Progesterone signaling in the normal breast and its implications for cancer

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Abstract (250)

Progesterone is considered the pregnancy hormone and acts on many different target tissues. Progesterone receptor (PR) signaling is important for normal development and the physiologic function of the breast and impinges on breast carcinogenesis. Both systemically and locally, in the breast epithelium, there are multiple layers of complexity to progesterone action, many of which have been revealed through experiments in mice. The hormone acts via its receptor expressed in a subset of cells, the sensor cells, in the breast epithelium with different signaling outcomes in individual cells eliciting distinct cell intrinsic and paracrine signaling involving different mediators for different intercellular interactions. PR expression itself is developmentally regulated and the biological outcome of PR signaling depends on the developmental stage of the mammary gland and the endocrine context. During both puberty and adulthood PR activates stem and progenitor cells through Wnt4-driven activation of the myoepithelium with downstream Adamts18-induced changes in ECM/BM. During estrous cycling and pregnancy, the hormone drives a major cell expansion through Rankl. At all stages PR signaling is closely tied to estrogen receptor α (ER) signaling. As the PR itself is a target gene of ER, the complex interactions are experimentally difficult to dissect and still poorly understood. *Ex vivo* models of the human breast and studies on biopsy samples show that major signaling axes are conserved across species. New intraductal xenograft models hold promise to provide a better understanding of PR signaling in the normal breast epithelium and in breast cancer development in the near future.

Introduction

A woman's risk to get breast cancer is affected by her reproductive history and hence exposure to reproductive hormones. An early full-term pregnancy has protective effects (MacMahon et al., 1970) whereas risk increases with the number of menstrual cycles she experiences during her life time, that is with shorter cycles, younger age at menarche, and later menopause (Colditz et al., 2004). Furthermore, exposure to exogenous hormones, as it occurs widely in the context of hormonal contraception and hormone replacement therapy, increases breast cancer risk. Finally, exposures to endocrine disruptors, in particular when they occur *in utero* and during the perinatal period, affect breast cancer risk, as reviewed in (Soto and Sonnenschein, 2015; Soto et al., 2013).

The role of the ovarian steroid, progesterone, is tightly linked to ER signaling and is just beginning to emerge from the shadow of its highly visible cousin 17-β-estradiol (Brisken, 2013). What we know about PR signaling in the normal breast and how this relates to breast carcinogenesis, will be examined here in its broader developmental and physiological context.

The particular sensitivity of the breast to carcinogenic insults is likely a consequence of its unique plasticity: the breast is the only organ that develops mostly after birth and can undergo repeated cycles of cell proliferation and differentiation during pregnancy without exhaustion. The two major ovarian

hormones, estrogens and progesterone, have important roles in orchestrating morphogenesis, cell proliferation and differentiation at different stages of development to ensure that the breast can produce copious amounts of milk for the offspring by the end of pregnancy.

To dissect the role of PR signaling in normal development and physiology, rodent models, in particular genetically engineered mouse models (GEMMs), have been instrumental. What we have learnt from these models and how these findings translate to human breast function and human breast cancer development will be discussed here.

Development and physiology of the breast

The hypothalamic–pituitary–ovarian axis controls female reproductive function and the development and physiology of the breast. The hypothalamic-pituitary clock determines the onset of puberty and the pituitary peptide hormones LH and FSH control ovarian function. The ovaries are the major source of estrogens and progesterone of which they secrete only trace amounts until puberty.

The breast is a skin appendage that develops through epithelial-mesenchymal interactions in the embryo (Veltmaat, 2017). At birth, rudimentary glandular tissue emanates from the nipples. This small gland grows isometrically with the rest of the body until puberty. The budding of the breasts, thelarche, heralds puberty and coincides with increased ovarian estrogen (E) and some progesterone (P) production. When sexual maturity is reached and ovulatory menstrual cycles are established, cyclical changes in hormone levels are established (Figure 1) with an estrogen peak occurring in the first half of the cycle, prior to ovulation, and a raise in progesterone during the second phase (luteal phase) that is accompanied by a less pronounced increase in estrogen levels and elevated testosterone levels (Skiba et al., 2019). When the egg is fertilized and implants, the corpus luteum will produce increasing levels of progesterone that will be further increased through placental progesterone production (Figure 1). If the egg is not fertilized, progesterone levels drop abruptly, menstrual bleeding occurs, and the next menstrual cycle begins.

Extensive longitudinal growth of the ducts with branching and expansion of the mammary fat occur during puberty. In the adult female, recurrent menstrual cycles elicit changes within the breast. Women may feel breasts engorge due to increased blood flow and subtle changes in epithelial cell morphology can be observed. The myoepithelium becomes more prominent, the ECM is distended (Vogel et al., 1981). Mitotic figures can be observed in the luminal epithelium during luteal whereas the breast is quiescent during follicular phase (Anderson et al., 1982; Ferguson and Anderson, 1981). Breast epithelial cell proliferation culminates during early and mid-pregnancy, driving further branching and alveologenesis thereby achieving an enormous increase in functional surface. Following differentiation into a milk-secreting organ, copious amounts of milk can be produced during lactation. Upon weaning, milk production stops and most of the epithelial structures disappear. No matter how many pregnancies occur, there is no evidence that the cell expansion potential of the breast epithelium gets exhausted. This process of extensive cell proliferation prior to lactogenic differentiation in largely stimulated by PR signaling as we will explain below.

PR signaling in the normal breast

Detailed insights into the complexities of PR signaling at the molecular level have been gained from studies with T47D cells, a breast cancer cell line established from the pleural effusion of a 54-year-old breast cancer patient (Keydar et al., 1979) and several other hormone-sensitive breast cancer cell line models, such as MCF-7 and ZR-75 cells and this work is reviewed elsewhere in this issue.

It has become apparent that PR signaling is context-dependent and that, in particular, growth factor receptor signaling and cell cycle kinases intersect with it at multiple levels (Dwyer et al., 2020; Hagan and Lange, 2014). As tumor cells have increased growth factor receptor signaling activity, the context in which progesterone will act in a cancer cell is likely very different from that of a normal breast epithelial cell and is unclear how representative findings in any cell line are for what happens in different patients' tumors.

Another major obstacle to our understanding of physiological PR signaling is the lack of cell line models for normal breast epithelial cells that are hormone-responsive. Primary breast epithelial cells can readily by obtained from reduction mammoplasty specimens (Stampfer and Bartley, 1985) but as soon as they are cultured *in vitro,* they lose hormone receptor expression. Moreover, upon passaging primary human breast epithelial cell cultures, which contain a mix of luminal and myoepithelial cells, the luminal cells, which proliferate more slowly *in vitro,* are lost and cells with a basal phenotype, DNp63+, ITGB1+ grow out. As a consequence, all immortalized HBEC cells lines and so-called normal breast cell lines, like MCF10A, represent a basal phenotype and have no hormone receptor expression.

The use of 3D-cultures with matrigel has demonstrated the importance of the correct structure in space and the role of the extracellular matrix (ECM) for the formation of a bi-layered epithelium and cellular differentiation (Aggeler et al., 1991) however, steroid hormone receptor expression is down modulated and progesterone target genes like *WNT4* and *RANKL* are not induced (Graham et al., 2009). Nevertheless, in these various elegant systems differential effects on the ECM (Liu et al., 2018) and on cell proliferation (Graham et al., 2009; Sokol et al., 2016) have been observed in response to hormone stimulation. In light of the complexities *in vivo* with changing hormone levels, hormones acting on many different tissues with many indirect effects on the breast, it has been a challenge to dissect the mechanisms of PR signaling in the breast epithelium.

Hormone ablation and replacement studies in rodents

The foundations of our understanding of the endocrine regulation of the mammary gland were laid in the late 1950s by hormone ablation and replacement experiments performed in rats, rabbits, and mice (Lyons, 1958; Nandi, 1958). Through surgical removal of the pituitary gland, the ovaries and the adrenal glands, animals were hormone-depleted; as a result, mammary gland development stalled and the

epithelium atrophied. In the triple abrogated animals, mammary gland growth was largely restored by injection of growth hormone (GH), E, and P. In the presence of GH, administration of E was sufficient to trigger the ductal outgrowth with characteristic terminal end buds and dichotomous branching that is seen during puberty (Figure 2). Progesterone had little effect in the absence of circulating estrogens, which may, at least in part, be due to very reduced expression of the PR, which is an ER target gene (Haslam and Shyamala, 1979; Kastner et al., 1990). Added to GH, E+P treatment resulted in extensive ductal side branching and alveolar bud formation driving the gland to a mid/late pregnancy like state (Figure 2). These experiments established P as driver of side branching and begged the question whether the changes elicited by hormone administration are attributable to PR signaling in the mammary gland and if so, in which cell type, or secondary to signals from other organs.

Lessons from the genetically engineered mouse models

The advent of genetically engineered mouse models (GEMMs) provided useful tools for the genetic dissection of biological functions. PR deficient mice (*PR*$^{-/-}$), both males and females, developed normally but female mutants had multiple reproductive problems, including failure to ovulate. Limited mammary gland development was observed (Lydon et al., 1995). To address to what extent this was due to lack of mammary epithelial PR signaling versus systemic effects caused by the germ line deletion, we adapted the technique of mammary epithelial transplants originally developed to propagate epithelium from mice bearing hyperplasia (DeOme, 1959). In prepubertal, 3-week-old mice, the rudimentary ductal tree can be surgically removed from the elongated inguinal mammary glands leaving behind a cleared fat pad. A piece of epithelium from a syngeneic mouse grafted to the center of such a cleared fat pad can grow into a new ductal tree that behaves like the endogenous epithelium but does not communicate with the nipple, hence lactation cannot occur. Engraftment of contralateral cleared fat pads with *WT* and *PR*$^{-/-}$ epithelium allows one to compare the two within the same normal hormonal milieu and hence to determine the effect of this single gene deletion specifically in the mammary epithelium. *PR*$^{-/-}$ epithelium filled the engrafted fat pad by dichotomous branching albeit with fewer branching points than the *WT* (Brisken et al., 1998; Rajaram et al., 2015). When the recipients were impregnated, *WT* grafts, like endogenous control glands, were full of alveoli at the end of pregnancy, whereas the *PR*$^{-/-}$ epithelium showed only dichotomous branching, no side branching nor any alveologenesis. Together with the endocrine ablation and replacement studies discussed above, these genetic tissue recombination experiments show that progesterone, acting through the mammary epithelial PR, drives the cell expansion during pregnancy that results in side branching and subsequent alveologenesis during recurrent estrous cycles and pregnancy.

The *PR* gene has two promoters resulting in translation from two different AUGs. The shorter PR-A isoform lacks a 165 amino acid stretch at the N-terminus that encodes a PR-B specific transactivation function (Sartorius et al., 1994) and shows different transcriptional activities (Richer et al., 2002). The two forms were reported to be differentially expressed during development with the A-form decreasing

during pregnancy (Aupperlee et al., 2005) and to be regulated in distinct ways by estrogens and progesterone and along the estrous cycle as assessed by IHC (Shyamala et al., 1998). Isoform-specific deletions revealed that PR-A is required for uterine function but not essential for the response of the mammary gland to progesterone (Mulac-Jericevic et al., 2000). *PR-B*$^{-/-}$ females were fertile; mammary gland development was deficient; more specifically, side branching during pregnancy was reduced albeit not completely as in *PR*$^{-/-}$ females. Moreover, in contrast to *PR*$^{-/-}$ mice, alveologenesis was observed in *PR-B*$^{-/-}$ females (Mulac-Jericevic et al., 2003). As different endocrine perturbances are encountered in the distinct *PR* mutant strains, which may confound the interpretation of the observed phenotypes, the mice were ovariectomized and subsequently treated with either E or E+P. The side branching elicited by E+P administration in *PR-A*$^{-/-}$ females was comparable to that observed in their *WT* controls, abrogated in *PR*$^{-/-}$ and reduced in *PR-B*$^{-/-}$ females (Mulac-Jericevic et al., 2003). Hence, PR-B is required for ductal side branching and PR-A is required for alveologenesis. This indicates that PR-A and PR-B have context-dependent roles in the mammary epithelium; whether these relate to specific transcriptional activation of specific target genes and/or different interactions with the ER, which has growth-stimulatory role during puberty and growth-inhibitory role in pregnancy (Cagnet et al., 2018) and or depends on the amount of ligands around remains to be determined.

A differential role for the two forms in human breast cancer is emerging with the PR-A form specifically upregulated in breast cancer as reviewed by Satorius and Horwitz in this issue.

Developmental control of PR expression

An additional level of complexity of PR signaling in normal physiology stems from the differential developmental regulation of the expression of the receptor itself. PR protein expression by immunohistochemistry is first detected around postnatal day 10 (Rajaram et al., 2015). During puberty, the receptor is expressed in most of the cells constituting the inner layer of the milk ducts, the luminal epithelial cells. In adulthood, its expression becomes more confined and only about 30-50% of the luminal cells are PR+, this proportion decreases further during pregnancy (Grimm et al., 2002). Analysis of the mammary glands of *PR*^{lacZ} reporter mice at different developmental stages revealed changing regulation of *PR* promoter with transcription already occurring in the ducts of the embryonic mammary gland (Ismail et al., 2002). During puberty, *LacZ* was widely transcribed in the luminal epithelial cells and body cells of the terminal end buds. B-galactosidase activity was not detected in cap cells nor myoepithelial cells, nor in periductal fibroblasts, adipocytes or endothelial cells. In the adult female, *PR* expression was attenuated and a nonuniform expression pattern was observed with high expression at the tips of the budding side branches and decreased activity by the end of pregnancy (Ismail et al., 2002) indicating that the discrete and changing expression patterns of the PR observed by IHC are determined at the transcriptional level.

Cell intrinsic and paracrine signaling

The variegated expression of ER and PR in the adult mammary gland is also observed in other animal species and in humans. It was first shown in the human breast that most of the proliferating cells in the normal breast epithelium are ER/PR negative by IHC (Clarke et al., 1997), an observation that confirmed in rats (Russo et al., 1999) and mice (Seagroves et al., 2000). Mixtures of mammary epithelial cells from *PR$^{-/-}$.ROSA26+* females with an excess of *WT.ROSA26-* mammary epithelial cells were used to reconstitute cleared mammary fat pads. The resulting chimeric glands showed that in the context of surrounding *WT* cells, *PR$^{-/-}$.ROSA26+* mammary epithelial cells were able to contribute to side branching and alveologenesis during pregnancy (Brisken et al., 1998). This established that progesterone-induced side branching and alveolar budding can be elicited by one or more paracrine signals and led to a model whereby PR+ cells, or a subset thereof, function as hormone sensors that act as organizers and translate the systemic signal into paracrine signals that enable neighboring PR negative cells to proliferate and activate stem and progenitor cells (Brisken and Duss, 2007) (Figure 3). This organizational structure is a means of coordinating the behavior of multiple cell types, as well as a way of propagating locally a fluctuating systemic signal over time.

Subsequently, a number of factors were identified as downstream mediators of PR signaling either by candidate approaches or through global gene expression profiling (Fernandez-Valdivia et al., 2008; Lain et al., 2013, 2015). Among the PR targets in the adult mouse mammary gland are a number of genes encoding secreted factors including Receptor activator of NFκB (Rankl) (Brisken et al., 2002; Cao et al., 2001; Mulac-Jericevic et al., 2003), Wnt4 (Brisken et al., 2000), Calcitonin (Ismail et al., 2004), CXCL-12 (Shiah et al., 2015), Amphiregulin (Aupperlee et al., 2013), and Adamts18 (Ataca et al., 2020). on Here, we will describe what has been revealed about the importance in mediating distinct biological aspects of PR signaling function *in vivo*. Of note, the rat model was used to identify PR targets by ChIP-seq and revealed that networks relating to cell cycle and FGF and ErbB signaling were enriched (Ding et al., 2019).

Cell proliferation: intrinsic and paracrine effects of PR signaling

Cell proliferation is the aspect that is easiest to dissect because it can readily by assessed by BrdU incorporation. Upon ovariectomy, cell proliferation in the mammary epithelium ceases and ducts become atrophic. Administration of estrogen to adult ovariectomized mice elicits little proliferation (Wang et al., 1990) but induces the expression of the PR. Co-administration of E and P causes extensive cell proliferation in the epithelium (Wang et al., 1990). This occurs in two waves; the first small wave, revealed by BrdU incorporation occurs in PR+ cells within 24 hours of stimulation, it is followed by a much larger wave of cell proliferation entailing PR negative cells. The first type of proliferation of ER/PR+ cells is cyclin D1-dependent; it fails to occur in *cyclin D1$^{-/-}$* epithelium, the second, larger wave of cell proliferation, occurring in cells which are PR negative, is Rankl-dependent (Beleut et al., 2010). The importance of Rankl in mediating the proliferative response to P was illustrated by MMTV-Rankl transgenics which show side branching and alveolar budding in the absence of progesterone stimulation

(Fernandez-Valdivia et al., 2009) and a very elegant GEMM, in which *Rankl* was specifically induced in the ER+ sensor cells of $PR^{-/-}$ mice and shown to rescue side branching and alveologenesis (Mukherjee et al., 2010).

Stem cell activation

The realization that the most frequent insertion site of the MMTV virus in the mammary epithelium, the *int-1* locus (Nusse and Varmus, 1982) harbored a gene with sequence homology to the drosophila *wingless* gene (*Wnt1*) lead early on to interest in Wnt signaling in mammary gland development and in the context of breast cancer. Overexpression of *Wnt1* in the mammary gland under the control of the MMTV LTR, drastically altered the mouse mammary gland resulting in a brush-like appearance of the milk ducts with excessive side branches and caused mammary tumors (Tsukamoto et al., 1988). Various Wnt transcripts were detected and shown to have developmentally-regulated expression pattern (Dale et al., 1996; Gavin and McMahon, 1992). Of these, specifically Wnt4 was induced by progesterone and to a lesser extent by estrogen in the mammary glands of ovariectomized adult mice (Brisken et al., 2000) and its overexpression in mammary epithelial cells mimicked the effects of *Wnt1* and elicited pregnancy-like changes (Bradbury et al., 1995). Ectopic expression of *Wnt1* by means of a *MMTV:Wnt1* transgene (Tsukamoto et al., 1988) overcame the side branching defect of $PR^{-/-}$ epithelia showing that Wnt signaling is important downstream of *PR*. Moreover, ectopic *Wnt-1* expression induced side branching of $PR^{-/-}$ epithelia by a paracrine mechanism as shown by generating chimeric epithelia of $PR^{-/-}$ and *MMTV-Wnt1*+ mammary epithelial cells (Brisken et al., 2000).

Evidence emerged that Wnt signaling pathway is important for adult stem cells in many organs (Reya and Clevers, 2005). The ability of individual mammary epithelial cells to give rise to mammospheres (Dontu et al., 2003) became a useful assay for stem cells and was shown to correlate with the ability to reconstitute mammary fat pads (Cicalese et al., 2009; Pece et al., 2010). Furthermore, stem cells were defined by FACS profiling based on the expression of ITGB1 or ITGA6 and functionally validated by limiting dilution assays (Shackleton et al., 2006; Stingl et al., 2006), which turned into the golden assay of mammary stem cells. By these assays Rankl, Wnt4 and Cxcl12/CxcR4 were shown to be important downstream of P in activating stem cells (Asselin-Labat et al., 2010; Joshi et al., 2010; Shiah et al., 2015).

However, it is important to consider that the expression of various integrins used as stem cell markers itself can be subject to hormonal regulation; their expression at the cell surface may be affected by changes in the ECM secondary to hormonal stimulation. Hence, an increase in cell populations expressing these markers does not necessarily reflect an increase in the number of stem cells but may reflect an increased expression of the specific antigens on the cell surface. In addition, a more complex issue lies in the cleared fat pad assay itself. Before any cell that is injected into the fat pad can unfold its stem cell potential, it needs to adjust to the non-physiological environment, to survive and interact with the cells and matrix of the mammary stroma. It is conceivable that a mutation impairs these abilities of

an otherwise perfect mammary stem cell. As a result, even the ideal mammary stem cell will not survive and/or not give rise to an outgrowth and therefore not be scored as a stem cell. Of note, both, cell survival and interactions with ECM and stromal cells, are dependent on integrin signaling and it is conceivable that the presence of these stem cell markers bestows an adhesion and survival advantage. In contrast, the luminal cells, at least some of which show plasticity and high proliferative capacity *in vivo* may simply not get to unfold their stem cell properties in this assay because they lack the integrins required for survival and interaction with the mammary matrix. In line with this, lineage tracing experiments have revealed plasticity of luminal cells (Van Keymeulen et al., 2011, 2017) and vice versa identified rare luminal cells that can give rise ER+ basal cells during pregnancy (Song et al., 2019).

A more comprehensive assay for the regenerative potential of the mammary epithelium is the serial transplantation assay, in which a piece of mammary epithelium is transplanted for 7 subsequent generations (Daniel, 1973). Comparison of different mutants by this assay revealed that *Wnt4* deletion had the most severe effect on the regenerative potential of the mammary epithelium followed by *PR* whereas *Rankl* deletion did not significantly affect the serial reconstitution ability (Rajaram et al., 2015). These findings indicate that PR signaling is required for the induction of stem cell potential and requires Wnt4 for this function. A likely explanation for the finding that *Wnt4* deletion causes a more severe defect than *PR* abrogation is that ER signaling is involved in the control of *Wnt4* expression during earlier developmental stages (Rajaram et al., 2015).

ECM/basal membrane restructuring

The PR target, *Wnt4*, activates canonical Wnt signaling in the myoepithelium as shown genetically with the use of a reporter of the canonical Wnt signaling target, *Axin2* (Lustig et al., 2002; Rajaram et al., 2015). Consistently, global gene expression changes resulting from conditional deletion of the direct PR target *Wnt4* in the mammary epithelium are more striking in the myoepithelial than in the luminal cell compartment (Ataca et al., 2020). Among the indirect progesterone targets in the myoepithelium, is Adamts18, a secreted protease, which provides a molecular link between the luminal PR signaling and changes in the ECM.

Deletion of *Adamts18* in the mammary epithelium results in delayed ductal outgrowth and side branching but normal alveologenesis, reminiscent of, but less severe than the phenotype of *Wnt4*$^{-/-}$ mammary epithelium. *Adamts18*$^{-/-}$ mammary epithelium has decreased serial transplantation capacity indicating that the protease affects stem cells. Analysis of ADAMTS18 interactors in the MCF10A cell line model by Mass Spectometry revealed that the protease interacts with multiple basal membrane proteins. In line with its role being pertinent to the basal membrane and the basal membrane being part of the stem cell niche *Adamts18* interacts genetically with the basal membrane specific *Collagen18A1* in mammary gland development and in controlling the regenerative potential of the mammary epithelium (Ataca et al., 2020).

Of note, major effects on accumulation of ECM proteins, like Fibronectin, Laminin, CollagenI and IV were observed specifically during puberty but not in the adult mammary gland suggesting that the role of PR in controlling stem cells is particularly important in puberty. However, additional, yet to be discerned changes in the ECM may be elicited by Adamts18 at later stages. In any case, the ability of progesterone to induce a secreted protease that can cleave Fibronectin (Ataca et al., 2020), which is central to ECM assembly, provides a mechanistic link between epithelial hormone signaling and stromal changes.

Stage-specific roles of PR signaling

Taken together, a picture emerges, in which PR signaling has at least three distinct roles in the normal mammary epithelium (Figure 4).The first is during puberty, when progesterone levels are low and PR expression is high and widespread in the mammary epithelium. At this stage, the hormone, contributes to the induction of *Wnt4* and *Adamts18* expression which are involved in controlling mammary stem cells and their niches. This axis is maintained during adulthood when signaling is recurrently activated during diestrus but possibly most important during puberty.

The second function is important in adulthood, with recurrent estrus cycles and during pregnancy; PR signaling stimulates some cell-intrinsic proliferation via Cyclin D1 and elicits the extensive cell proliferation that is required to establish side branches by inducing Rankl expression. At this stage, all Rankl expressing cells are PR+, however, not all the PR+ cells express detectable levels of Rankl (Beleut et al., 2010), similarly, all *Wnt4* expressing cells are PR+, yet, not all the PR+ cells express *Wnt4* (Rajaram et al., 2015). This points to the possibility that different subsets of PR+ cells have different assignments in the progesterone orchestra and begs the questions whether the different transcriptional outcomes reflect stochastic events or depend on different cellular contexts laid down by distinct epigenetic profiles.

The third role of the PR is during the last third of pregnancy and relates to alveologenesis and requires PR-A. In *PR-B*$^{-/-}$ females, *Rankl* and *Wnt4* induction by E+P in ovariectomized adult females is abrogated, yet, alveologenesis occurs in *PR-B*$^{-/-}$ females but not in *PR*$^{-/-}$ females (Mulac-Jericevic et al., 2000, 2003). This indicates that the presence of PR-A isoform is sufficient for alveologenesis to occur and that alveologenesis occurs independent of the paracrine mediators *Rankl* and *Wnt4*. It is tempting to speculate that this may relate to PR-A isoform specific interactions with the ER, which has an important but barely understood inhibitory role in this process (Cagnet et al., 2018).

Implications for breast cancer

It is intuitive that induction of progesterone-dependent activities such as stem/progenitor cell activation, cell proliferation, and ECM modifications, if they occur similarly in the human breast in the context of recurrent luteal phases and/or progestin containing treatments, may promote carcinogenesis in the breast. This could, at least partly, explain why menstrual cycles and hormonal contraception, as well as

combined HRT, all of which result in PR signaling, increase BC risk. But are the same signals operating in the human breast?

Studies with reduction mammoplasty-derived normal breast epithelial cells cultured in Matrigel, revealed that progesterone stimulates cell proliferation and increases the number of progenitor cells (Graham et al., 2009). However, neither *RANKL* nor *WNT4* expression were induced in this model. To address whether the same pathways are pertinent in the human breast epithelium, we developed an *ex vivo* approach to test hormone response. Fresh reduction mammoplasty specimens were mechanically disrupted and enzymatically digested to isolate tissue microstructures that consist of epithelia and associated stromal cells (Sflomos et al., 2015; Tanos, 2013). Keeping the intercellular connections intact preserved the hormone response for at least 72 hours. In this *ex vivo* model, progesterone induced both *RANKL* and *WNT4* transcripts. Moreover, functionally, progesterone-induced cell proliferation was inhibited in the presence of the recombinant decoy receptor osteoprotegerin (rOPG) (Tanos, 2013), a finding confirmed in *ex vivo* experiments with tissue from *BRCA1* mutation carriers (Nolan et al., 2016). As observed in the mouse mammary gland, RANKL protein localized exclusively to PR+ cells but not all PR+ cells showed detectable RANKL expression (Tanos, 2013). Importantly, the physiological relevance of the finding in the *ex vivo* model was shown through analysis of normal breast biopsies. The RANKL protein was not detected by IHC in the breast epithelium from women in follicular phase but in samples from women with the high serum progesterone levels characteristic of luteal phase; furthermore its expression was higher and more widespread in samples from pregnant women who have yet higher progesterone levels (Tanos, 2013).

A study on tumor and contralateral normal breast tissue samples from premenopausal breast cancer patients with menstrual cycle staging through hormone measurements, confirmed the increase in *RANKL* expression related to high progesterone levels in the normal breast (Hu et al., 2014). Furthermore, together with other studies it revealed that *RANKL* transcripts are also regulated in ER/PR+ tumors (Haynes et al., 2013, 2014). Interestingly, in the normal breast epithelium increased mitotic activity has been reported to occur during luteal phase when progesterone levels are high (Anderson et al., 1982; Ferguson and Anderson, 1981; Pardo et al., 2014) but ER+ tumors proliferation-associated genes were decreased when progesterone levels peaked (Haynes et al., 2014, 2019). It needs to be evaluated whether this discrepancy may be related to different experimental approaches or whether this is an indication that PR signaling and cell cycle control are wired differently in normal breast epithelial cells and cancer cells.

Most insights into physiologic gene expression changes in the human breast epithelium comes from the work of Susan Clare and her colleagues, who drove the efforts leading to the Susan Komen tissue bank, which is a collection of normal breast tissue from healthy volunteers of different ages and races (Sherman et al., 2012). Hormone levels at the time of tissue sampling can be determined and samples thereby be attributed to follicular versus luteal phases of the menstrual cycle. When 20 samples from healthy breast tissue donors were used to isolate mammary epithelium by laser capture microdissection

and RNA sequencing was performed, 255 genes were found to be differentially expressed in luteal phase versus follicular with 221 increased in the luteal phase (Pardo et al., 2014). Most of them related to DNA replication, DNA damage, and mitosis. Both *RANKL* and *WNT4* were found to be increased in luteal phase together with a third paracrine factor *EPIREG*, known to bind ErbB1 and ErbB4, reviewed in (Riese and Cullum, 2014).

Furthermore, the secreted proteases, *MMP3* and *ADAMTS9* were significantly increased. RNA from myoepithelial cells represent a smaller fraction than luminal cell RNA and this may have precluded that *ADAMTS18* reached significance in this relatively small data set. The protease was induced by progesterone in xenografted human breast epithelial cells (Ataca et al., 2020). These findings may have important clinical implications; mammographic density is the single most important risk factor for breast cancer (Boyd et al., 2007; McCormack and dos Santos Silva, 2006) and has been correlated with progestin intake levels (Greendale et al., 2003; Vachon et al., 2002). While the role of the individual proteases remains to be dissected, they may provide insights into the molecular mechanisms underlying increased breast density and offer new targets for preventive strategies.

Together, these findings suggest that the organizational principles and major signaling axes are conserved between mice and humans and that hence recurrent activation of PR signaling may promote breast carcinogenesis.

An additional pathway that emerged from studies with human cells and tissue sections is the GHR signaling. GH is induced by progesterone in the breast epithelium in cells in direct vicinity of PR+ cells and was shown to act on ER-/PR- progenitor cells, which express the GHR (Lombardi et al., 2014) Interestingly, the GH receptor transcript was also increased during the luteal phase (Pardo et al., 2014). An interesting scenario was proposed that pituitary GH may stimulate mammary stem cells during pubertal growth whereas in the adult organism when systemic GH release largely subsided, GH can be produced locally under the control of PR signaling for progenitor cell expansion (Lombardi et al., 2014).

Another question of outstanding clinical importance is the role of PR signaling alterations in the context of endocrine resistance and metastasis. Decreased or lost expression of PR, independent of ER signaling activity (Kim et al., 2006; Piva et al., 2014), and PR mutations have been reported in ER+ metastatic breast cancer (Fowler et al., 2020).

Open questions, challenges and outlook

Given its preeminent role in tumor promoting activities, PR signaling is clearly an attractive target for cancer preventive strategies. To what extent the mechanisms we discussed are operational in tumor cells and whether it may also be useful to target the receptor in cancer is debated while estrogen is widely accepted as a driving force of breast cancer. Evidence that progesterone may stimulate cancer stem cells has accumulated, reviewed in (Axlund and Sartorius, 2012), however *RANKL* and *WNT4* are not induced by progesterone in most ER+/PR+ breast cancer cell lines and in various xenograft models, progesterone antagonizes E2-induced growth (Mohammed et al., 2015; Singhal et al., 2016). It is important to better

understand how the two players, ER and PR interact with each other when they synergize in many ways in the normal breast epithelium and if and/or how their interactions change during carcinogenesis? How does the change in ratio of the A- and B- isoforms affect PR signaling and its interactions with ER? Relative expression of the PR-A isoform is increased in tumor cells, how this affects PR signaling and/or interactions with other family members like the androgen and the glucocorticoid receptor?

The dissociation between HR expression and cell proliferation described in the normal breast is not observed in tumors where most of the proliferating cells are ER+ and by extension PR+ (Clarke et al., 1997). Indeed, abrogation of the dissociation between hormone receptor expression and cell proliferation is part of the earliest alterations that characterizes precursor lesions that show uniform ER/PR expression. The variegated expression characteristic of the adult breast epithelium is lost and all luminal cells express ER (Lee et al., 2007). How is this alteration brought about and how does it affect PR signaling? Throughout this review we distinguished PR+ and PR- luminal cells. This widely used dichotomy, however, is based on the somewhat arbitrary interpretation of IHC. Tissue recombination studies with mammary epithelial cells from different *Esr1* mutant GEMMs revealed that the ER is functionally important in cells that are ER negative by IHC. Indeed, about 50% of these ER-IHC negative cells were found to express detectable levels of *Esr1* transcript suggesting that there are at least 3 distinct luminal cell population with regards to ER status: IHC+ RNA+, IHC- RNA+, and IHC-/RNA; alternatively, there may be a gradient of ER positivity (Cagnet et al., 2018). The same scenario may apply to PR expression as well and may complicate the interpretation of PR signaling activities in the mammary gland when these are assessed across the epithelium. It prompts the question of whether PR expression levels are a stable feature of distinct cell populations or merely a transient characteristic that can fluctuate over time; i.e. low level expression may reflect recent signaling activities, as PR transcription is under negative control by PR signaling (Alexander et al., 1989). Expression of the PR in a subset of myoepithelial/progenitor cells has been reported (Hilton et al., 2014). Ever more powerful single cell analyses approaches are likely to provide the key to understanding the intercellular heterogeneity of PR signaling.

The *ex vivo* models increasingly used to study hormone signaling (Sflomos et al., 2015) have severe limitations when it comes to biochemical analyses, they are highly variable and because of the complex and changing cell composition difficult to interpret. However, combined with single cell analyses new opportunities arise and new culture approaches may extend their lifetime (C. Brito, personal communication). Furthermore, the possibility to xenograft hormone-sensitive tumor cells and normal human breast epithelial cells (Sflomos et al., 2016) to the milk ducts of immunocompromised mice and to study them there opens new experimental opportunities that will allow to address some of these questions in the near future.

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Declaration of interest:

We declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure legends:

Figure 1: Scheme of hormonal changes during a woman's life cycle.

Scheme showing the plasma concentrations of the two ovarian hormones E (red) and P (blue) over the lifetime of a woman as a function of reproductive stage of a woman. Data are based on studies on puberty (Elmlinger et al., 2005), during menstrual cycle, menopause (Kratz et al., 2004) and in pregnancy (Abbassi-Ghanavati et al., 2009).

Figure 2: Hormone induced changes in the mouse mammary gland.

Scheme depicting the morphogenetic changes elicited by E and E+P administration in mice ovariectomized in this case just around puberty. The rudimentary ductal system present at birth is induced to branch dichotomously. At the tips of the rapidly growing ducts terminal end buds (TEBs) form. The ducts bifurcate until the entire fat pad is filled. The administration of E+P elicits extensive side branching.

Figure 3: The progesterone signaling hub in the adult mammary epithelium

Progesterone, upon binding to its receptor in the ER+/PR+ sensor cells (blue) activates different signaling pathways. It can stimulate cell intrinsic proliferation by a cyclin D1-dependent mechanism (blue) and induce secreted factors like Amphiregulin, CXCl12, or Calcitonin (blue). Distinct PR+ cells induce Wnt4, which acts on the myoepithelium where it activateds canonical Wnt signaling, which results in the expression of the sereted protease Adamts18 that cleaves fibronectin. As a result the ECM, part of the stem cells niche is biochemically altered with resulting activation of the hippo signalling pathway and increased transcription of FGFR signaling components (red). In other PR+ cells, Rankl is induced that induces the proliferation of neighboring ER-/PR- responder cells (green).

Figure 4: Stage-specific outcome of PR signaling

Scheme of mammary gland development highlighting different functions of PR at different developmental stages. During puberty (1) PR signaling drives directly Wnt4 and indirectly Adamts18 expression both of which are important for the activation of mammary stem cells. In the adult mammary gland, during diestrous and during pregnancy (2), PR-B signaling relies on cyclinD1, Rankl and Wnt4 ot drive the extension cell proliferation that results in side branching. During the last third of pregnancy (3) there is a role for PR-A in alveologenesis revealed through genetic experiments. The underlying mechanisms are still poorly understood.

References:

Abbassi-Ghanavati, M., Greer, L.G., and Cunningham, F.G. (2009). Pregnancy and Laboratory Studies: A Reference Table for Clinicians. Obstet. Gynecol. *114*, 1326–1331.

Aggeler, J., Ward, J., Blackie, L., Barcellos-Hoff, M., Streuli, C., and Bissell, M. (1991). Cytodifferentiation of mouse mammary epithelial cells cultured on a reconstituted basement membrane reveals striking similarities to development in vivo. J Cell Sci *99*, p407-17.

Alexander, I.E., Clarke, C.L., Shine, J., and Sutherland, R.L. (1989). Progestin inhibition of progesterone receptor gene expression in human breast cancer cells. Mol. Endocrinol. Baltim. Md *3*, 1377–1386.

Anderson, T.J., Ferguson, D.J., and Raab, G.M. (1982). Cell turnover in the 'resting' human breast: influence of parity, contraceptive pill, age and laterality. Br J Cancer *46*.

Asselin-Labat, M.L., Vaillant, F., Sheridan, J.M., Pal, B., Wu, D., Simpson, E.R., Yasuda, H., Smyth, G.K., Martin, T.J., Lindeman, G.J., et al. (2010). Control of mammary stem cell function by steroid hormone signalling. Nature *465*, 798–802.

Ataca, D., Aouad, P., Constantin, C., Laszlo, C., Beleut, M., Shamseddin, M., Rajaram, R.D., Jeitziner, R., Mead, T.J., Caikovski, M., et al. (2020). The secreted protease Adamts18 links hormone action to activation of the mammary stem cell niche. Nat. Commun. *11*, 1571.

Aupperlee, M.D., Smith, K.T., Kariagina, A., and Haslam, S.Z. (2005). Progesterone receptor isoforms A and B: temporal and spatial differences in expression during murine mammary gland development. Endocrinology *146*, 3577–3588.

Aupperlee, M.D., Leipprandt, J.R., Bennett, J.M., Schwartz, R.C., and Haslam, S.Z. (2013). Amphiregulin mediates progesterone-induced mammary ductal development during puberty. Breast Cancer Res *15*, R44.

Axlund, S.D., and Sartorius, C.A. (2012). Progesterone regulation of stem and progenitor cells in normal and malignant breast. Mol. Cell. Endocrinol. *357*, 71–79.

Beleut, M., Rajaram, R.D., Caikovski, M., Ayyanan, A., Germano, D., Choi, Y., Schneider, P., and Brisken, C. (2010). Two distinct mechanisms underlie progesterone-induced proliferation in the mammary gland. Proc Natl Acad Sci U A *107*, 2989–2994.

Boyd, N.F., Guo, H., Martin, L.J., Sun, L., Stone, J., Fishell, E., Jong, R.A., Hislop, G., Chiarelli, A., Minkin, S., et al. (2007). Mammographic density and the risk and detection of breast cancer. N Engl J Med *356*, 227–236.

Bradbury, J.M., Edwards, P.A., Niemeyer, C.C., and Dale, T.C. (1995). Wnt-4 expression induces a pregnancy-like growth pattern in reconstituted mammary glands in virgin mice. Dev Biol *170*, 553–563.

Brisken, C. (2013). Progesterone signalling in breast cancer: a neglected hormone coming into the limelight. Nat Rev Cancer *13*, 385–396.

Brisken, C., and Duss, S. (2007). Stem cells and the stem cell niche in the breast: an integrated hormonal and developmental perspective. Stem Cell Rev *3*, 147–156.

Brisken, C., Park, S., Vass, T., Lydon, J.P., O'Malley, B.W., and Weinberg, R.A. (1998). A paracrine role for the epithelial progesterone receptor in mammary gland development. Proc Natl Acad Sci U A *95*, 5076–5081.

Brisken, C., Heineman, A., Chavarria, T., Elenbaas, B., Tan, J., Dey, S., McMahon, J., McMahon, A., and Weinberg, R. (2000). Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. Genes Dev *14*, p650-4.

Brisken, C., Ayyannan, A., Nguyen, C., Heineman, A., Reinhardt, F., Tan, J., Dey, S.K., Dotto, G.P., and Weinberg, R.A. (2002). IGF-2 is a mediator of prolactin-induced morphogenesis in the breast. Dev Cell *3*, 877–887.

Cagnet, S., Ataca, D., Sflomos, G., Aouad, P., Schuepbach-Mallepell, S., Hugues, H., Krust, A., Ayyanan, A., Scabia, V., and Brisken, C. (2018). Oestrogen receptor α AF-1 and AF-2 domains have cell population-specific functions in the mammary epithelium. Nat. Commun. *9*, 4723.

Cao, Y., Bonizzi, G., Seagroves, T.N., Greten, F.R., Johnson, R., Schmidt, E.V., and Karin, M. (2001). IKKalpha provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. Cell *107*, 763–775.

Cicalese, A., Bonizzi, G., Pasi, C.E., Faretta, M., Ronzoni, S., Giulini, B., Brisken, C., Minucci, S., Di Fiore, P.P., and Pelicci, P.G. (2009). The tumor suppressor p53 regulates polarity of self-renewing divisions in mammary stem cells. Cell *138*, 1083–1095.

Clarke, R.B., Howell, A., Potten, C.S., and Anderson, E. (1997). Dissociation between steroid receptor expression and cell proliferation in the human breast. Cancer Res *57*, 4987-91.

Colditz, G.A., Rosner, B.A., Chen, W.Y., Holmes, M.D., and Hankinson, S.E. (2004). Risk factors for breast cancer according to estrogen and progesterone receptor status. J Natl Cancer Inst *96*, 218–228.

Dale, T.C., Weber-Hall, S.J., Smith, K., Huguet, E.L., Jayatilake, H., Gusterson, B.A., Shuttleworth, G., O'Hare, M., and Harris, A.L. (1996). Compartment switching of WNT-2 expression in human breast tumors. Cancer Res *56*, 4320–4323.

Daniel, C.W. (1973). Finite growth span of mouse mammary gland serially propagated in vivo. Experientia *29*, 1422–1424.

DeOme, K.B. (1959). Development of Mammary Tumors from Hyperplastic Alveolar Nodiles Transplanted into Gland-free Mammary fat pads of female C3H Mice. Cancer Res *19*, 511–520.

Ding, L., Shunkwiler, L.B., Harper, N.W., Zhao, Y., Hinohara, K., Huh, S.J., Ekram, M.B., Guz, J., Kern, M.J., Awgulewitsch, A., et al. (2019). Deletion of Cdkn1b in ACI rats leads to increased proliferation and pregnancy-associated changes in the mammary gland due to perturbed systemic endocrine environment. PLoS Genet. *15*, e1008002.

Dontu, G., Abdallah, W.M., Foley, J.M., Jackson, K.W., Clarke, M.F., Kawamura, M.J., and Wicha, M.S. (2003). In vitro propagation and transcriptional profiling of human mammary stem/progenitor cells. Genes Dev *17*, 1253–1270.

Dwyer, A.R., Truong, T.H., Ostrander, J.H., and Lange, C.A. (2020). Steroid Receptors as MAPK Signaling Sensors in Breast Cancer: Let the Fates Decide. J. Mol. Endocrinol.

Elmlinger, M.W., Kühnel, W., and Ranke, M.B. (2005). Reference Ranges for Serum Concentrations of Lutropin (LH), Follitropin (FSH), Estradiol (E2), Prolactin, Progesterone, Sex Hormone-Binding Globulin (SHBG), Dehydroepiandrosterone Sulfate (DHEAS), Cortisol and Ferritin in Neonates, Children and Young Adults. Clin. Chem. Lab. Med. *40*, 1151–1160.

Ferguson, D.J., and Anderson, T.J. (1981). Morphological evaluation of cell turnover in relation to the menstrual cycle in the 'resting' human breast. Br J Cancer *44*.

609 Fernandez-Valdivia, R., Mukherjee, A., Creighton, C.J., Buser, A.C., DeMayo, F.J., Edwards, D.P.,
610 and Lydon, J.P. (2008). Transcriptional response of the murine mammary gland to acute progesterone
611 exposure. Endocrinology *149*, 6236–6250.

612 Fernandez-Valdivia, R., Mukherjee, A., Ying, Y., Li, J., Paquet, M., DeMayo, F.J., and Lydon, J.P.
613 (2009). The RANKL signaling axis is sufficient to elicit ductal side-branching and alveologenesis in
614 the mammary gland of the virgin mouse. Dev Biol *328*, 127–139.

615 Fowler, A.M., Salem, K., DeGrave, M., Ong, I.M., Rassman, S., Powers, G.L., Kumar, M., Michel,
616 C.J., and Mahajan, A.M. (2020). Progesterone Receptor Gene Variants in Metastatic Estrogen
617 Receptor Positive Breast Cancer. Horm. Cancer *11*, 63–75.

618 Gavin, B.J., and McMahon, A.P. (1992). Differential regulation of the Wnt gene family during
619 pregnancy and lactation suggests a role in postnatal development of the mammary gland. Mol Cell
620 Biol *12*, 2418–2423.

621 Graham, J.D., Mote, P.A., Salagame, U., van Dijk, J.H., Balleine, R.L., Huschtscha, L.I., Reddel,
622 R.R., and Clarke, C.L. (2009). DNA replication licensing and progenitor numbers are increased by
623 progesterone in normal human breast. Endocrinology *150*, 3318–3326.

624 Greendale, G.A., Reboussin, B.A., Slone, S., Wasilauskas, C., Pike, M.C., and Ursin, G. (2003).
625 Postmenopausal hormone therapy and change in mammographic density. J Natl Cancer Inst *95*, 30–
626 37.

627 Grimm, S.L., Seagroves, T.N., Kabotyanski, E.B., Hovey, R.C., Vonderhaar, B.K., Lydon, J.P.,
628 Miyoshi, K., Hennighausen, L., Ormandy, C.J., Lee, A.V., et al. (2002). Disruption of steroid and
629 prolactin receptor patterning in the mammary gland correlates with a block in lobuloalveolar
630 development. Mol Endocrinol *16*, 2675–2691.

631 Hagan, C.R., and Lange, C.A. (2014). Molecular determinants of context-dependent progesterone
632 receptor action in breast cancer. BMC Med. *12*, 32.

633 Haslam, S., and Shyamala, G. (1979). Effect of oestradiol on progesterone receptors in normal
634 mammary glands and its relationship with lactation. Biochem J *182*, p127-31.

635 Haynes, B.P., Viale, G., Galimberti, V., Rotmensz, N., Gibelli, B., A'Hern, R., Smith, I.E., and
636 Dowsett, M. (2013). Expression of key oestrogen-regulated genes differs substantially across the
637 menstrual cycle in oestrogen receptor-positive primary breast cancer. Breast Cancer Res. Treat. *138*,
638 157–165.

Haynes, B.P., Viale, G., Galimberti, V., Rotmensz, N., Gibelli, B., Smith, I.E., and Dowsett, M. (2014). Differences in expression of proliferation-associated genes and RANKL across the menstrual cycle in estrogen receptor-positive primary breast cancer. Breast Cancer Res Treat *148*, 327–335.

Haynes, B.P., Ginsburg, O., Gao, Q., Folkerd, E., Afentakis, M., Buus, R., Quang, L.H., Thi Han, P., Khoa, P.H., Dinh, N.V., et al. (2019). Menstrual cycle associated changes in hormone-related gene expression in oestrogen receptor positive breast cancer. NPJ Breast Cancer *5*, 42.

Hilton, H.N., Santucci, N., Silvestri, A., Kantimm, S., Huschtscha, L.I., Graham, J.D., and Clarke, C.L. (2014). Progesterone stimulates progenitor cells in normal human breast and breast cancer cells. Breast Cancer Res Treat *143*, 423–433.

Hu, H., Wang, J., Gupta, A., Shidfar, A., Branstetter, D., Lee, O., Ivancic, D., Sullivan, M., Chatterton, R.T., Jr., Dougall, W.C., et al. (2014). RANKL expression in normal and malignant breast tissue responds to progesterone and is up-regulated during the luteal phase. Breast Cancer Res Treat *146*, 515–523.

Ismail, P.M., Li, J., DeMayo, F.J., O'Malley, B.W., and Lydon, J.P. (2002). A novel LacZ reporter mouse reveals complex regulation of the progesterone receptor promoter during mammary gland development. Mol. Endocrinol. Baltim. Md *16*, 2475–2489.

Ismail, P.M., DeMayo, F.J., Amato, P., and Lydon, J.P. (2004). Progesterone induction of calcitonin expression in the murine mammary gland. J Endocrinol *180*, 287–295.

Joshi, P.A., Jackson, H.W., Beristain, A.G., Di Grappa, M.A., Mote, P.A., Clarke, C.L., Stingl, J., Waterhouse, P.D., and Khokha, R. (2010). Progesterone induces adult mammary stem cell expansion. Nature *465*, 803–807.

Kastner, P., Krust, A., Turcotte, B., Stropp, U., Tora, L., Gronemeyer, H., and Chambon, P. (1990). Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. EMBO J. *9*, 1603–1614.

Keydar, I., Chen, L., Karby, S., Weiss, F.R., Delarea, J., Radu, M., Chaitcik, S., and Brenner, H.J. (1979). Establishment and characterization of a cell line of human breast carcinoma origin. Eur. J. Cancer *15*, 659–670.

Kim, H.-J., Cui, X., Hilsenbeck, S.G., and Lee, A.V. (2006). Progesterone receptor loss correlates with human epidermal growth factor receptor 2 overexpression in estrogen receptor-positive breast cancer. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. *12*, 1013s–1018s.

669 Kratz, A., Ferraro, M., Sluss, P.M., and Lewandrowski, K.B. (2004). Case records of the
670 Massachusetts General Hospital. Weekly clinicopathological exercises. Laboratory reference values.
671 N Engl J Med *351*, 1548–1563.

672 Lain, A.R., Creighton, C.J., and Conneely, O.M. (2013). Research resource: progesterone receptor
673 targetome underlying mammary gland branching morphogenesis. Mol Endocrinol *27*, 1743–1761.

674 Lain, A.R., Creighton, C.J., and Conneely, O.M. (2015). Research Resource : Progesterone Receptor
675 Targetome. *27*, 1743–1761.

676 Lee, S., Medina, D., Tsimelzon, A., Mohsin, S.K., Mao, S., Wu, Y., and Allred, D.C. (2007).
677 Alterations of gene expression in the development of early hyperplastic precursors of breast cancer.
678 Am J Pathol *171*, 252–262.

679 Liu, Z., Speroni, L., Quinn, K.P., Alonzo, C., Pouli, D., Zhang, Y., Stuntz, E., Sonnenschein, C., Soto,
680 A.M., and Georgakoudi, I. (2018). 3D organizational mapping of collagen fibers elucidates matrix
681 remodeling in a hormone-sensitive 3D breast tissue model. Biomaterials *179*, 96–108.

682 Lombardi, S., Honeth, G., Ginestier, C., Shinomiya, I., Marlow, R., Buchupalli, B., Gazinska, P.,
683 Brown, J., Catchpole, S., Liu, S., et al. (2014). Growth Hormone Is Secreted by Normal Breast
684 Epithelium upon Progesterone Stimulation and Increases Proliferation of Stem/Progenitor Cells. Stem
685 Cell Rep. *2*, 780–793.

686 Lustig, B., Jerchow, B., Sachs, M., Weiler, S., Pietsch, T., Karsten, U., van de Wetering, M., Clevers,
687 H., Schlag, P.M., Birchmeier, W., et al. (2002). Negative feedback loop of Wnt signaling through
688 upregulation of conductin/axin2 in colorectal and liver tumors. Mol Cell Biol *22*, 1184–1193.

689 Lydon, J., De, M.F., Funk, C., Mani, S., Hughes, A., Montgomery, C.J., Shyamala, G., Conneely, O.,
690 and O'Malley, B. (1995). Mice lacking progesterone receptor exhibit pleiotropic reproductive
691 abnormalities. Genes Dev *9*, p2266-78.

692 Lyons, W.R. (1958). Hormonal synergism in mammary growth. Proc R Soc Lond Ser B 303–325.

693 MacMahon, B., Lin, T.M., Lowe, C.R., Mirra, A.P., Ravnihar, B., Salber, E.J., Trichopoulos, D.,
694 Valaoras, V.G., and Yuasa, S. (1970). Lactation and cancer of the breast. A summary of an
695 international study. Bull World Health Organ *42*, 185–194.

696 Mangelsdorf, D.J., Thummel, C., Beato, M., Herrlich, P., Schütz, G., Umesono, K., Blumberg, B.,
697 Kastner, P., Mark, M., Chambon, P., et al. (1995). The nuclear receptor superfamily: the second
698 decade. Cell *83*, 835–839.

McCormack, V.A., and dos Santos Silva, I. (2006). Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. Cancer Epidemiol Biomark. Prev *15*, 1159–1169.

Mohammed, H., Russell, I.A., Stark, R., Rueda, O.M., Hickey, T.E., Tarulli, G.A., Serandour, A.A., Birrell, S.N., Bruna, A., Saadi, A., et al. (2015). Progesterone receptor modulates ERalpha action in breast cancer. Nature *523*, 313–317.

Mukherjee, A., Soyal, S.M., Li, J., Ying, Y., He, B., DeMayo, F.J., and Lydon, J.P. (2010). Targeting RANKL to a specific subset of murine mammary epithelial cells induces ordered branching morphogenesis and alveologenesis in the absence of progesterone receptor expression. FASEB J *24*, 4408–4419.

Mulac-Jericevic, B., Mullinax, R.A., DeMayo, F.J., Lydon, J.P., and Conneely, O.M. (2000). Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. Science *289*, 1751-4.

Mulac-Jericevic, B., Lydon, J.P., DeMayo, F.J., and Conneely, O.M. (2003). Defective mammary gland morphogenesis in mice lacking the progesterone receptor B isoform. Proc Natl Acad Sci U A *100*, 9744–9749.

Nandi, S. (1958). Endocrine control of mammary gland development and function in the C3H/HeCrgl mouse. J Natl Cancer Inst 1039–1063.

Nolan, E., Vaillant, F., Branstetter, D., Pal, B., Giner, G., Whitehead, L., Lok, S.W., Mann, G.B., Kathleen Cuningham Foundation Consortium for Research into Familial Breast Cancer (kConFab), Rohrbach, K., et al. (2016). RANK ligand as a potential target for breast cancer prevention in BRCA1-mutation carriers. Nat. Med. *22*, 933–939.

Nusse, R., and Varmus, H. (1982). Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. Cell *31*, p99-109.

Pardo, I., Lillemoe, H.A., Blosser, R.J., Choi, M., Sauder, C.A., Doxey, D.K., Mathieson, T., Hancock, B.A., Baptiste, D., Atale, R., et al. (2014). Next-generation transcriptome sequencing of the premenopausal breast epithelium using specimens from a normal human breast tissue bank. Breast Cancer Res *16*, R26.

Pece, S., Tosoni, D., Confalonieri, S., Mazzarol, G., Vecchi, M., Ronzoni, S., Bernard, L., Viale, G., Pelicci, P.G., and Di Fiore, P.P. (2010). Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. Cell *140*, 62–73.

729 Piva, M., Domenici, G., Iriondo, O., Rábano, M., Simões, B.M., Comaills, V., Barredo, I., López-
730 Ruiz, J.A., Zabalza, I., Kypta, R., et al. (2014). Sox2 promotes tamoxifen resistance in breast cancer
731 cells. EMBO Mol. Med. *6*, 66–79.

732 Rajaram, R.D., Buric, D., Caikovski, M., Ayyanan, A., Rougemont, J., Shan, J., Vainio, S.J., Yalcin-
733 Ozuysal, O., and Brisken, C. (2015). Progesterone and Wnt4 control mammary stem cells via
734 myoepithelial crosstalk. EMBO J *34*, 641–652.

735 Reya, T., and Clevers, H. (2005). Wnt signalling in stem cells and cancer. Nature *434*, 843–850.

736 Richer, J.K., Jacobsen, B.M., Manning, N.G., Abel, M.G., Wolf, D.M., and Horwitz, K.B. (2002).
737 Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. J.
738 Biol. Chem. *277*, 5209–5218.

739 Riese, D.J., and Cullum, R.L. (2014). Epiregulin: roles in normal physiology and cancer. Semin. Cell
740 Dev. Biol. *28*, 49–56.

741 Russo, J., Ao, X., Grill, C., and Russo, I.H. (1999). Pattern of distribution of cells positive for estrogen
742 receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland [In
743 Process Citation]. Breast Cancer Res Treat *53*, 217–227.

744 Sartorius, C.A., Melville, M.Y., Hovland, A.R., Tung, L., Takimoto, G.S., and Horwitz, K.B. (1994).
745 A third transactivation function (AF3) of human progesterone receptors located in the unique N-
746 terminal segment of the B-isoform. Mol Endocrinol *8*, 1347–1360.

747 Seagroves, T.N., Lydon, J.P., Hovey, R.C., Vonderhaar, B.K., and Rosen, J.M. (2000). C/EBPbeta
748 (CCAAT/enhancer binding protein) controls cell fate determination during mammary gland
749 development. Mol Endocrinol *14*, 359-68.

750 Sflomos, G., Shamsheddin, M., and Brisken, C. (2015). An ex vivo model to study hormone action in
751 the human breast. J Vis Exp e52436.

752 Sflomos, G., Dormoy, V., Metsalu, T., Jeitziner, R., Battista, L., Scabia, V., Raffoul, W., Delaloye,
753 J.F., Treboux, A., Fiche, M., et al. (2016). A Preclinical Model for ERalpha-Positive Breast Cancer
754 Points to the Epithelial Microenvironment as Determinant of Luminal Phenotype and Hormone
755 Response. Cancer Cell *29*, 407–422.

756 Shackleton, M., Vaillant, F., Simpson, K.J., Stingl, J., Smyth, G.K., Asselin-Labat, M.L., Wu, L.,
757 Lindeman, G.J., and Visvader, J.E. (2006). Generation of a functional mammary gland from a single
758 stem cell. Nature *439*, 84–88.

Sherman, M.E., Figueroa, J.D., Henry, J.E., Clare, S.E., Rufenbarger, C., and Storniolo, A.M. (2012). The Susan G. Komen for the Cure Tissue Bank at the IU Simon Cancer Center: a unique resource for defining the "molecular histology" of the breast. Cancer Prev. Res. Phila. Pa *5*, 528–535.

Shiah, Y.J., Tharmapalan, P., Casey, A.E., Joshi, P.A., McKee, T.D., Jackson, H.W., Beristain, A.G., Chan-Seng-Yue, M.A., Bader, G.D., Lydon, J.P., et al. (2015). A Progesterone-CXCR4 Axis Controls Mammary Progenitor Cell Fate in the Adult Gland CXCR4 Function in Mammary Progenitors. Stem Cell Rep.

Shyamala, G., Yang, X., Silberstein, G., Barcellos-Hoff, M.H., and Dale, E. (1998). Transgenic mice carrying an imbalance in the native ratio of A to B forms of progesterone receptor exhibit developmental abnormalities in mammary glands. Proc Natl Acad Sci U A *95*, 696–701.

Singhal, H., Greene, M.E., Tarulli, G., Zarnke, A.L., Bourgo, R.J., Laine, M., Chang, Y.-F., Ma, S., Dembo, A.G., Raj, G.V., et al. (2016). Genomic agonism and phenotypic antagonism between estrogen and progesterone receptors in breast cancer. Sci. Adv. *2*, e1501924.

Skiba, M.A., Bell, R.J., Islam, R.M., Handelsman, D.J., Desai, R., and Davis, S.R. (2019). Androgens During the Reproductive Years: What Is Normal for Women? J. Clin. Endocrinol. Metab. *104*, 5382–5392.

Sokol, E.S., Miller, D.H., Breggia, A., Spencer, K.C., Arendt, L.M., and Gupta, P.B. (2016). Growth of human breast tissues from patient cells in 3D hydrogel scaffolds. Breast Cancer Res. BCR *18*, 19.

Song, W., Wang, R., Jiang, W., Yin, Q., Peng, G., Yang, R., Yu, Q.C., Chen, J., Li, J., Cheung, T.H., et al. (2019). Hormones induce the formation of luminal-derived basal cells in the mammary gland. Cell Res. *29*, 206–220.

Soto, A.M., and Sonnenschein, C. (2015). Endocrine disruptors: DDT, endocrine disruption and breast cancer. Nat. Rev. Endocrinol. *11*, 507–508.

Soto, A.M., Brisken, C., Schaeberle, C., and Sonnenschein, C. (2013). Does cancer start in the womb? altered mammary gland development and predisposition to breast cancer due to in utero exposure to endocrine disruptors. J Mammary Gland Biol Neoplasia *18*, 199–208.

Stampfer, M.R., and Bartley, J.C. (1985). Induction of transformation and continuous cell lines from normal human mammary epithelial cells after exposure to benzo[a]pyrene. Proc Natl Acad Sci U A *82*, 2394–2398.

Stingl, J., Eirew, P., Ricketson, I., Shackleton, M., Vaillant, F., Choi, D., Li, H.I., and Eaves, C.J. (2006). Purification and unique properties of mammary epithelial stem cells. Nature *439*, 993–997.

790 Tanos, T., Sflomos, G.,.Echeverria,P.C.,. Ayyanan, A.,. Gutierrez,
791 M.,.Delaloye,J.F.,.Raffoul,W.,.Fiche,M.,. Dougall, W.,. Schneider, P.,. Yalcin-Ozuysal, O.,. Brisken,
792 C. (2013). Progesterone/RANKL is a major regulatory axis in the human breast. Sci. Transl. Med. *5*.

793 Tsukamoto, A., Grosschedl, R., Guzman, R., Parslow, T., and Varmus, H. (1988). Expression of the
794 int-1 gene in transgenic mice is associated with mammary gland hyperplasia and adenocarcinomas in
795 male and female mice. Cell *55*, p619-25.

796 Vachon, C.M., Sellers, T.A., Vierkant, R.A., Wu, F.F., and Brandt, K.R. (2002). Case-control study of
797 increased mammographic breast density response to hormone replacement therapy. Cancer Epidemiol
798 Biomark. Prev *11*, 1382–1388.

799 Van Keymeulen, A., Rocha, A.S., Ousset, M., Beck, B., Bouvencourt, G., Rock, J., Sharma, N.,
800 Dekoninck, S., and Blanpain, C. (2011). Distinct stem cells contribute to mammary gland
801 development and maintenance. Nature *479*, 189–193.

802 Van Keymeulen, A., Fioramonti, M., Centonze, A., Bouvencourt, G., Achouri, Y., and Blanpain, C.
803 (2017). Lineage-Restricted Mammary Stem Cells Sustain the Development, Homeostasis, and
804 Regeneration of the Estrogen Receptor Positive Lineage. Cell Rep. *20*, 1525–1532.

805 Veltmaat, J.M. (2017). Prenatal Mammary Gland Development in the Mouse: Research Models and
806 Techniques for Its Study from Past to Present. Methods Mol. Biol. Clifton NJ *1501*, 21–76.

807 Vogel, P.M., Georgiade, N.G., Fetter, B.F., Vogel, F.S., and McCarty, K.S. (1981). The correlation of
808 histologic changes in the human breast with the menstrual cycle. Am. J. Pathol. *104*, 23–34.

809 Wang, S., Counterman, L.J., and Haslam, S.Z. (1990). Progesterone action in normal mouse mammary
810 gland. Endocrinology *127*, 2183–2189.

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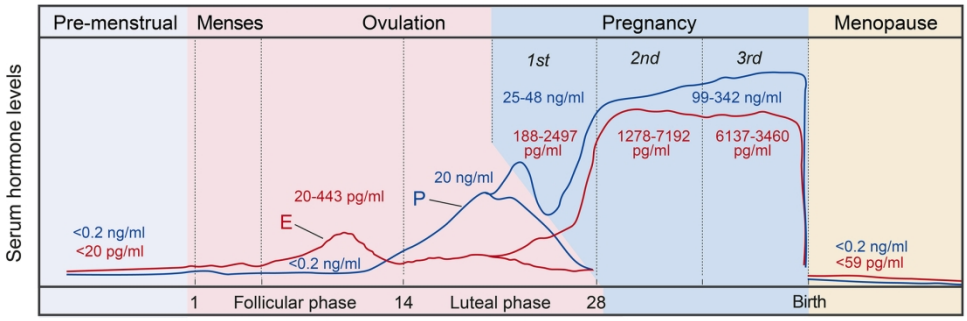

Figure 1: Scheme of hormonal changes during a woman's life cycle.
Scheme showing the plasma concentrations of the two ovarian hormones E (red) and P (blue) over the lifetime of a woman as a function of reproductive stage of a woman. Data are based on studies on puberty (Elmlinger et al., 2005), during menstrual cycle, menopause (Kratz et al., 2004) and in pregnancy (Abbassi-Ghanavati et al., 2009).

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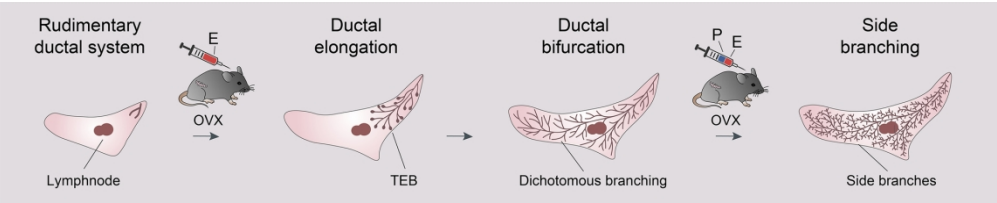

Figure 2: Hormone induced changes in the mouse mammary gland.
Scheme depicting the morphogenetic changes elicited by E and E+P administration in mice ovariectomized in this case just around puberty. The rudimentary ductal system present at birth is induced to branch dichotomously. At the tips of the rapidly growing ducts terminal end buds (TEBs) form. The ducts bifurcate until the entire fat pad is filled. The administration of E+P elicits extensive side branching.

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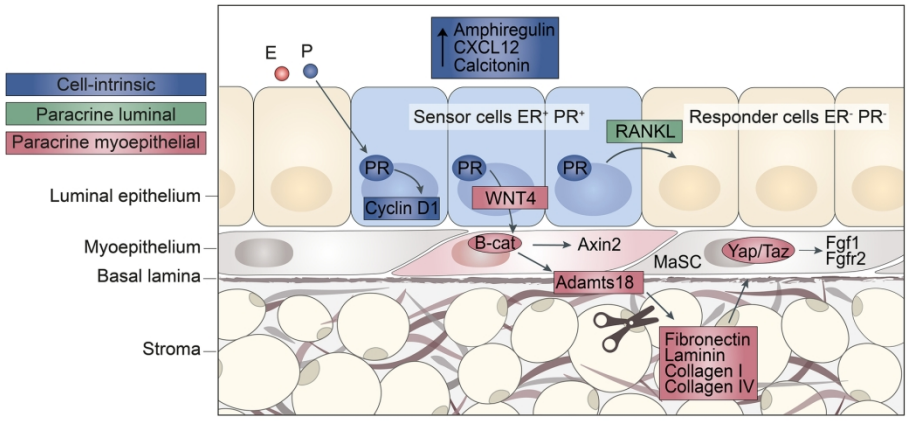

Figure 3: The progesterone signaling hub in the adult mammary epitheliumProgesterone, upon binding to its receptor in the ER+/PR+ sensor cells (blue) activates different signaling pathways. It can stimulate cell intrinsic proliferation by a cyclin D1-dependent mechanism (blue) and induce secreted factors like Amphiregulin, CXCl12, or Calcitonin (blue). Distinct PR+ cells induce Wnt4, which acts on the myoepithelium where it activateds canonical Wnt signaling, which results in the expression of the sereted protease Adamts18 that cleaves fibronectin. As a result the ECM, part of the stem cells niche is biochemically altered with resulting activation of the hippo signalling pathway and increased transcription of FGFR signaling components (red). In other PR+ cells, Rankl is induced that induces the proliferation of neighboring ER-/PR- responder cells (green).

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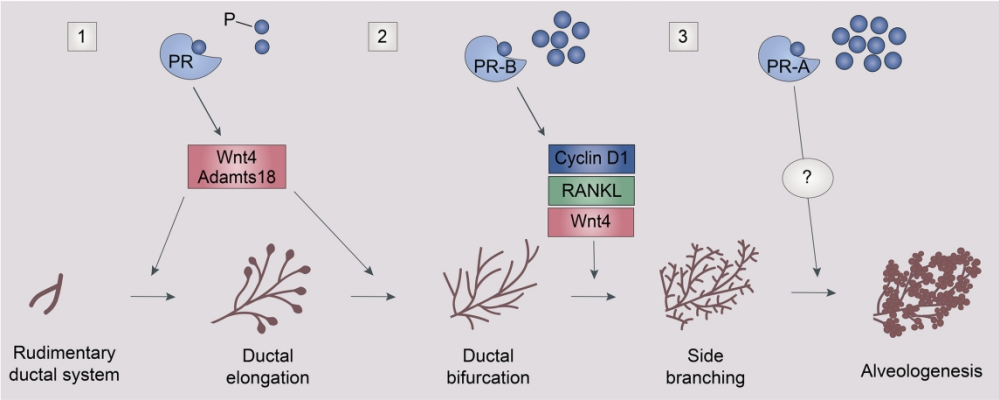

Figure 4: Stage-specific outcome of PR signaling
Scheme of mammary gland development highlighting different functions of PR at different developmental stages. During puberty (1) PR signaling drives directly Wnt4 and indirectly Adamts18 expression both of which are important for the activation of mammary stem cells. In the adult mammary gland, during diestrous and during pregnancy (2), PR-B signaling relies on cyclinD1, Rankl and Wnt4 ot drive the extension cell proliferation that results in side branching. During the last third of pregnancy (3) there is a role for PR-A in alveologenesis revealed through genetic experiments. The underlying mechanisms are still poorly understood.

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