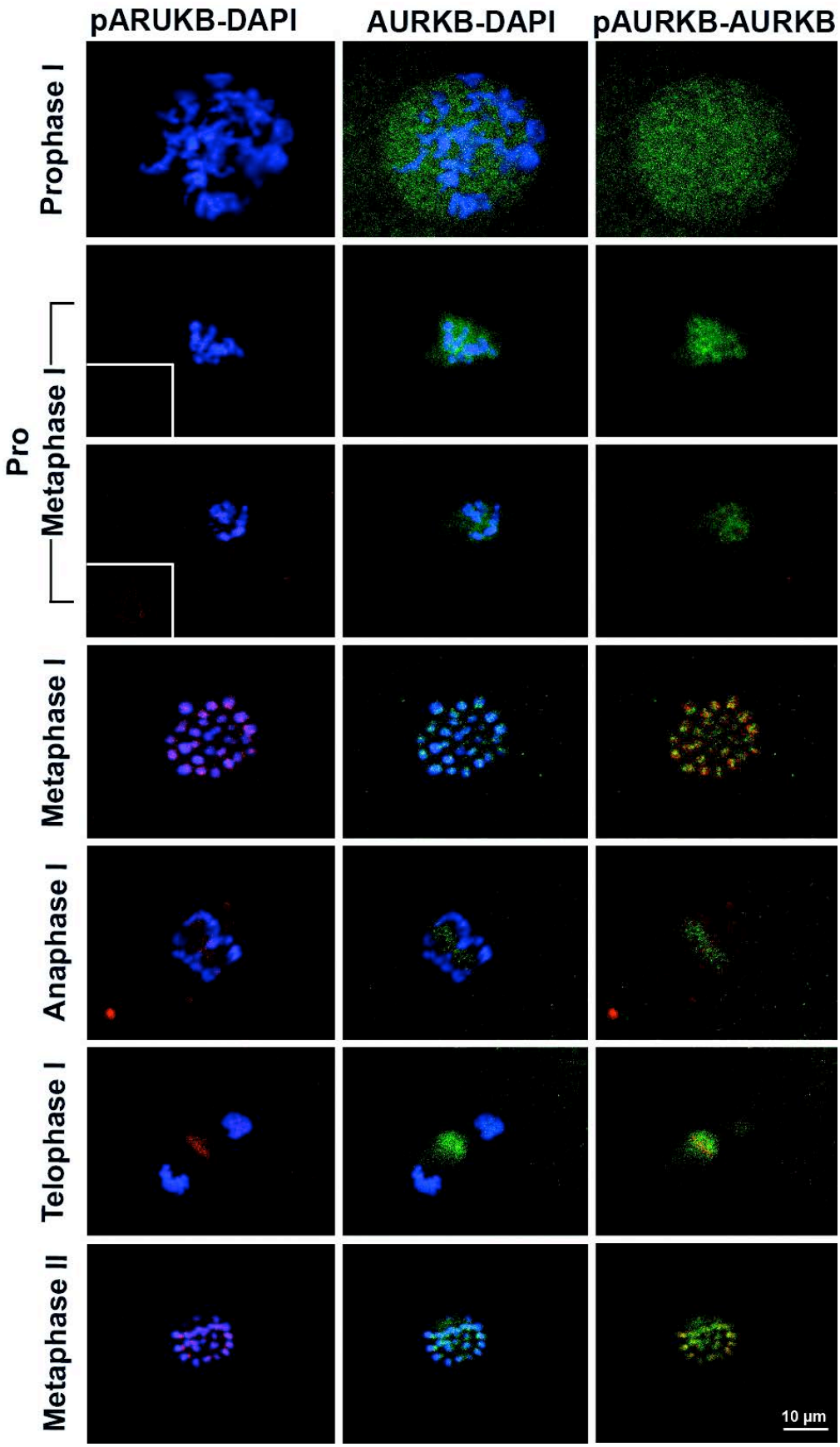


Figure 14 (legend on next page)

Figure 14. Colocalization of AURKB and phosphorylated AURKB during bovine oocyte maturation. Representative confocal images showing the relationship between AURKB and the active (phosphorylated) form of AURKB (pAURKB) from the prophase I (GV stage) to the methaphase II stages of meiosis. pAURKB (red) and AURKB (green) immunostaining are merged with DAPI (DNA; blue) counterstained image. In the right column pAURKB and AURKB images are merged to reveal sites of their colocalization (orange). Images were cropped to show the area containing the chromatin. The inserts in the prometaphase stage are images of pAURKB staining. This immunocytochemical analysis was conducted on a total of 239 oocytes (38 in prophase I; 18 in prometaphase I; 43 in metaphase I; 41 in anaphase I; 53 in telophase I and 46 in metaphase II.

PGRMC1 and Oocyte Maturation

To begin to assess the role of PGRMC1 on oocyte maturation, GV stage oocytes within their cumulus cell mass were injected with antibody to PGRMC1 while controls were injected with either IgG or PBS. After 24h, only 22% (n=68) of the oocytes injected with the PGRMC1 antibody progressed to metaphase II compared to 74% (n=39; $p < 0.05$) and 68% (n=43; $p < 0.05$) of PBS or IgG-injected oocytes, respectively (Figure 15A). A high proportion of PGRMC1 antibody-injected oocytes (57.4%) arrested in prometaphase I stage. Some of the PGRMC1 antibody-injected oocytes progressed beyond prometaphase I as 5.8 (n=4), 8.8 (n=6) and 22% (n=15) reached metaphase I, anaphase/telophase I and metaphase II respectively, with most of these possessed chromosomes that were disorganized and scattered throughout the ooplasm (compare Figures 15C with 15B).

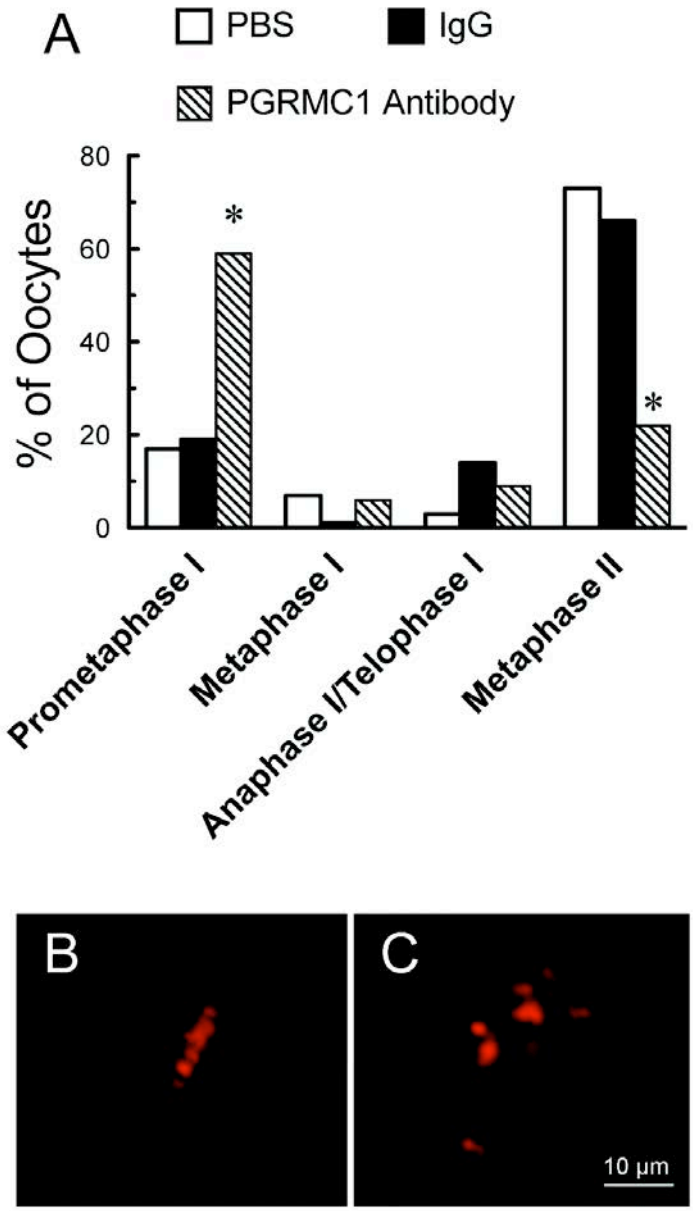


Figure 15. Effect of PGRMC1 antibody injection on bovine oocyte maturation. * indicates a value that is significantly difference ($p<0.05$) from either PBS or IgG. After 24 h of culture, oocytes were stained with propididum iodide (red) to reveal the chromosomes. Panel B shows chromosomes that are precisely arranged in a metaphase plate (i.e. control) while panel C shows a bovine oocyte 24 h after being injected with PGRMC1 antibody. Note that the chromosomes are not aligned and disorganized.

DISCUSSION

The present study confirms and expands a previous mRNA-based observation [130] by demonstrating that PGRMC1 is present in bovine oocytes as a ≈ 26 kDa protein. This is consistent with PGRMC1 western blots obtained from lysates of other organs and cell types such as porcine liver [40], human and rat granulosa and luteal cells [28, 101], mouse and human smooth muscle, uterus and placenta [115]. The present study also shows that PGRMC1 is present at the GV- and MII-stage oocyte and is associated with male and female pronucleus formation of the zygote and is highly expressed in blastocysts. Specifically prior to fusion of the pronuclei, PGRMC1 localizes to several foci that appear to be within 'nucleolar precursor bodies (NPBs)' [162]. NPBs have been observed at the pronuclei of bovine zygotes and apparently function in nucleolus formation [163]. Moreover, NPBs are the sites of ribosomal RNA synthesis with rRNA encoding polypeptides they comprise ribosomes and therefore are essential for the synthesis of proteins that ultimately direct embryonic development [164].

Because fertilization and embryonic development are dependent on successful oocyte maturation, the present work focused more intensely on changes in PGRMC1 after the resumption of meiosis. These studies, utilizing confocal microscopy, reveal that PGRMC1 starts to concentrate on the chromatin. This becomes more evident at metaphase I, where PGRMC1 is restricted to the centromeric region of bivalent chromatids. During anaphase I and telophase I, PGRMC1 dissociates from the chromosomes and localizes in the midzone and midbody, respectively. At metaphase II, PGRMC1 again concentrates at the centromeric region of the chromosomes. These temporal changes in the localization of PGRMC1 during oocyte maturation suggest an important role for PGRMC1 in the process that governs meiotic progression of the oocyte.

That PGRMC1 localizing to the chromosomes is important, is supported by the finding that PGRMC1 colocalizes with AURKB at specific stages of the oocyte meiotic

progression. It is well known that AURKB is expressed during oocyte meiosis in cows [153], mice [151, 152, 155] and pigs [150]. In general, our observations on AURKB are in agreement with the known changes in the localization of AURKB that occur during oocyte meiotic division. The one notable exception is that Uzbekova and co-authors [153] failed to detect AURKB within the GV, while our studies did. The use of a different AURKB antibody and/or a different fixation protocol likely account for the differences observed in these two studies.

While the issue of AURKB within the GV remains to be resolved, it is important to appreciate that AURKB is a serine/threonine protein kinase that regulates many processes during mitosis and meiosis [155, 165, 166]. In meiosis, AURKB is involved in chiasma resolution and its activation (i.e. phosphorylation on Thr-232) is correlated with cytokinesis and first polar body formation [155, 165]. In addition treatment with a pharmacological inhibition of AURKB results in the failure to extrude the first polar body. Furthermore in porcine oocytes, AURKB activity increases after prometaphase I reaching a maximum at metaphase I and thereafter is maintained until metaphase II [150]. The present studies are consistent with these observations and further reveal that during telophase I AURKB localizes within the spindle midbody. Interestingly not all of the AURKB within the spindle midbody is active. Rather only the AURKB at the center of the midbody is active (phosphorylated). Interestingly the active fraction of AURKB appears to colocalize with PGRMC1. This implies that both PGRMC1 and AURKB are involved in the mechanism by which asymmetric cytokinesis occurs during first polar body formation.

The fact that PGRMC1 only appears to colocalize with active AURKB raises the issue of whether PGRMC1 is essential for the activation of AURKB. Recently a fluorescence resonance energy transfer (FRET)-based assay was used to assess phospho-Thr^{232} AURKB activity in anaphase HeLa cells. Similar to our observations in the bovine oocycte, this study revealed that most of the AURKB activity localized to the center

of the midbody [167]. These authors suggest that the spindle or spindle-associated proteins are responsible for activating AURKB. Although a previous study indicated that PGRMC1 is associated with the mitotic spindle [129], a role of PGRMC1 in activating AURKB during mitosis and meiosis can be only hypothesized and considerably more studies must be conducted to resolve this issue.

In addition, PGRMC1 may be involved in meiotic events that precede polar body formation, since the present data demonstrate that PGRMC1 associates with chromatin and colocalizes with AURKB at metaphase I and metaphase II. AURKB is a component of the chromosomal passenger complex (CPC), which in part consists of the inner centromere protein, INCENP, to which AURKB is physically associated [145]. Furthermore, altered AURKB expression has been suggested to increase the frequency of non-disjunction and aneuploidy in mammalian oocytes [155] while an over-expression prevents chromosome misalignment during meiosis of mouse oocytes [151]. Collectively, these findings are consistent with the concept that the interaction between PGRMC1 and AURKB plays a role in AURKB's ability to promote homologous chromosome alignment and segregation.

This concept is supported by two observations. First, the present work demonstrates that PGRMC1 concentrates at foci on the chromatin of prometaphase I-staged oocytes and at this time, AURKB starts to be phosphorylated. As meiosis progresses, both PGRMC1 and the phosphorylated form of AURKB become concentrated on the centromeric region. Since pharmacological inhibition of AURKB activity leads to abnormal chromosome congression and alterations in the formation of the chromosomal plate [152, 155], this implicates that PGRMC1-AURKB interaction is involved in the mechanism by which chromosomal separation is mediated.

The second and probably more important observation supporting a functional role for PGRMC1 in oocyte meiotic maturation is provided by the PGRMC1 antibody injection

study. In this study, PGRMC1 antibody injection at GV stage results in the improper positioning of the chromatin, affecting the ability of the chromosomes to condense properly and resolve bivalents. While these observations support a role for PGRMC1 in meiosis, they also demonstrate the need to define the exact nature of the relationship between PGRMC1 and active AURKB.

The present studies also supports the hypothesis that changes in the level of PGRMC1 expression or its genetic structure could predispose oocytes to undergo abnormal meiotic division, which would lead to errors in chromosome segregation and ultimately aneuploid embryos. Interestingly, there are at least two conditions in women in which PGRMC1 is altered. The first is a reduction in the level of PGRMC1 expression observed in women with an X;autosome translocation (i.e. [t(X;11)(q24;q13)]). This X-chromosome translocation likely depletes one copy of *PGRMC1*, thereby accounting for the reduced level of PGRMC1 in these women [6]. The second condition involves a single base mutation in which the histidine at residue 165 is changed to arginine. Although this mutation does not change the level of PGRMC1 expression, it does reduce its functional activity by approximately 50% [6]. Both of these genetic alterations in PGRMC1 are detected in woman with premature ovarian failure [6]. Whether these alterations in PGRMC1 expression or genetic structures adversely affects the functional capacity of the oocyte remains to be determined.

In summary these data demonstrate that PGRMC1 is expressed in bovine oocytes, zygotes and blastocysts. Our data also suggest an important role for PGRMC1 in oocyte maturation that may be specifically related to the mechanism by which chromosomes segregate and the first polar body extruded. These studies also imply that PGRMC1 and AURKB interact at specific points in the meiotic cascade to insure that errors in spindle formation, chromosome segregation and cytokinesis do not occur. These possibilities are currently under investigation.

10. Part III

Oocytes isolated from dairy cows with reduced ovarian reserve have a high frequency of aneuploidy and alterations in the localization of Progesterone Receptor Membrane Component-1 and Aurora Kinase-B

SUMMARY

Oocytes isolated from cows of reproductive age with reduced antral follicle counts (AFC) have a diminished capacity of embryonic development, which may be related to alterations in the mechanism that directs the proper segregation of chromosomes. Since we demonstrated that Progesterone Receptor Membrane Component 1 (PGRMC1) is involved in chromosome congression and metaphase II (MII) plate formation, the present study was designed to determine 1) if the decrease in oocyte developmental competence observed in dairy cows with a reduced AFC is due to a higher incidence of aneuploidy and 2) whether alterations in PGRMC1 contributes to the incidence of aneuploidy. Oocytes from ovaries with reduced AFC and aged-matched controls were matured in vitro and the occurrence of aneuploidy determined as well as the mRNA level and localization of PGRMC1. Although oocytes from ovaries with reduced AFC were capable of undergoing meiosis in vitro, these oocytes showed a 3-fold increase in aneuploidy compared to oocytes isolated from control ovaries ($P<0.05$). Although *Pgrmc1* mRNA levels were not altered, PGRMC1 and AURKB failed to localize to precise focal points on MII chromosomes of oocytes from ovaries with reduced AFC. Further, when oocytes of control ovaries were cultured with an inhibitor of AURKB activity, their MII plate was disrupted and PGRMC1 was not properly localized to the chromosomes. These results suggest that alterations in PGRMC1 and/or AURKB localization account in part for the increased aneuploidy and low development competence of oocytes from ovaries with reduced AFC.

INTRODUCTION

Premature ovarian senescence as estimated by a reduced number of antral follicle count (AFC) is a common cause of infertility in both woman and cattle [10, 168]. Low AFC has been described in women in peri-menopausal period [169, 170] as well as in young infertile and subfertile women affected by premature ovarian failure [9, 10] suggesting that the factors that reduce follicular reserve also affect the quality of the remaining oocytes [171].

Recently, we demonstrated that about 5% of culled dairy cows 4-8 years old had a reduced AFC compared to aged-matched controls [11]. Moreover, oocytes isolated from low AFC ovaries had limited developmental capability compared to aged-matched controls [11, 172, 173]. The mechanism responsible for the reduced developmental competence of these oocytes is still unclear and it may be related to alterations in the mechanism that directs the proper segregation of chromosomes.

During oocyte meiosis, the cell division machinery must function sequentially during a reductive (meiosis I) and equational (meiosis II) cell division to ultimately produce a haploid oocyte that can be fertilized and give rise to the next generation. Errors in this process can result in aneuploidy, which can have adverse reproductive outcomes such as infertility, miscarriages, and birth defects [174-177].

Aurora kinase B (AURKB) is a component of the chromosomal passenger complex, which together with the spindle assembly checkpoint ensures faithful chromosome segregation [144, 178]. During mitosis, AURKB plays a critical role in chromosome-microtubule interactions [146]. This kinase is localizes to the kinetochores from prophase to metaphase and relocates to the central spindle and the mid-body during cytokinesis [147]. Alterations in the localization of AURKB are implicated in defects of centrosome function, spindle assembly and chromosomal instability [148, 149].

Interestingly, Progesterone Receptor Membrane Component 1 (PGRMC1) and the phosphorylated (active) form of AURKB co-localize at the centromere of chromosomes in MII oocytes [179]. Moreover, an injection of a PGRMC1 antibody impairs the ability of oocytes to successfully mature and causes abnormalities in chromosomal segregation such as chromosomes misalignment and disorganization [179], suggesting that PGRMC1 plays a key role in chromosomes congression.

Therefore, the present study was designed to determine 1) if the decrease in oocyte developmental competence observed in dairy cows with a reduced AFC is due to a higher incidence of aneuploidy and 2) whether alterations in PGRMC1 and AURKB localization contributes to the incidence of aneuploidy.

MATERIAL AND METHODS

Oocyte Collection and Culture

All the chemicals used in this study were purchased from Sigma Chemical Company (St. Louis, MO) except for those specifically mentioned. Bovine ovaries were recovered at the abattoir (INALCA S.p.A., Ospedaletto Lodigiano, LO, IT 2270M CE, Italy) from pubertal females (4-8 years old) subjected to routine veterinary inspection and in accordance to the specific health requirements stated in Council Directive 89/556/ECC and subsequent modifications. As previously stated [180] ovaries were transported at 26°C and all subsequent procedures were performed at 35-38°C. From each slaughtered animal, ovaries were isolated and classified into the two previously described categories [11, 172, 173]: ovaries with reduced AFC (<10 mid-sized antral follicles of 2-6 mm) and age-matched controls (>10 follicles of 2-6 mm).

Cumulus-oocyte complexes (COCs) were retrieved from mid-sized antral follicles (2-6 mm) and examined under a stereomicroscope. Only COCs medium-brown in color, with five or more complete layers of cumulus cells with oocytes with finely granulated homogenous ooplasm were used [180].

Groups of 15-30 COCs were in vitro matured in TCM-199 supplemented with 0.68 mM L-glutamine, 25 mM $NaHCO_3$, 0.4% BSA fatty acid free, 0.2 mM sodium pyruvate and 0.1 IU/ml of recombinant human FSH (r-hFSH, Gonal-F, Serono, Rome, Italy) in humidified air under 5% CO_2 at 38.5°C as previously described [156].

Aneuploidy Assessment

Karyotype analysis was performed as previously described [181] with some modifications and all chromosome preparation procedure was performed at 26°C, unless

otherwise stated [182]. After IVM, oocytes were mechanically separated from cumulus cells by 5 minutes vortexing. Only oocytes with polar body were selected for karyotype analysis and incubated for 20 minutes. Each oocyte was placed in a hypotonic solution (0.075 M KCl) for 20 minutes to induce nuclear swelling. Subsequently oocytes were gently transferred to a well with the first fixative (methanol:acetic acid:distilled water, 5:1:4, v/v) for 5 minutes. Then they were dropped from 5 cm to a wet slide heated at 37°C and 45 degree sloped. When the drop was spread out, the oocytes were covered with the second fixative (methanol: acetic acid, 3:1, v/v) and then the slides were immersed in a coplin jar containing the second fixative for 10 minutes. After that they were transferred to a second jar containing the third fixative (methanol : acetic acid : distilled water, 3:3:1, v/v) for 1 minute then the slides were slowly removed and air-dried overnight. Chromosomes staining was performed by a 4% GIEMSA solution for 15 minutes. Then the slides were extensively washed with tap and distilled water and air-dried at 37°C for 30 minutes and mounted. Slides were examined by light microscopy (40X magnification) and chromosomes number for each metaphase plate was counted using Leica CW4000 Karyo software (Leica Microsystems, Wetzlar, Germany). Two investigators examined each chromosome preparation.

Real Time PCR for PGRMC1

The levels of *Pgrmc1* mRNA expression were assessed in oocytes from ovaries with Low AFC and from ovaries of age-match control group, at the time of collection from the follicle (GV stage) and after 24 h of IVM (MII stage) using a real-time multiplex PCR assay. COCs were collected and cultured as described above and cumulus cells were mechanically removed by the use of vortex. Denuded oocytes were stored in RNAlater solution (Ambion, Life Technologies) until assayed. Total RNA was extracted from groups of 20 oocytes using the RNeasy Plus Mini Kit (Quiagen) according to the manufacturer

instruction. Total RNA was then retro-transcribed with oligo dT using the M-MLV Reverse Transcriptase System (Invitrogen, Life Technologies). A total amount of cDNA equivalent to 6 oocytes was used in each amplification reaction. Reactions were run in triplicates on 4 different biological replicates for each experimental group. Amplification was performed with specific primers and TaqMan probes for bovine *Pgrmc1* (NCBI RefSeq NM_001075133.1; Forward primer: 5'-CTGGAAGAGATGCATCCAGA -3'; Reverse primer: 5'- GAGATCCCAGTCACTCAGGGT-3'; probe: 5' d FAM-TCCGACCTCACTCCTGCCCA- BHQ-1 3') and *β actin* (NCBI RefSeq: NM_173979.3; Forward primer: 5'- CACTCTTCCAGCCTTCCTTC -3'; Reverse primer: 5'-GGATGTCCACGTCACACTTC -3',probe: 5' CAL Fluor Gold 540-TGCCACAGGACTCCATGCCC- BHQ-1 3') using an CFX96 PCR Thermal Cycler (Bio-Rad, Hercules, CA, USA). Beta Actin was used as internal standard and relative level of *Pgrmc1* mRNA was determined using the ΔCT method.

Immunofluorescence Staining

Indirect immunofluorescence was carried out to evaluate the cellular localization of PGRMC1 and AURKB in MII stage- paraformaldehyde fixed oocytes, as previously described [132, 159]. The samples were incubated overnight at 4°C either with a rabbit polyclonal anti-PGRMC1 (dilution 1: 50, Prestige Antibodies by Sigma) or a mouse monoclonal anti-AIM-1 antibody (dilution 1: 50, Aurora B and Ipl1-like midbody associated protein; BD Transduction Laboratories, Franklin Lakes, NJ, USA). Secondary antibodies used were: TRITC labeled donkey anti rabbit antibody (dilution 1:100; Vector Laboratories, Inc., Burlingame, CA, USA) or with Alexa Fluor 488 labeled donkey anti mouse antibody (dilution 1:500; Invitrogen, Life Technologies, Carlsbad, CA, USA) for 30 min at room temperature. The samples were mounted on slides in the antifade medium Vecta Shield (Vector Laboratories) supplemented with 1 μg/ml DAPI. In each experiment

negative controls were performed by omitting the primary antibodies and did not reveal any staining.

Samples stained for PGRMC1 were analyzed on a C1si confocal laser-scanning microscope (Nikon Corp. Tokyo, Japan) with a X60 objective. Z-stacks were compiled with 0.25 µm intervals encompassing the entire MII plate. PGRMC1 localization was classified as regular when PGRMC1 localized at the centromeric region of each chromosome or irregular when presented one or more of the following aspects: more than one point on a chromosome, shape different from the punctuated, not in the centromeric region, lack of PGRMC1. Samples stained for AURKB were analyzed on an epifluorescence microscope (Eclipse E600, Nikon) equipped with a X60 objective, a digital camera and a deconvolution software (NIS elements Imaging Software, Nikon). AURKB localization was classified as regular or irregular according to the criteria adopted for PGRMC1 localization.

ZM447439 Treatment

ZM447439 was dissolved in dimethyl sulfoxide (DMSO) at 10 mM and stored in aliquots at -20°C. Appropriate concentrations were prepared in culture medium so that the final concentration of DMSO never exceeded 1 µl/ml. This DMSO concentration has been previously reported to be not effective on bovine oocyte maturation and fertilization [179, 183].

In a first set of experiments GV stage oocytes were cultured in presence of the Aurora Kinase activity inhibitor ZM447439 (Tocris, Ellisville, MO, USA) in concentrations ranging from 0 to 10 µM. After 24 h, COCs were freed of cumulus cells, fixed and stained with DAPI 1 µg/ml. Samples were analyzed on a epifluorescence microscope (Eclipse E600, Nikon) for polar body extrusion, maturation rate and chromosome alignment. In a second

set of experiments GV stage oocytes were cultured in presence of 5 μM ZM447439, then fixed and stained for PGRMC1 immunolocalization, as described above.

All the MII stage oocytes analyzed, either following 3-dimension reconstruction of the metaphasic plate by confocal microscopy or with conventional fluorescence microscopy, that failed to align all their chromosome along the equator of the metaphasic plate were classified as “misaligned”, as previously described [151].

Statistical analysis

All the experiments were repeated three to five times. Observations from all the experiments were pooled. Statistical significance was determined by Fisher's exact test. Values of $p < 0.05$ were considered significant.

RESULTS

Maturation rates were similar between oocytes isolated from reduced AFC (89%, 67/75) and age-match control ovaries (94%, 119/127). Importantly, karyotype analysis (Figure 16) indicated that although oocytes from ovaries with reduced AFC were capable of undergoing meiosis in vitro, these oocytes showed a 3-fold increase in aneuploidy compared to oocytes isolated from aged-matched control ovaries (Figure 16A). The analysis of karyotypes detected both hyperploid and hypoploid (Figure 16B) oocytes within each group but there was not a difference in the percentage of hypoploid or hyperploid oocytes between the reduced AFC and age-match control groups. Moreover, no specific chromosome-dependence aneuploidy was detected.

Because PGRMC1 plays a role in the formation of the MII plate, *Pgrmc1* mRNA levels and the colocalization of PGRMC1 with AURKB was monitored at the GV and at the MII stage. *Pgrmc1* mRNA levels were not altered in either GV- or MII-staged oocytes of ovaries with reduced ovarian reserve when compared to the age-match control group (Supplemental Figure 1). Moreover, PGRMC1 and AURKB colocalized at the centromeric region of MII stage bovine oocyte, as confirmed in a control experiment that corroborated our previous observations (Figure 17). However, PGRMC1 failed to localize to precise focal points on MII chromosomes in a significantly higher percentage of oocytes isolated from reduced AFC ovaries compared to oocytes of age-match control ovaries (Figure 18 A and 18 B). Similarly, the percentage of oocytes in which AURKB failed to properly localize on the MII chromosomes was significantly higher in oocytes from ovaries with reduced AFC compared to oocytes isolated from ovaries of aged-matched controls (Figure 19 A and 19 B).

To further investigate the relationship between PGRMC1 and the AURKB function during oocyte maturation, oocytes isolated from control ovaries were matured in the presence of increasing concentrations of ZM447439 [151], an AURKB inhibitor [184]. At

all concentrations tested (1 to 10 μM), the percentages of oocytes that reached MII with polar body extrusion after 24 hr of treatment did not differ from control oocytes (Table 4), which is consistent with a previous report [155]. However at the concentrations of 5 and 10 μM, a significantly higher percentage of oocytes exhibited misaligned chromosomes when compared to controls (Table 3). In another set of experiment, when oocytes of age-match control ovaries were cultured with 5 μM of ZM447439 the chromosomal organization of the MII plate was disrupted (Figure 20) and PGRMC1 was not properly localized to the chromosomes (Table 4) even though the ability of oocytes to mature was not affected (Table 4).

Table 3: Effect of ZM447439 on meiotic progression, polar body extrusion and chromosome alignment.

ZM447439 Concentration	N	MII (%)	PB Extrusion (% of MII)	Misalignment (% of MII)
0	55	47 (85.5)	47 (100)	14 (29.8)[a]
1 μM	53	41 (77.4)	40 (97.6)	17 (41.5)[a]
2 μM	58	42 (72.4)	42 (100)	20 (47.6)[a]
5μM	54	39 (72.2)	37 (94.9)	32 (82.1)[b]
10 μM	56	42 (75.0)	38 (90.5)	39 (92.9)[b]

N represents the number of oocytes analyzed in each group. Data were analyzed by Fisher's exact test. a, b: values with different superscripts within columns are significantly different (a, b: $p<0.0001$).

Table 4: Effect of ZM447439 on PGRMC1 localization.

ZM447439 Concentration	N	MII (%)	Misalignment (% of MII)	Irregular PGRMC1 localization (% of MII)
0	21	20 (95.2)	10 (50)[a]	9 (45)[a]
5 μM	26	22 (84.6)	20 (90.1)[b]	20 (90.1)[b]

N represents the number of oocytes analyzed in each group. Data were analyzed by Fisher's exact test. a, b: values with different superscripts within columns are significantly different (a, b: p=0.0001).

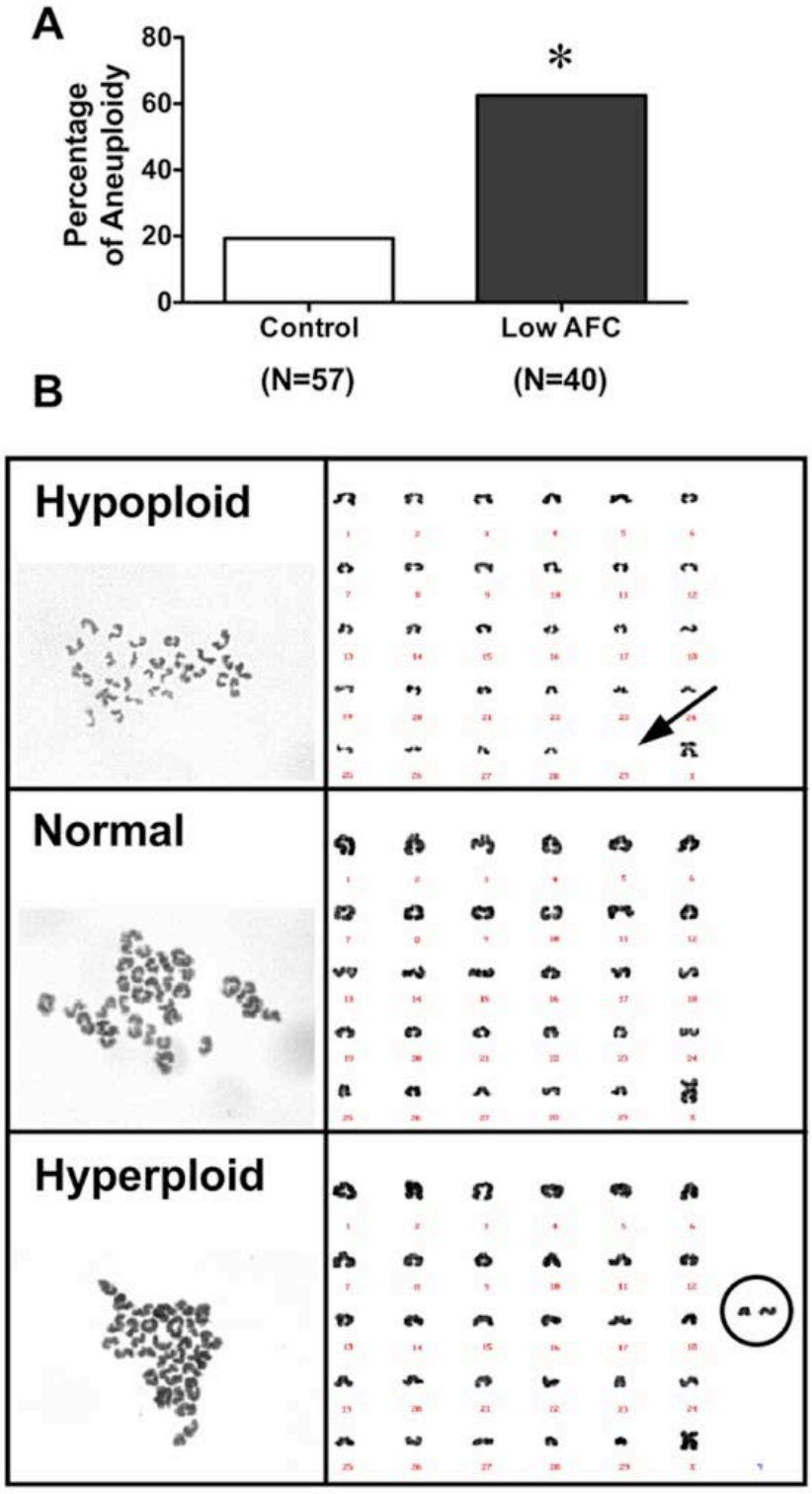
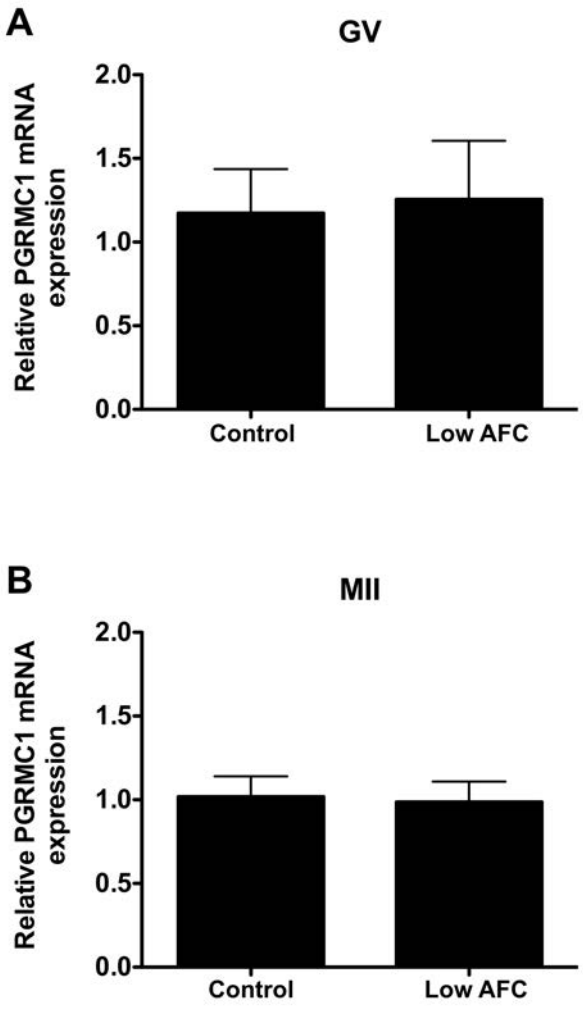


Figure 16. **A**: Percentage of aneuploidy in oocytes isolated from control and Low AFC ovaries. Data were analyzed by Fisher's exact test (asterisk indicates $p<0.05$). **B**: Representative images of hypoploid, normal and hyperploid karyograms.



Supplemental Figure 1. PGRMC1 mRNA expression levels in Control and Low AFC oocytes. Relative PGRMC1 mRNA expression in GV stage (A) and MII stage (B) Control and Low AFC oocytes. Data were calculated by the ΔCT using beta Actin as internal standard.

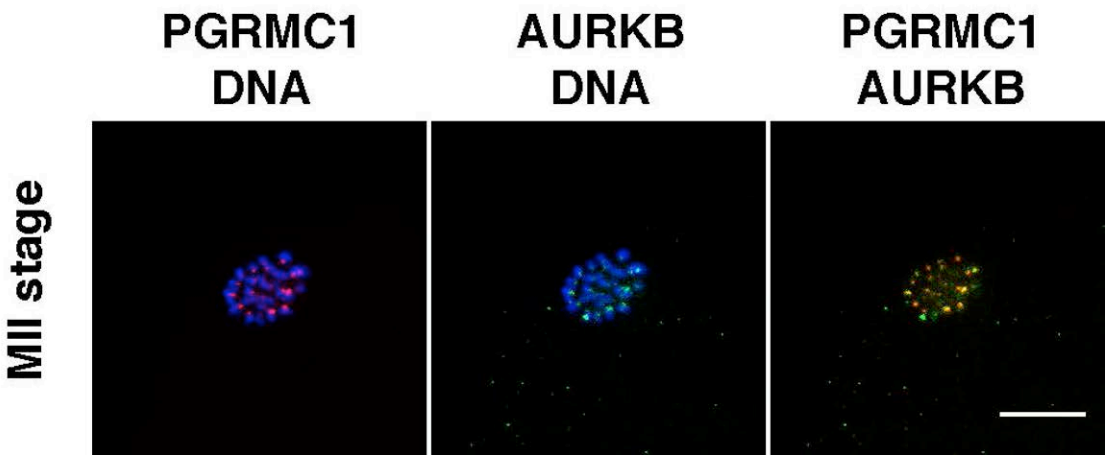


Figure 17: PGRMC1 and AURKB co-localization on centromere of chromosomes in metaphase II control oocytes. Red: PGRMC1; green: AURKB. Bar 10 μm.

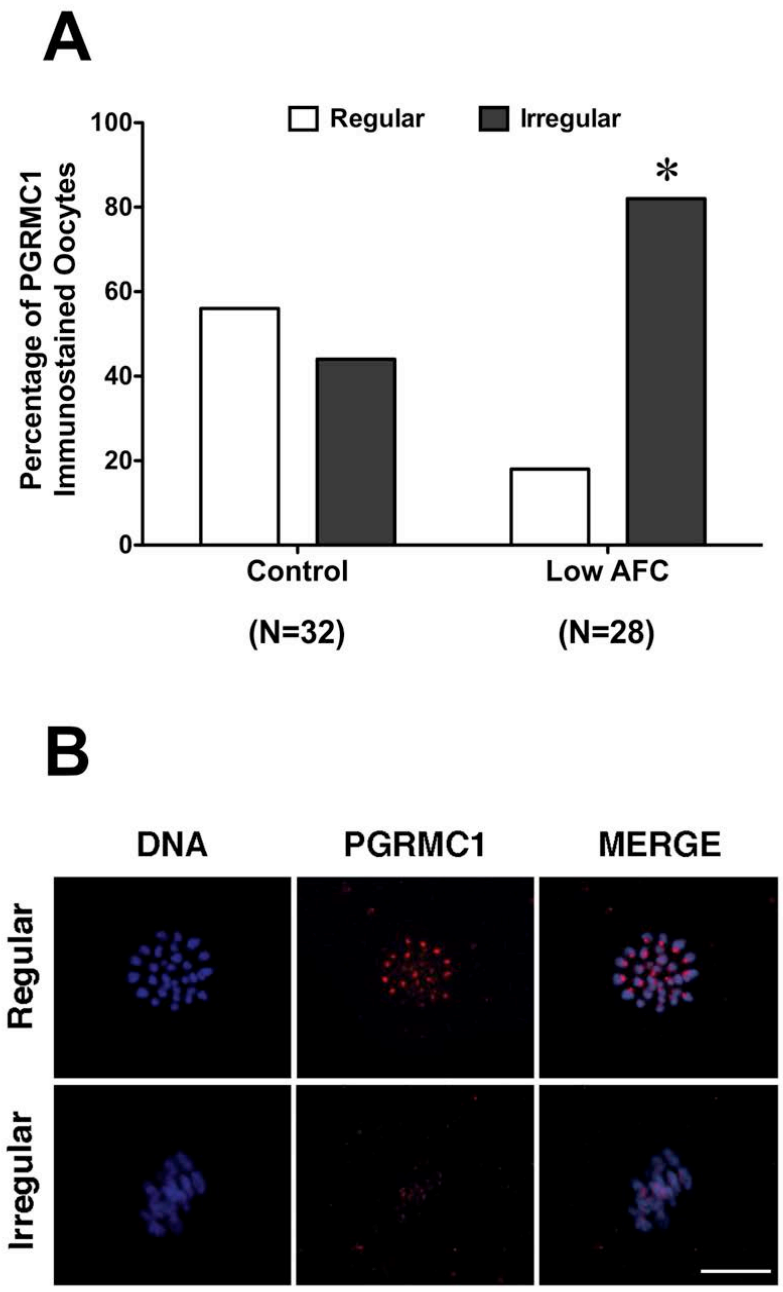


Figure 18: PGRMC1 localization in oocytes isolated from Low AFC and age-match control ovaries. A: percentage of oocytes isolated from control and low antral follicle count ovaries showing regular and irregular PGRMC1 localization. Data were analyzed by Fisher's exact test (asterisk indicates $p<0.05$). B: representative images of regular and irregular localization of PGRMC1 in MII stage oocytes. Blue: DNA; Red: PGRMC1. Bar 10 µm.

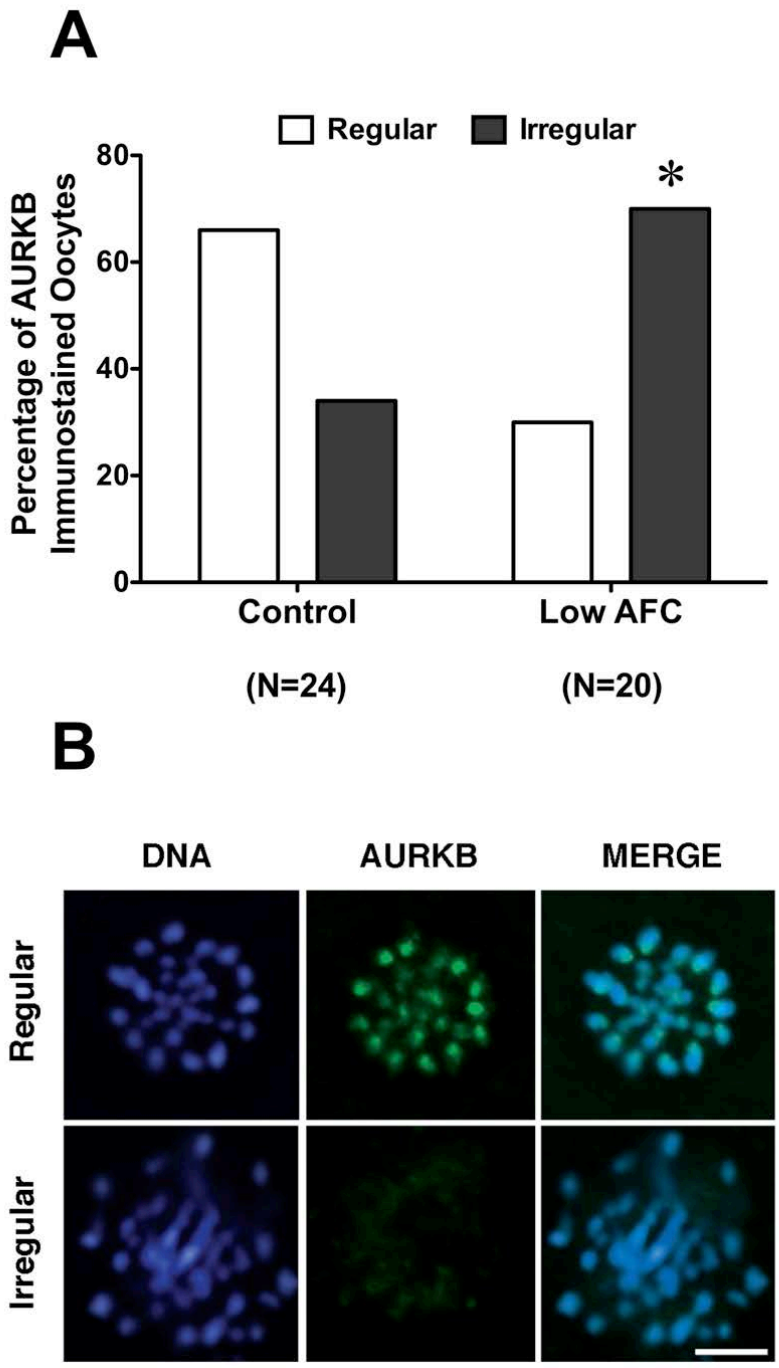


Figure 19: AURKB localization in oocytes isolated from Low AFC and age-match control ovaries. A: percentage of oocytes isolated from control and low antral follicle count ovaries showing regular and irregular AURKB localization. Data were analyzed by Fisher's exact test (asterisk indicates $p<0.05$) B: representative images of regular and irregular localization of AURKB in MII stage oocytes. Blue: DNA; Green: AURKB. Bar 5 μm.

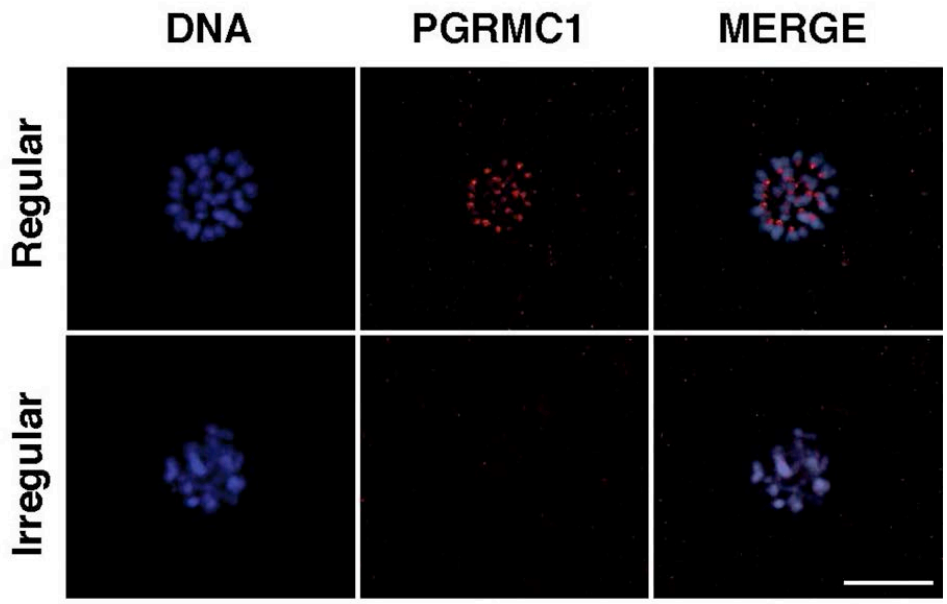


Figure 20: PGRMC1 localization in oocytes of control ovaries treated with the AURKB inhibitor ZM447439. Representative images of regular and irregular localization of PGRMC1 in MII stage oocytes. Blue: DNA; Red: PGRMC1. Bar 10 µm.

DISCUSSION

During oocyte meiotic division, defects in spindle formation and/or function can generate chromosome instability and aneuploidy, a condition that is the major cause of defective early embryonic development, miscarriages and birth defects [7]. While errors in chromosomal segregation can occur at any time during embryonic development, aneuploidy occurs frequently during oocyte meiotic division with about 20% of human oocytes having chromosomal segregation errors [185]. While aneuploidy is an important cause of reproductive wastage, the factors that promote chromosomal segregation errors have not been clearly defined. In mice the frequency of chromosomal segregation errors (i.e. aneuploidy) increases with the gradual depletion of ovarian follicle reserve, which is associated with increasing chronological age [8]. The present work also reveals a relationship between ovarian reserve and chromosomal segregation errors by demonstrating that oocytes, collected from cows with reduced ovarian reserve (i.e. reduced AFC) have a 3-fold increase in aneuploidy compared with oocytes collected from cows with a normal or high AFC. In contrast to the well known age-related increase in chromosomal segregation errors, the present study examined animal of the same approximate age. Therefore, there may be some defect(s) within oocytes isolated from ovaries with premature decreases in ovarian reserve that are distinctly different from age-related alterations.

As evidence of functional molecular alterations in oocytes harvested from ovaries with reduced ovarian reserve, the present study reveals that PGRMC1 fails to properly associate with the MII chromosomes of oocytes isolated from cows with a reduced ovarian reserve. This inability to target PGRMC1 to the MII chromosomes occurs even through *Pgrmc1* is expressed at levels similar to that of oocytes with normal ovarian reserve. Moreover, failure of PGRMC1 to associate with the MII chromosomes likely accounts, at least in part, for their higher incidence of aneuploidy, since disrupting PGRMC1's action

with an antibody to PGRMC1 interferes with the formation of the MII plate [132].

Although PGRMC1 influences the formation of the MII plate, its mechanism of action is not known. PGRMC1 co-localizes with the active (Thr-232 phosphorylated) form of AURKB. AURKB is gradually activated during oocyte maturation and phosphorylates essential proteins that are involved in chromosome segregation such as the meiotic cohesin protein Rec8 [186], which functions to hold sister chromatids together [187].

Although AURKB colocalizes with PGRMC1, a functional relationship between these two proteins has not been established. The present study demonstrates that the AURKB inhibitor, ZM447439, causes chromosome misalignment and alters PGRMC1's localization at centromeres in oocytes obtained from ovaries with high or normal AFC. This implies that AURKB activity is required to properly localize PGRMC1 to the MII chromosomes. However, some caution must be exercised in drawing this conclusion, since ZM447439 could inhibit AURKA or AURKC as well as AURKB. Importantly all the concentrations tested, ZM447439 did not inhibit maturation and polar body extrusion, which are known to be dependent on AURKA [151, 155]. This argues that ZM447439 does not inhibit AURKA even at the maximum concentration used in this study (10 μM). Interestingly, the expression of the AURKC isoform is negligible in immature and mature bovine oocytes [153]. It is likely then that even though the inhibition of AURKC cannot be completely excluded, the biological effect of ZM447439 should be principally ascribed to the inhibition of AURKB and it is therefore logical to conclude that AURKB is required for PGRMC1 to properly localize to the MII chromosomes.

Because our data indicate that *Pgrmc1* mRNA levels were not reduced in oocytes of ovaries with reduced AFC, the hypothesis that PGRMC1 regulates oocyte maturation through the association with AURKB raises the question as to whether post-translational modification of PGRMC1 could be regulated by AURKB. Several proteomic-based studies indicate that PGRMC1 can be phosphorylated at several serine residues [188, 189]. In

fact, phosphorylated PGRMC1 is associated with the mitotic spindle [5, 129]. Since AURKB is a serine/threonine kinase [190], it is possible that AURKB either directly or indirectly phosphorylates PGRMC1 with phosphorylation directing PGRMC1 to the MII chromosomes. This hypothesis is currently being assessed.

In addition to phosphorylation, recent studies reveal that PGRMC1 is sumoylated in ovarian cells [191]. Sumoylation is an important post-translational modification in which small ubiquitin-related modifier (SUMO) proteins are covalently attached to lysine residues in their substrate proteins. Substrate modification by sumoylation can alter protein–protein interactions, change protein intracellular localization or direct changes in the activities of the protein to which SUMO is attached [192]. Importantly, sumoylation may play an essential role in regulating mouse oocyte meiosis, since SUMO1 or more likely proteins that are covalently coupled to SUMO1 localize to the spindle poles in prometaphase I, metaphase I and II, and around the separating chromosomes in anaphase I and telophase I, while SUMO2/3 is mainly concentrated near the centromeres [193]. These observations are consistent with the concept that part of mechanism that localizes PGRMC1 to MII chromosomes involves both phosphorylation and sumoylation.

In conclusion, our data is supports the hypothesis that PGRMC1 and AURKB interaction during oocyte meiosis plays an essential role in the process of chromosome segregation, because the inhibition of AURKB causes changes in the localization of PGRMC1 and alterations in the MII chromosomal plate. Moreover, alteration in the localization of PGRMC1 and AURKB could account in part for the increased aneuploidy and low development competence of oocytes of ovaries isolated from cows with reduced ovarian reserve.

11. CONCLUSIONS

Reproductive efficiency in dairy cows is decreasing worldwide. The root cause of the declining fertility is probably a combination of a variety of physiological and management factors that have an additive effect on reproductive efficiency [2]. These factors include increasing in milk production and herd size, greater use of confinement housing, labor shortages and higher inbreeding percentages. In addition, the reproductive physiology of dairy cattle has also changed in response to genetic selection for milk production. Compared with traditional dairy cows, modern dairy cows have longer intervals to first ovulation, a higher incidence of anestrus and abnormal luteal phases, lower blood progesterone and IGF-I concentrations, higher incidence of multiple ovulations and twinning rates as well as greater embryonic loss [1]. Declining fertility represents an obstacle in maintaining profitability of dairy farms [1-3]. It has been estimated that a slight increase in pregnancy rates results in a significant increase in profitability that could make the difference in whether a family farm remains in operation [194, 195]. These findings suggest that defining the factors and mechanisms that contribute to oocyte and embryo quality is essential for improving female fertility. In particular, basic knowledge of which proteins within the oocyte regulate meiosis, oocyte fertilizability and developmental potential would be advantageous.

Starting from these observations, our studies were conducted to test the hypothesis that **PGRMC1** is one of the key factors that regulate mammalian oocyte quality and therefore female fertility. Initial indications that PGRMC1 participates in progesterone signaling in the reproductive system come from studies in which PGRMC1 expression was silenced using siRNA in ovarian cells. These experiments demonstrated that Progesterone's ability to inhibit ovarian cells from undergoing apoptosis in vitro is dependent on PGRMC1 [119], indicating that PGRMC1 plays an essential role in promoting the survival of ovarian cell in

vitro. This led us to start our investigations on the function of PGRMC1 in bovine fertility.

Initial experiments were conducted to determine the presence and localization of PGRMC1 in various compartments of the bovine female reproductive organs, during the follicular and luteal phases of the estrous cycle. Importantly, these studies revealed the presence of PGRMC1 in the nucleus of bovine oocytes. Further studies revealed that PGRMC1 is present in both GV- and MII-stage oocyte, is associated with male and female pronuclei in the zygote and is highly expressed in the blastocysts, with typical localization at each of these stages.

Since fertilization and embryonic development are mainly dependent on the completion of oocyte maturation, we focused our attention on PGRMC1's role during this important step. Our localization as well as our functional data suggest an important role of PGRMC1 in oocyte maturation that may be specifically related to the mechanism by which chromosomes segregate and the first polar body extruded. Moreover, we have started to elucidate the mechanism by which PGRMC1 could act as a regulator of oocyte meiosis. In fact we demonstrated not only that PGRMC1 co-localize with the active form of AURKB, but also that the activities of these two molecules may be somehow related. In fact, inhibition of AURKB causes changes in the localization of PGRMC1 and alterations in the MII chromosomal plate. Furthermore, our data suggest that alteration in the localization of PGRMC1 and AURKB could account in part for the increased aneuploidy and low development competence of oocytes of ovaries isolated from cows with reduced ovarian reserve.

In conclusion, the present study raise important questions regarding the role of PGRMC1 in the regulation of oocyte maturation and the loss of fertility of dairy cows. Despite the mechanism through which PGRMC1 regulates these processes is still unknown and remains to be deeply investigated, our data indicate that functional alterations of PGRMC1 could impair meiosis and reduce development competence of

oocytes. Finally, understanding these mechanisms in cattle could provide insight into how these processes are regulated in other species, including humans.

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13. Acknowledgements

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15. Pubblications

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