Reproductive tissue selective actions of progesterone receptors

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Abstract

The steroid hormone, progesterone, plays a central coordinate role in diverse events associated with female reproduction. In humans and other vertebrates, the biological activity of progesterone is mediated by modulation of the transcriptional activity of two progesterone receptors, PR-A and PR-B. These receptors arise from the same gene and exhibit both overlapping and distinct transcriptional activities *in vitro*. To delineate the individual roles of PR-A and PR-B *in vivo*, we have generated mouse models in which expression of a single PR isoform has been ablated. Analysis of the reproductive phenotypes of these mice has indicated that PR-A and PR-B mediate mostly distinct but partially overlapping reproductive responses to progesterone. While selective ablation of the PR-A protein (PR-A knockout mice, PRAKO mice) shows normal mammary gland response to progesterone but severe uterine hyperplasia and ovarian abnormalities, ablation of PR-B protein (PRBKO mice) does not affect biological responses of the ovary or uterus to progesterone but results in reduced pregnancy-associated mammary gland morphogenesis. The distinct tissue-specific reproductive responses to progesterone exhibited by these isoforms are due to regulation of distinct subsets of progesterone-dependent target genes by the individual PR isoforms. This review will summarize our current understanding of the selective contribution of PR isoforms to the cellular and molecular actions of progesterone in reproductive tissues.

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Progesterone receptors

The steroid hormone, progesterone, is a key modulator of normal reproductive functions. These include ovulation, uterine and mammary gland development and the neuro-behavioral expression associated with sexual responsiveness (Clarke & Sutherland 1990, Lydon *et al.* 1995). The physiological effects of progesterone are mediated by interaction of the hormone with two specific intracellular progesterone receptors (PRs) termed PR-A and PR-B. Progesterone receptors are members of a large family of structurally related gene products known as the nuclear receptor (NR) superfamily (Evans 1988, O'Malley & Conneely 1992, Tsai & O'Malley 1994a, Mangelsdorf *et al.* 1995) of transcription factors. NRs regulate gene transcription by discriminative binding to DNA regulatory sequences as well as by specific interactions with co-activator and/or co-repressor proteins to regulate the activity of the RNA polymerase complex (McKenna & O'Malley 2001).

PRs are expressed from a single gene as a result of transcription from two alternative promoters (Kastner *et al.* 1990, Kraus & Katzenellenbogen 1993) and translation initiation at two alternative AUG initiation codons

(Conneely *et al.* 1989). The production of these two isoforms is conserved in a number of vertebrate species including humans and rodents (Lessey *et al.* 1983, Conneely *et al.* 1989, Shyamala *et al.* 1990, Giangrande & McDonnell 1999) and the ratios of the individual isoforms vary in reproductive tissues as a consequence of developmental (Shyamala *et al.* 1990) and hormonal (Duffy *et al.* 1997) status and during carcinogenesis (Graham *et al.* 1996).

Binding of progesterone to PRs induces a significant conformational change on the receptor proteins (Allan *et al.* 1992a,b) that results in dimerization of two ligand receptor complexes (Tsai *et al.* 1988, Tsai & O'Malley 1991), increased receptor phosphorylation (Weigel *et al.* 1995), binding of receptor dimers to specific hormone responsive DNA elements located in the promoter regions of target genes (Gronemeyer 1991, Tsai & O'Malley 1994b), and interaction of the receptor complex with specific coactivator proteins and general transcription factors (Onate *et al.* 1995, Kamei *et al.* 1996) to form a productive transcription initiation complex on specific target gene promoters. The overall structural features of the PRs that are responsible for these activities are well defined

Figure 1 Structural organization of the human PR-A and PR-B isoforms. Numbers denote the amino acid position in each protein. AF-1, AF-2 and AF-3 are activation domains; DBD, DNA binding domain; LBD, ligand binding domain; ID, inhibitory domain; DIM, sequences important for receptor dimerization.

(Fig. 1). The amino terminal or A/B region of PRs is the most hypervariable region in terms of both size and amino acid sequence among members of the superfamily. This region contains transactivation domains (AF-1 and AF-3) that recruit coactivator proteins to the receptor to modulate the level and promoter specificity of target gene activation (Tora *et al.* 1988, Dobson *et al.* 1989, Meyer *et al.* 1992, Sartorius *et al.* 1994) as well an inhibitory domain (ID) responsible for recruitment of transcriptional inhibitory corepressor proteins (Giangrande *et al.* 1997). The most conserved region (C) is the DNA binding domain (DBD) that, in the case of PRs, is centrally located. This domain consists of approximately 66–68 amino acids and is composed of two type II zinc finger structures that facilitate binding of the receptor to specific cis-acting DNA sequences and are the hallmark of the nuclear receptor superfamily (Luisi *et al.* 1991, Freedman 1992). A highly conserved ligand binding domain (LBD) is located on the carboxy terminal side of the DBD. In addition to its progesterone binding function, this region contains an additional transactivation domain (AF-2) required for hormone-dependent coactivator recruitment, sequences important for interaction of inactive receptors with heat shock proteins and for receptor dimerization (DIM) (Pratt *et al.* 1988, Webster *et al.* 1988, Fawell *et al.* 1990, Vegeto *et al.* 1992).

In the early 1990s, *in vitro* biochemical studies revealed that the two PR isoforms displayed quite distinct transactivational properties that are specific to both the cell type and the target gene promoter used (Tora *et al.* 1988, Meyer *et al.* 1992, Vegeto *et al.* 1993, Hovland *et al.* 1998). Specifically, PR-B has been shown to function as a strong activator of transcription of several PR-dependent promoters and in a variety of cell types in which PR-A is inactive. In addition, when the PR-A and PR-B proteins are co-expressed in cultured cells, the PR-A can repress the activity of PR-B as well as the activity of other nuclear receptors (Giangrande & McDonnell 1999). Further, the PR-A and PR-B proteins also respond differently to progesterone antagonists (reviewed in Giangrande & McDonnell 1999). While antagonist bound PR-A is inactive, antagonist bound PR-B can be converted to a strongly active transcription factor. When expressed in equimolar ratios in cells, the PR-A and PR-B proteins can dimerize

and bind DNA as three distinct species: A:A or B:B homodimers or A:B heterodimers. The differential transactivation properties contributed to these complexes by the presence or absence of the PR-B-specific AF-3 domain is likely to contribute to the complete repertoire of physiological responses to progesterone.

While it is well established that PRs mediate the transcription regulatory effects of progesterone, the signal transduction properties of PRs are not restricted to respond to their steroidal ligand. Studies using tissue culture systems have revealed that PRs as well as some other nuclear receptors can be activated in a ligand-independent manner by stimuli that modulate intracellular kinase activity (Denner *et al.* 1990, Aronica & Katzenellenbogen 1991, 1993, Beck *et al.* 1993, Sartorius *et al.* 1993). The physiological relevance of a progesterone-independent mechanism of receptor activation has been substantiated *in vivo*: PR-mediated lordosis behavior exhibited by rats (Mani *et al.* 2001) and mice (Mani *et al.* 1996) can be stimulated either in response to progesterone or in the absence of progesterone by dopamine-activated intracellular signaling pathways.

In addition to its PR-mediated genomic actions, progesterone also stimulates cellular responses that are independent of the transcription regulatory function of PRs by at least two distinct mechanisms. The first is PR dependent and involves PR-mediated activation of intracellular phosphorylation cascades, at least one of which is the Src/Ras/MAP kinase pathway, to modulate cellular responses to the hormone (Boonyaratanakornkit *et al.* 2001, Ballare *et al.* 2003). The second mechanism is independent of PRs and appears to be mediated by interactions with specific membrane receptors for progesterone. Plasma membrane-associated progesterone-specific receptors have been isolated and cloned from a range of tissues and from a number of species (Bramley 2003) and are identified as G protein-coupled receptors distinct from classical intracellular PRs (Zhu *et al.* 2003). Rapid non-genomic effects of progesterone have been implicated in modulation of the sperm acrosome reaction (Bronson *et al.* 1999, Patrat *et al.* 2000), modulation of neuronal excitability (Genazzani *et al.* 2000, Lambert *et al.* 2003), prevention of preterm labor (Grazzini *et al.* 1998), and *Xenopus* oocyte maturation (Bayaa *et al.* 2000). While several of these responses appear to be regulated in a PR-independent manner, the contribution of PR-mediated non-genomic signaling to the physiological activities of progesterone remains poorly understood.

Progesterone receptor knockout (PRKO) models demonstrate tissue specific roles of PRs in reproductive tissues

Phenotypic studies of the PRKO mouse model in which the expression of both isoforms was inhibited by null mutation of the PR gene provided compelling evidence of

an essential role of PRs in all aspects of female reproduction. Specifically, female mice lacking both PRs exhibit impaired sexual behavior and neuroendocrine gonadotropin regulation, anovulation, uterine dysfunction and impaired pregnancy-associated mammary gland morphogenesis (Lydon *et al.* 1995, Mani *et al.* 1996, Chappell *et al.* 1999, Tibbetts *et al.* 1999). Furthermore, studies of the PRKO mouse reveal that PRs also play an essential role in regulation of thymic involution during pregnancy (Tibbetts *et al.* 1999) and in the cardiovascular system through regulation of endothelial and vascular smooth muscle cell proliferation and response to vascular injury (Vazquez *et al.* 1999).

The differences in transcriptional activities and coregulator interactions between the PR-A and PR-B observed *in vitro* predicted that these proteins may mediate different physiological responses to progesterone. In addition, the selective ability of PR-A to inhibit transcriptional responses induced by both PR-B and the estrogen receptors suggested that PR-A has the capacity to diminish overall progesterone responsiveness in certain tissues as well as contribute to the antiestrogenic activities of progesterone previously observed in the uterus (Lydon *et al.* 1995). However, physiological validation of the functional differences between the PR-A and PR-B isoforms has been hampered due to a lack of information on the specific cell types that express each isoform *in vivo* and a lack of appropriate animal models in which to dissect their selective functions.

The observation that PR-A and PR-B are produced by translation at two distinct AUG signals encoded by a single gene predicted that mutation of either ATG codon in the PR gene would result in selective ablation of expression of a single isoform *in vivo* (Conneely *et al.* 1989, Kastner *et al.* 1990). Thus, the CRE-loxP gene targeting approach in embryonic stem cells was used to introduce a point mutation into the PR gene at the ATG codon encoding Met 1 (M1L) in order to specifically ablate expression of the PR-B protein, and at the ATG encoding Met 166 (M166A) in order to ablate expression of PR-A (Mulac-Jericevic *et al.* 2000, 2003). This strategy has provided a powerful model system to examine the selective expression of each isoform *in situ* as well as to assess the selective contributions of PR-A and PR-B in their normal cellular context to the physiological functions of progesterone.

PRs and ovarian function

Luteinizing hormone (LH), the primary signal for rupture of preovulatory ovarian follicles leading to ovulation, stimulates transient expression of PR mRNA and proteins in granulosa cells (Natraj & Richards 1993, Park-Sarge & Mayo 1994) and the antiprogesterin, RU486, inhibits ovulation (Loutradis *et al.* 1991). Definitive proof that PRs are essential mediators of ovulation has been provided by

analysis of the ovarian phenotype of the PRKO mouse. Analysis of this model revealed that PRs are required specifically for LH-dependent follicular rupture leading to ovulation but not for differentiation of granulosa cells to form a corpus luteum (luteinization) (Lydon *et al.* 1995). Follicular rupture requires induction of a prostaglandin-mediated inflammatory response to LH as well as tissue degradation at the apex of the preovulatory follicle, an event that is mediated by matrix proteinases (Espey 1994). Analysis of the expression of potential mediators of ovulation in PRKO mice has demonstrated that LH-induced regulation of Cox-2, an enzyme that catalyses the production of prostaglandins and is essential for ovulation, is unaffected (Robker *et al.* 2000). In contrast, the expression of two metalloproteinases, ADAMTS-1 (a disintegrin and metalloproteinase with thrombospondin motifs) and cathepsin-L (a lysosomal cysteine protease) is inhibited in mural granulosa cells of the mature follicle (Tetel *et al.* 1999, Robker *et al.* 2000) in PRKO mice. One of these proteases, ADAMTS-1, is essential for ovulation (Robker *et al.* 2000) suggesting that this protein may represent a critical mediator of the progesterone-induced ovulatory event.

Both the PR-A and PR-B proteins are induced in preovulatory follicles in response to LH stimulation (Natraj & Richards 1993). Analysis of PRAKO mice, in which PR-A is ablated, showed that ovulation is severely impaired but unlike in PRKO mice, is not completely absent (Mulac-Jericevic *et al.* 2000). Histological analysis of the ovaries of PRAKO mice showed numerous mature anovulatory follicles that contained an intact oocyte and were arrested at a similar stage to that previously observed in PRKO mice. In contrast, ovulation is unaffected in PRBKO mice indicating that PR-A expression is both necessary and sufficient to mediate the ovulatory response to progesterone (Mulac-Jericevic *et al.* 2003). The ovulatory defects in PRAKO mice must therefore be due to an inability of PR-B to regulate signaling pathways necessary for follicular rupture that are as yet unidentified. The observation that the PR-A and PR-B proteins are not functionally redundant in the ovary provides physiological validation of previous studies in tissue culture demonstrating that these transcription factors have different functional activities. From a mechanistic standpoint, the observation that PR-A alone is sufficient to support normal ovulation indicates that heterodimeric interactions between the PR-A and PR-B proteins are not required for regulation of essential progesterin-responsive target genes associated with ovulation. With the exception of the above-mentioned proteases, the PR-dependent signaling pathways that mediate follicular rupture have not yet been elucidated. Because the PRKO mouse has a specific defect in follicular rupture while luteinization is maintained, differential array analysis using this model provides an excellent system to delineate the signaling pathways regulated by PRs that are specific to follicular rupture. The identification of these genes should facilitate the identification of PR isoform

selective target genes that are essential for ovulation, in addition to providing important new information on the molecular mechanisms of progesterone-induced follicular rupture.

PRs and uterine function

Progesterone plays a critical role during early pregnancy in preparation of the uterine epithelium for receptivity and in differentiation of endometrial stromal cells to a decidual phenotype that supports the development of the implanting embryo. Furthermore, progesterone is a potent antagonist of estrogen-induced proliferation in the uterine epithelium.

The uterus of PRKO mouse fails to support implantation after embryo transfer and is unresponsive to a decidual stimulus (Lydon *et al.* 1995). In PRKO mice, epithelial cells in the uterus become hyperplastic as a result of unopposed proliferative estrogen action. PRs are expressed in the epithelial, stromal and myometrial compartments of the uterus and their spatiotemporal expression within these compartments is regulated by both estrogen and progesterone (Tibbetts *et al.* 1998) and undergoes dynamic changes during the estrous cycle and early pregnancy (Tan *et al.* 1999). This intercompartmental regulation of PR expression is essential for the appropriate regulation of uterine gene expression (Kurita *et al.* 1998). Recent analysis of the effects of PR ablation on the expression of several genes previously implicated in progesterone-dependent uterine implantation have shown that the defects observed in PRKO mice are associated with inhibition of expression of several epithelial markers of uterine receptivity (Mulac-Jericevic *et al.* 2000) and at least one essential stromal mediator of decidualization, *hoxa-10* (Lim *et al.* 1999).

Analysis of progesterone-dependent uterine function in PRAKO and PRBKO mice has revealed that PR-A and PR-B have distinct functions in the uterus (Mulac-Jericevic *et al.* 2000, 2003). In PRAKO mice, progesterone-induced differentiation of endometrial stromal cells to a decidual phenotype is inhibited, suggesting that PR-A plays a crucial role in decidualization of the stroma prior to implantation (Mulac-Jericevic *et al.* 2000). In contrast, analysis of uterine function in PRBKO mice has shown that expression of PR-A is both necessary and sufficient to mediate both the antiproliferative- and implantation-associated responses to progesterone (Mulac-Jericevic *et al.* 2003). The uterine defects observed in PRAKO mice are due to an inability of PR-B to regulate a subset of PR-dependent target genes rather than to differences in the spatiotemporal expression of PR-A relative to PR-B in the uterus (Mulac-Jericevic *et al.* 2000).

Surprisingly, selective activation of PR-B in the uterus of PRAKO mice resulted in an abnormal progesterone-dependent induction of epithelial cell proliferation in contrast to its ability to inhibit estrogen-induced proliferation in the wild-type uterus (Mulac-Jericevic *et al.* 2000). This gain of

PR-B-dependent proliferative activity upon removal of PR-A indicates that PR-A is required not only to inhibit estrogen-induced hyperplasia of the uterus but also to limit potentially adverse proliferative effects of the PR-B protein.

In the human endometrium, the levels of PR-A and PR-B are differentially regulated during the reproductive cycle (Mangal *et al.* 1997, Mote *et al.* 2000). The physiological importance of maintaining the correct relative expression levels of PR isoforms in the uterus is indicated by the identification of aberrant ratios of PR isoforms in human endometrial cancers and the recent identification of a functional polymorphism in the human PR promoter that results in increased expression of the human PR-B isoform and is associated with increased risk of endometrial cancer (Arnett-Mansfield *et al.* 2001, De Vivo *et al.* 2002). Given the opposing effects of the PR isoforms in the uterus, one could predict that aberrant changes in the relative spatiotemporal expression patterns of PR isoforms in the uterus could play an important role in determining appropriate responsiveness to progestin therapy in the treatment of uterine epithelial hyperplasias.

PRs and mammary gland morphogenesis

Mammary gland development is predominantly postnatal and is controlled by a complex interplay of endocrine hormones, in particular estrogen, progesterone and prolactin, acting together with locally acting growth factors (Anderson 2002, Soyak *et al.* 2002). Postnatal mammary gland development involves two distinct growth phases that are initiated at the onset of puberty and pregnancy respectively. At puberty, estrogen promotes ductal elongation and dichotomous branching to the limits of the mammary fat pad. At adulthood, the virgin gland becomes relatively quiescent with the exception of minimal side branching and alveolar budding that occur over time as a result of the cyclic rise of ovarian steroids during the estrous cycle. At pregnancy, exposure to progesterone and prolactin (PRL) results in extensive epithelial proliferation, increased dichotomous side branching and differentiation of milk-filled alveolar lobules. At weaning, removal of the suckling stimulus elicits involution of the lobular alveolar system through apoptosis and matrix degrading proteinase-mediated remodeling. At the end of the involution process, the postnatal developmental cycle of mammary gland development is completed and the mammary gland resembles the general architecture of the pre-pregnant mammary gland (Anderson 2002, Soyak *et al.* 2002).

Null mutation of both PR isoforms in PRKO mice has demonstrated that PRs are specifically required for pregnancy-associated ductal proliferation and lobuloalveolar differentiation of the mammary epithelium. Mammary glands of PRKO mice failed to develop the pregnancy-associated side-branching of the ductal epithelium with attendant lobular alveolar differentiation despite normal postpubertal mammary gland morphogenesis of the virgin

Table 1 Components of female reproductive system and phenotype identified in progesterone receptor knockout mouse models.

Component	Genotype	Phenotype
Ovaries	PRKO PRAKO PRBKO	Inability to ovulate Severely impaired ovulation Normal ovulation
Uterus	PRKO PRAKO PRBKO	Impaired implantation/decidualization/infertility Impaired implantation/decidualization/infertility Normal implantation/decidualization
Mammary gland	PRKO PRAKO PRBKO	Impaired mammary gland development Normal progesterone-induced mammary gland morphogenesis Reduced pregnancy-associated side-branching and lobuloalveolar development

mice (Lydon *et al.* 1995, Seagroves *et al.* 2000). Thus, in contrast to its antiproliferative role in the uterus, progesterone is an essential pregnancy-associated proliferative stimulus in the mammary gland.

PRs are expressed exclusively in the mammary epithelium (Seagroves *et al.* 2000, Sivaraman *et al.* 2001, Ismail *et al.* 2002) and development of the mammary gland from juvenile to adult state is associated with a change in the pattern of expression of PRs from a uniform to a scattered pattern of expression in a subset of epithelial cells (Seagroves *et al.* 2000, Grimm *et al.* 2002). Recent studies have shown that PR-expressing cells are segregated from proliferating cells in the normal mammary glands of both rodents and humans (Clarke *et al.* 1997, Seagroves *et al.* 2000, Ismail *et al.* 2002). Consistent with this finding, the proliferative responses of the ductal and alveolar epithelium to progesterone are associated with local induction of PR-dependent growth factors that act in a paracrine manner on PR-negative cells to control their proliferation (Brisken *et al.* 1998, 2000). In contrast to the normal mammary gland, segregation of the steroid receptor expressing cells from proliferating cells is lost in mammary epithelial cells that have been exposed to carcinogen (Sivaraman *et al.* 2001) and in cells of breast tumors (Graham & Clarke 2002). This aberrant change in pattern of receptor expression is likely to contribute to abnormal growth of breast cancer cells.

Both isoforms of PR are expressed in the mammary gland of the virgin mouse (Shyamala *et al.* 1990) and during pregnancy (Fantl *et al.* 1999) and the levels of PR-A protein exceed those of the PR-B isoform. Progesterone-activated PR-B in PRAKO mice elicits side branching and lobular alveolar development in the mammary gland comparable to that of wild-type mammary gland (Mulac-Jericevic *et al.* 2000). Thus, PR-B is sufficient to elicit normal proliferation and differentiation of the mammary epithelium in response to progesterone and neither process appears to require functional expression of the PR-A protein. In contrast, recent analysis of the mammary glands of PRBKO mice has shown reduced pregnancy-associated ductal side branching and lobuloalveolar development as a consequence of decreased ductal and alveolar epithelial cell proliferation and increased apoptosis of alveolar

epithelium (Mulac-Jericevic *et al.* 2003). Despite these defects, PR-A retains its normal segregated spatiotemporal pattern relative to proliferating cells in PRBKO mice and is expressed at a higher level than that observed for PR-B in PRAKO mice. Examination of the molecular genetic signaling pathways that are differentially regulated by PRs in the mammary gland showed that the defects observed in PRBKO mice are associated with a PR-B isoform-selective regulation of the receptor activator of nuclear factor kappa B (NF κ B) ligand (RANKL) signaling pathway (Mulac-Jericevic *et al.* 2003) which is essential for alveologenesis (Fata *et al.* 2000). In contrast, progesterone-dependent activation of secreted growth factor, wnt4, a key mediator of branching morphogenesis (Brisken *et al.* 2000), is normally regulated by either PR-A or PR-B (Mulac-Jericevic *et al.* 2003). Thus PR-A and PR-B appear to regulate both overlapping and distinct subsets of progesterone-dependent signaling pathways required for mammary gland development.

Conclusion

Analysis of the phenotypic consequences of selective ablation of the PR-A or PR-B isoforms in mice has facilitated a comprehensive analysis of the selective spatiotemporal expression of these receptors in the reproductive tract and mammary gland as well as elucidation of their individual contributions to the established reproductive activities of progesterone. These studies have revealed that PR-A and PR-B exhibit mostly distinct but partially overlapping hormonal responses (Table 1). PR-A activation is both necessary and sufficient for establishment and maintenance of pregnancy but elicits reduced mammary gland morphogenic responses to hormonal stimulation relative to PR-B. In contrast, PR-B activation is insufficient to support female fertility but is a potent proliferative mediator in the mammary gland and, most surprisingly, in the uterus. The distinct tissue-selective activities of PR-A and PR-B observed *in vivo* in these studies support the conclusion that modulation of PR activity using isoform-selective progestins may contribute towards elimination of adverse tissue-specific side effects of progestins.

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