Ovarian, Hypophyseal and Hypothalamic Hormones Coordinate Mammary Gland Remodeling in Adult *Lagostomus maximus*: a Rodent that Shows Pseudo-Ovulation at Mid-Gestation

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Abstract

Adult female mammary glands go through extensive tissue remodeling during pregnancy, lactation and after the weaning of the neonates. Here we characterize mammary gland morphology of adult females of *Lagostomus maximus*, a hystricomorph rodent with a pseudo-ovulatory event at mid-gestation, and describe how the glandular tissue changes its architecture in response to variations of the hormonal environment. At mid-gestation, pseudo-ovulation is seen as an essential event increasing the number of secondary corpora lutea and thus rising the circulating levels of progesterone that help to maintain pregnancy to term. As a side effect, mammary gland development is favored early during the long-lasting pregnancy of *L. maximus*, preparing females for the nutritional need of fully developed pups in this k-strategist species.

Keywords: mammary gland, prolactin, estradiol, progesterone, *Lagostomus maximus*

1. Introduction

Lactation has evolved as a vital part of the mammalian reproduction strategy [1]. During this process, ovarian, hypophyseal and hypothalamic hormones together with a myriad of factors synchronize actions for the growing and remodeling of the mammary glands. Over the past years, our understanding on how this complex hormone-driven process coordinate mecha-

nisms to guide mammary glands throughout growth-lactation-regression cycles has greatly improved. Nonetheless, there is still much to be learned about their roles in the development of each of the structural components of this organ.

The vast majority of mammary glands investigations have been performed in mice and rats. However, many aspects still remain unfulfilled covered by these conventional animal models since they differ considerably in mammary glands development and types of breast cancer from women [2, 3]. On the other hand, studies performed on unconventional rodents such as guinea pigs and hamsters that share with humans some endocrine and reproductive biology aspects have contributed to a better understanding of human physiology and disease [4], particularly on some reproductive tumors [5, 6].

Figure 1. Adult female plains vizcacha (*Lagostomus maximus*) nursing a pup. Credit: J. Halperin, Universidad Maimónides.

The South American plains vizcacha, *Lagostomus maximus*, is a hystricomorph rodent closely related to guinea pig (**Figure 1**) [7]. This species has attracted significant attention in the reproductive research field since female ovaries exhibit exceptional and unique characteristics among rodents. Females display natural massive poly-ovulation that can go up to 800 oocytes per cycle, the highest ovulatory rate so far recorded for a mammal, as a result of an unusual constitutive suppression of apoptosis that greatly decreases intra-ovarian oocyte dismissal caused by follicular atresia [8–12]. In addition, gestation lasts 154 ± 6 days [8], an unusually

long period for a rodent and one of the longest recorded among hystricomorphs. Moreover, pregnant females exhibit an ovulatory event at mid-pregnancy that leads to a considerable number of secondary corpora lutea with oocyte retention (i.e., pseudo-ovulation) and to an important rise of the progesterone levels [10, 11, 13]. This boost up in the circulating progesterone may contribute to an accurate maintenance of the uterus and embryo development up to the end of pregnancy [11, 14]. Given that ovarian hormones modulate growth and development of post-pubertal mammary glands, the reproductive peculiarities of the ovaries of *L. maximus* make of this species an interesting model to examine the mammary glands morphology according to its reproductive status.

The purpose of this chapter is to give a brief representation of the morphological changes that occur in the mammary glands between pregnancies under the action of ovarian, hypophyseal and hypothalamic hormones of adult plains vizcachas.

2. The mammary gland morphology of vizcachas

Adult female vizcachas have two pairs of functional mammary glands located below the ventral skin and laterally on the thorax. The skin epidermis is formed by a stratified squamous and keratinized epithelium which rests on a layer of dense collagenous connective tissue that contains hair follicles, sweat glands and fibroblasts. From the opening of the nipple and into the mammary glands, the number of epithelial layers decreases until it reaches a two-layer epithelium which upholsters each branched tubulo-alveolar gland [15].

The mammary gland secretory parenchyma is divided into lobes and then lobules by connective tissue septa. Lobules are formed by intralobular ducts that connect to an interlobular duct which finally empty into the lactiferous duct. The lactiferous duct is the excretory duct of each lobe and connects to the opening nipple to allow the release of milk during lactation. Before reaching the opening nipple, the lactiferous duct lumen forms a lactiferous sinus that functions as a reservoir for milk during lactation.

The mammary gland epithelium that coats the ducts is composed by an inner layer of secretory cells and an outer layer of myoepithelial cells which lies on the basement membrane that separates parenchymal and stromal compartments. The surrounding stroma is mainly composed by connective tissue, endothelial vessels, fibroblasts and immune cells. Unlike to what have been described for mouse and rat, mammary glands of adult vizcachas have a poor fat content [15]. General morphology and a detailed description of each cellular component of adult mammary glands of *L. maximus* are depicted in **Figure 2**.

As adult females transit throughout pregnancy and lactation, the mammary gland develops a more elaborated structure as a result of proliferation, branching and differentiation of the ductal tree. The extent of the development of the ductal network is closely related to the female reproductive status and the hormonal milieu.
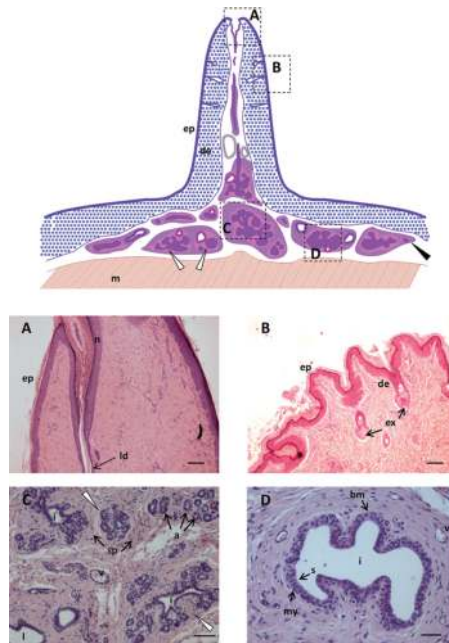
Figure 2. General morphology of the mammary gland of female *L. maximus*. Up: schematic draw depicts the general morphology of a post-pubertal mammary gland of *L. maximus*. Bottom: representative photomicrographs of each cellular component of the mammary glands. (A) Lactiferous duct epithelium is continuous with the stratified squamous and keratinized skin epidermis. (B) Underlying dermis is formed by a layer of dense collagenous connective tissue containing hair follicles, sweat glands and fibroblasts. (C) Secretory parenchyma composed by intralobular ducts forming lobules that join into lobes. Connective tissue septa surround each lobule. (D) Epithelial lining of the ductal network is made up of a luminal secretory cell layer and a basal myoepithelial cell layer which rests on a basal membrane that separates the epithelium from the surrounding stroma. a, secretory alveolus; bm, basal membrane; de, dermis; ep, epidermis; ex, exocrine gland; i, intralobular duct; I, interlobular duct; m, muscle; my, myoepithelial cell layer; n, nipple opening; s, secretory cell layer; sp, connective tissue septum; v, blood vessel. Filled arrowhead: lobes. Empty arrowhead: lobules. Scale bar is 150 μm for photos A and B; 100 μm for photo C and 25 μm for photo D.

3. Hormonal regulation of mammary glands growth and development according to the vizcacha reproductive status

3.1. Cycling

Short before the breeding season, mammary glands of non-pregnant adult vizcachas are in a "resting" state and present predominance of stromal connective tissue over the rudimentary ductal tree, which is mainly characterized by a few ducts and scarce secretory alveoli. At this stage, circulating estradiol can be high if the animal is ovulating. Yet, expression of estrogen receptor β, ERβ, is weak, and ERα is almost absent in the mammary glands (**Figure 3**). These observations could be interpreted as an indication that estradiol does not play an important role in the metabolism of cycling mammary glands of *L. maximus*. Such hypothesis is opposed

to what have been previously shown in mammary glands of virgin mice [16–18]. Those reports demonstrated that estradiol is a crucial regulator of branching through both of its receptors, being ERα the more important for mammary glands development. Considering that our group of cycling vizcachas is composed by adult females captured in their natural environment, they most likely have gone through one or more pregnancies prior to the capture. Their mammary glands have already experienced pregnancy-lactation-regression cycles. Moreover, although at this resting state there is no secretory activity in the mammary glands, some ducts still show residual milk fat globules in their lumen which is indicative of a recent lactation. These evidences support the idea that these are not virgin females and so, their mammary glands are already mature. Their ductal network, even in a resting status, already comprises secondary branching. Nevertheless, the normal expression of ERα and ERβ in mammary gland of virgin vizcachas is still pending. Just then, we will be able to confirm the role of those receptors in the regulation of mammary gland secondary branching.
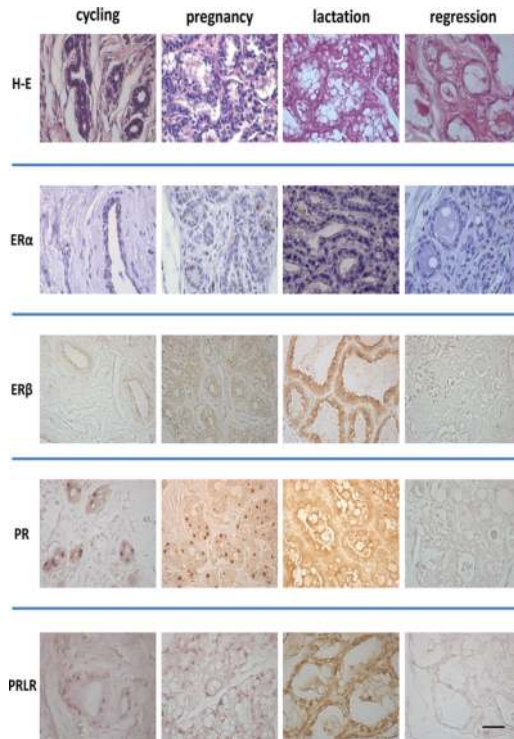

Figure 3. Hormonal regulation of the vizcacha mammary gland development according to the reproductive status. Representative photomicrographs of mammary gland sections of adult vizcachas at cycling, pregnancy, lactation and regression status. H-E, hematoxilyn-eosin; ERα, estrogen receptor α ERβ, estrogen receptor β PR, progesterone receptor; PRLR, prolactin receptor. Immunoreactivity is shown in brown and only for ERα hematoxylin-counterstained nuclei in blue. All photomicrographs have the same magnification. Scale bar is depicted in the last photo (bottom right) and represents 25 μm.

It is well established that in response to ovarian steroids at the onset of cyclicity, the mammary gland enlarges, the ducts undergo rapid extension and branching, and the mammary epithelial cells fill the mammary fat pad. It is also known that, in cycling females, prolactin (PRL) is only indirectly involved in the formation of ductal side branching by promoting luteal progesterone synthesis, as evident by the restoration of ductal branching in PRL knockout females treated with progesterone [19, 20]. In accordance with these references, we did not detected PRL receptor (PRLR) expression in membrane of ductal epithelium of cycling vizcachas (**Figure 3**). Yet, we detected a conspicuous PRLR mark in nuclei if ductal epithelium. It has been proposed that polypeptide ligands like PRL and their receptors may translocate into the nucleus and regulate the expression of specific transcription factors [21]. Our results suggest that the role of PRL over mammary glands may not be restricted to its known trophic effect during pregnant and lactation phases, but it also could be modulating other physiological processes in mammary glands of non-pregnant animals. In fact, it has been shown that intact transmembrane PRLR localizes in the nucleus of human breast carcinoma cells where it functions as a co-activator through interaction with the latent transcription factor Stat5a and the high mobility group N2 protein (HMGN2) and contributes to the expression of the ER and progesterone receptor (PR) [22, 23].

3.2. Pregnancy

During this stage, mammary glands have to undergo further development and morphological changes in preparation for nutrition of neonates. It has been already established that progesterone induces extensive side-branching and alveologenesis and, in combination with PRL, promotes the differentiation of the alveoli, which are the structures that synthesize and secrete milk during lactation [24].

Along pregnancy, mammary glands of vizcachas increase the parenchymal-stromal ratio as well as the vascularization that surrounds each lobule. We observed that, during the first half of pregnancy of *L. maximus*, there is an increase in branching and elongation of the ductal tree accompanied by an increased expression of PRLR and of progesterone receptor (PR) expression in nuclei of secretory epithelium (**Figure 3**). Bulbous terminal end buds (TBEs) formed at the tip of growing ducts during ductal morphogenesis, now proliferate and bifurcate generating new branches. TEBs show multiple layers of epithelium implying a high proliferative rate of this cell population during gestation. Particularly, after pseudo-ovulation takes place, mammary ductal network becomes noticeably more ramified: the alveolar buds located at the end of the branches progressively cleave and differentiate into individual alveoli which occupy the majority of the fat pad (**Figure 3**) [15].

L. maximus shows two well defined phases during pregnancy: before and after pseudo-ovulation. In the first half of pregnancy, around day 70 of gestation, circulating progesterone gradually decreases as a result of normal luteolysis. Approximately at day 90, when circulating progesterone reaches its minimum level, a new wave of follicular recruitment, pseudo-ovulation and luteinization occurs and the released luteal progesterone progressively increases its levels throughout the second half of gestation [25].

Considering that progesterone is known as a key factor in the regulation of post-pubertal mammary gland development, it is interesting to note that although its levels drastically change throughout pregnancy of *L. maximus*, the pattern of PR immunoreactivity in the secretory alveoli of mammary glands remains relatively constant (**Figure 3**). The enhancement of the circulating progesterone as a result of the pseudo-ovulation has been mostly related to its critical role in the maintenance of the uterus and embryo development up to the end of gestation since by this time most embryos are being resorbed through a natural selective abortion process [10, 14]. Nevertheless, although progesterone fluctuates during gestation, its levels might be enough to induce extensive side-branching and alveologenesis in mammary glands of pregnant vizcachas.
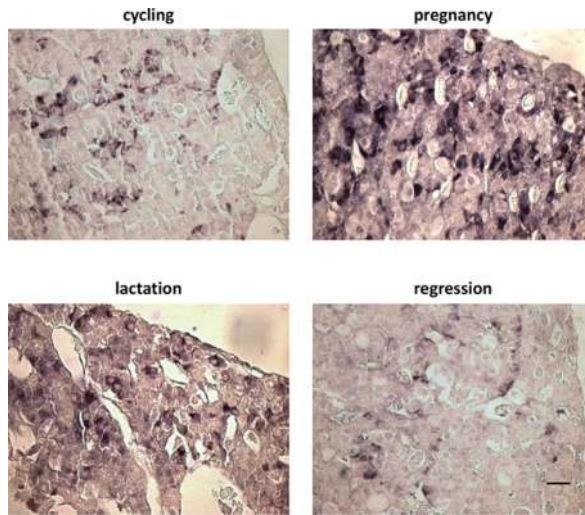

Figure 4. PRL hypophyseal content according to the developmental status of the mammary gland of female vizcachas. Representative photomicrographs of adenohypophysis sections of adult vizcachas whose mammary glands are at cycling, pregnancy, lactation and regression status. Immunoreactivity is shown in black. All photomicrographs have the same magnification. Scale bar is depicted in the last photo (bottom right) and represents 25 µm.

Right before parturition, alveolar epithelial cells are enlarged due to a high content of milk fat globules. These alveoli will ultimately become milk-secreting lobules during lactation. As expected along this reproductive stage, the expression of PRLR in the secretory alveolar cells of mammary glands strongly increases in tune with the hypophyseal PRL content of pregnant vizcachas (**Figures 3** and **4**) [15, 26, 27]. On the other hand, even though it has been described that PRL regulates mammary epithelial cell proliferation also via autocrine/paracrine mechanisms [28, 29], we could not detect PRL expression in mammary glands of *L. maximus* neither at protein nor at mRNA level (not shown).

Interestingly, our data shows that, at the peri-pseudo-ovulation interval (approximately between days 90 and 100 of gestation), circulating estradiol peaks and both ERα and ERβ increase their expression in mammary glands (**Figure 3**). ERα localizes in nuclei of both

secretory epithelia and stromal cells located immediately beneath of it, supporting the idea of a paracrine role for this transcription factor [17]. Moreover, these data correlate with the accelerated ductal proliferation, branching and alveolar differentiation of mammary glands toward the end of gestation [15]. It has been described that besides its role in pubertal branching, ERα is also essential in alveologenesis during pregnancy and lactation [30]. As for ERβ, it has been reported its requirement for normal lobuloalveolar development during pregnancy rather than for prepubertal growth [31].

Both PRL and luteinizing hormone (LH) are intimately linked to estradiol expression. As result of the hypothalamic-hypophyseal-gonadal axis re-activation in adult pregnant vizcachas, serum LH significantly raises, targets the ovaries and triggers pseudo-ovulation. From there and up to the end of pregnancy, whereas LH gradually decreases, hypophyseal PRL concentration progressively increases up to parturition and remains high during lactation. It has been demonstrated that estrogens target lactotrophs and stimulate PRL gene expression and release, enhance storage capacity and increase cell proliferation [32]. Our preliminary results in adenohypophysis of vizcacha show that hypophyseal ERα is highly expressed at term-gestating females [33]. Last but not the least, at the time of pseudo-ovulation, expression of both hypothalamic PR and gonadotropin-releasing hormone (GnRH) markedly increases. This strongly suggests a role of the hypothalamic-hypophyseal-gonadal axis in the modulation of ovulation during gestation in *L. maximus* [13]. Knowing that ovarian hormones are key players in adult mammary gland growth and development, we could hypothesize that GnRH may play an indirect role in mammary gland remodeling. Moreover, in the near future, we should direct our efforts to elucidate ERα modulation over hypophyseal PRL expression in both pregnant and lactating vizcachas.

3.3. Lactation

At this stage, milk-secreting alveoli occupy most of the lobule in the mammary glands of *L. maximus*. As late pregnancy transitioned to lactation, mammary glands consist almost completely of secretory epithelium forming the alveolar structures with lumens full of milk fat globules and milk (**Figure 3**). The magnitude of the dramatic change in the mammary gland architecture is pointed out by the difference in mammary gland weight and size. It has been already described that the fully developed lactating mammary gland in a mouse is seven to 10 times heavier than the mature virgin gland [34].

The secretory epithelial cells of mammary glands during the lactation phase are cuboidal and visibly polarized. The cell nucleus is positioned basally, and the cytoplasm is vacuolated and full of milk droplets. The lumen of alveoli and ducts are full of milk as well. The contraction of myoepithelial cells that surround alveoli helps to empty their content into the interlobular ducts. A very thin connective tissue sheath surrounds each alveolus. We observed the presence of immune cells in the stromal connective tissue and within the milk into the alveoli and ducts. No differences were observed in the morphology between anterior and posterior mammary glands. Anterior and posterior glands are highly branched and full of milk. In fact, we observed that pup suckling occurs indistinctly among the nipples. Lactating females exhibit only one

milk patch beneath the skin along the milk line that contains both anterior and posterior nipples [15].

PRL has been well characterized as a terminal differentiation factor of the mammary epithelial cells and for synthesis of milk components during lactation [35]. While mammary glands of *L. maximus* go through a lactation phase, PRLR alveolar expression reaches its highest level which correlates with a high content of hypophyseal PRL (**Figures 3** and **4**).

During lactation, mammary gland expression of PR is much stronger than in any other reproductive state and such expression shifts to the cytoplasm of alveolar cells although some nuclei still show positivity for this receptor (**Figure 3**). This could indicate that the PR antibody used in our experiments recognizes both isoforms of PR (PRA and PRB) which have been described co-expressing in mammary glands of mice at late pregnancy [36].

3.4. Regression

Weaning of the litter triggers the process of regression, whereby the mammary gland is remodeled back to its pre-pregnancy state. Mammary gland regression is a period of intensive tissue remodeling. During milk stasis, mammary gland epithelial cells change from a secretory cuboidal to a nonsecretory squamous epithelium. One of the aspects that characterized this stage in *L. maximus* is the detachment of alveolar epithelial cells that shed into the lumen. The structure of the gland displays major changes: alveoli start to collapse, basement membrane becomes fragmented and connective tissue, mostly fibroblast and some adipocytes, start to refill (**Figure 3**). Apoptotic cells, cellular debris and milk components must be cleared for normal regression to proceeds. It is notorious the presence of polymorphonuclear cells in the stroma, infiltrated in the secretory epithelia and in the lumen of the alveoli and ducts of regressing mammary glands of *L. maximus* [15]. Interestingly, it has been described that besides the classical phagocytosis carried out by macrophages, "nonprofessional phagocytes" such as epithelial cells, endothelial cells and fibroblasts also have the capability to participate in the removal of neighboring cells that have undergone apoptosis [34].

These mechanisms that ultimately lead to the regression of the gland are not synchronized in the entirety of the gland of vizcachas. Whereas some lobules display their ductal network disorganized and massive epithelial cell death, other lobules still show alveolar epithelial cells with cytoplasmic fat droplets and alveoli and intralobular ducts with milk remains [15]. This is consistent with the fact that, in natural involution, pups will continue to suckle intermittently as they move to a solid diet. Therefore, in natural involution, mammary gland remodeling proceeds in an unsynchronized fashion with different areas of the gland undergoing involution at different times [34].

The values of circulating ovarian hormones and the expression of their receptors in regressing mammary glands of *L. maximus* notoriously decrease compared to lactation and pregnancy stages. It is almost as if it were a necessary condition to allow mammary gland to go through the remodeling associated with this stage. Strikingly, our preliminary data show that GnRH content at medial basal hypothalamus is higher during the regression stage compared to full term pregnant vizcachas (1.2 ± 0.1 and 0.48 ± 0.08 pg/μg total proteins, respectively). This is

very interesting considering a recent report published by Rieanrakwong and col. [37] that shows that involution is also dependent on mammary gonadotropin-releasing hormone expression that is suppressed by PRL during lactation.

4. Concluding remarks

Although other rodents, such as mice and rats, show an enhanced mammary gland development toward the end of gestation, plains vizcachas also exhibit a pseudo-ovulation event at midterm that causes a sharp rise in circulating progesterone and estradiol which correlates with an augment in the expression of ERα, ERβ and PRLR in mammary glands. These events correlate with the development of a more elaborated and differentiated ductal network and pinpoint a possible relation between the hypothalamic-hypophyseal-gonadal reactivation axis at mid-gestation and the accelerated mammary gland branching and alveolar differentiation of *L. maximus*. Pseudo-ovulation at mid-gestation, which is thought to rescue distal fetuses from selective abortion, influences a precocious development of the mammary gland, preparing females to face the nutritional demand of fully developed newborn in this seasonal-breeding species.

Acknowledgements

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