Endocrine, *vol. 15, no. 3, 263–269, August 2001* 0969–711X/01/15:263–269/$11.75

Relaxin Has a Minor Role in Rat Mammary Gland Growth and Differentiation During Pregnancy

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Growth and differentiation of mammary gland is associated with numerous hormones and a variety of cell-cell, cell-matrix interactions. This study addressed the role of relaxin (Rlx) on these processes. Morphologic and biochemical changes that occur throughout the second half of pregnancy are reported. Temporal patterns and spatial distributions of markers useful to evaluate proliferation, secretion, and collagen remodeling were established. To evaluate the role of Rlx, an ablation/replacement animal model was used. Considering Rlx secretion pattern, two periods were selected: d 11 through d 13, and d 20 through d 23. In the stroma, the extracellular compartment showed changes associated with the lack of Rlx. Collagen remodeling within the lobuloalveolar structure, measured by a significant increase in collagen birefringence, decreased at d 12, d 21, and d 22. Parenchymal structures were less sensitive to the absence of Rlx than stroma. Epithelial cell proliferation was lower in Rlx-deficient rats only at d 12, and α-lactalbumin expression decreased at d 21 and d 22. Both lobuloalveolar diameter and percentage of area occupied by these structures showed no changes. In the absence of Rlx, some of the studied markers showed statistically significant differences in scattered days; these do not make clear trends. No differences were found on d 23 on any of the studied parameters suggesting that compensatory mechanisms might be activated to overcome the effects of the absence of Rlx. Unlike the critical role of Rlx either in uterine cervix dilation or in nipple development during rat pregnancy, Rlx had a minor role in growth and differentiation of rat mammary gland.

Key Words: Mammary gland; relaxin; α-lactalbumin; proliferation; collagen remodeling.

Received April 9, 2001; Revised July 9, 2001; Accepted July 9, 2001.

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Introduction

Growth and differentiation of mammary gland is associated with the action of numerous hormonal systems and a variety of cell-cell, cell-matrix interactions. In rodents, estrogen (E) and progesterone (P_4) are both required for mammary cell proliferation and lobuloalveolar differentiation during pregnancy. However, other hormones (e.g., prolactin [PRL], growth hormone, somatomammotropin, placental lactogen) are also necessary to achieve complete mammary development. Both the precise roles of each hormone and their relative importance remain unclear *(1,2)*.

Relaxin (Rlx) has also been implicated in lobuloalveolar development of mammary gland. The administration of Rlx in combination with lactogenic hormones, to ovariectomized (OVX) and/or hypophysectomized nonpregnant rats promoted growth and lobulation of mammary gland as well as an increase in total DNA content *(3–5)*. In addition, specific binding for Rlx in epithelial cells of the mammary glands and, in epithelial cells, smooth muscle and skin of the nipple was reported *(6)*. Administration of anti-Rlx monoclonal antiserum to pregnant rats disrupted the development of lactiferous ducts and nipples and increased the production of collagen fibers *(7,8)*. On the other hand, in vitro studies with whole mammary glands in culture showed that Rlx is not required for normal lobuloalveolar development *(9)*.

In addition to the aforementioned controversies, although many physiologic effects of circulating Rlx are well established, little is known concerning the interaction between Rlx and ovarian steroids in the control of epithelial and stromal proliferation of rat mammary gland. Since it has been demonstrated that the composition of extracellular matrix plays a key role in the control of mammary epithelial proliferation and differentiation *(10)*, studies to address hormonal control of matrix remodeling in mammary development during pregnancy are necessary. Two fundamental steps toward understanding the specific role of E, P_4, and Rlx in the control of these processes are the study of proliferation and dif-ferentiation of epithelial and stromal compartments of

lobuloalveolar structures, and the evaluation of collagen remodeling in mammary stroma during pregnancy. To undertake this task, the experimental model of OVX rat during pregnancy with partial hormone replacement therapy was used *(11)*.

Results

Intact Pregnant Rats (Controls)

To evaluate growth and development of lobuloalveolar mammary gland structures, the diameter and percentage of area occupied by the lobuloalveolar structures were measured, and the proliferation pattern was established. As expected, there was a progressive increase in both the percentage of area occupied by lobuloalveolar structures (data not shown) and lobuloalveolar diameter from d 9 to parturition (Fig. 1A). From d 9 to d 13, lower-diameter values were found; at d 14 of pregnancy, a significant increase in diameter was observed. Lobuloalveolar diameters remained unchanged until d 22. At d 23, when the amount of milk secreted to the lumen increases significantly, the highest values were found. Representative lobuloalveolar structures from d 9 and d 22 are shown in Fig. 2A and B, respectively.

The proliferation pattern of mammary epithelial cells throughout the second half of pregnancy is shown in Fig. 1B. Higher proliferation indexes were found from d 9 to d 12; at d 13 proliferation index decreased abruptly and continued to decrease until parturition. Stromal cells (fibroblasts, adipocytes, and endothelium) displayed extremely low proliferation indexes that were not modified throughout the second half of pregnancy; infiltrating cells were not included in any quantitation (data not shown). Representative images of bromodeoxyuridine (BrdU) incorporation in mammary glands at d 9 and d 22 are shown in Fig. 2C and D, respectively.

To evaluate epithelial cell differentiation and milk-protein expression, the volume fraction of α-lactalbumin-positive cells was measured. α-Lactalbumin-positive cells increased throughout pregnancy (Figs. 1C and 2E,F), and near parturition (d 22–d 23) the highest values were found. Detectable levels of α-lactalbumin were found on any evaluated day; however, a high variability among individual animals was observed from d 9 to d 14 when the lowest α-lactalbumin values were obtained.

Since it has been demonstrated that the composition of extracellular matrix plays a key role in control of the epithelial proliferation and differentiation of mammary gland, collagen remodeling was evaluated. Collagen fibers of lobuloalveolar structure from d 9 to d 13 (Figs. 1D and 2I) were significantly more organized than in the other stages studied (Figs. 1D and 2J). From d 14 to d 23, the intensity and area of collagen birefringence decreased significantly (compared with former days), showing a remarkable collagen remodeling at term (Fig. 2J).

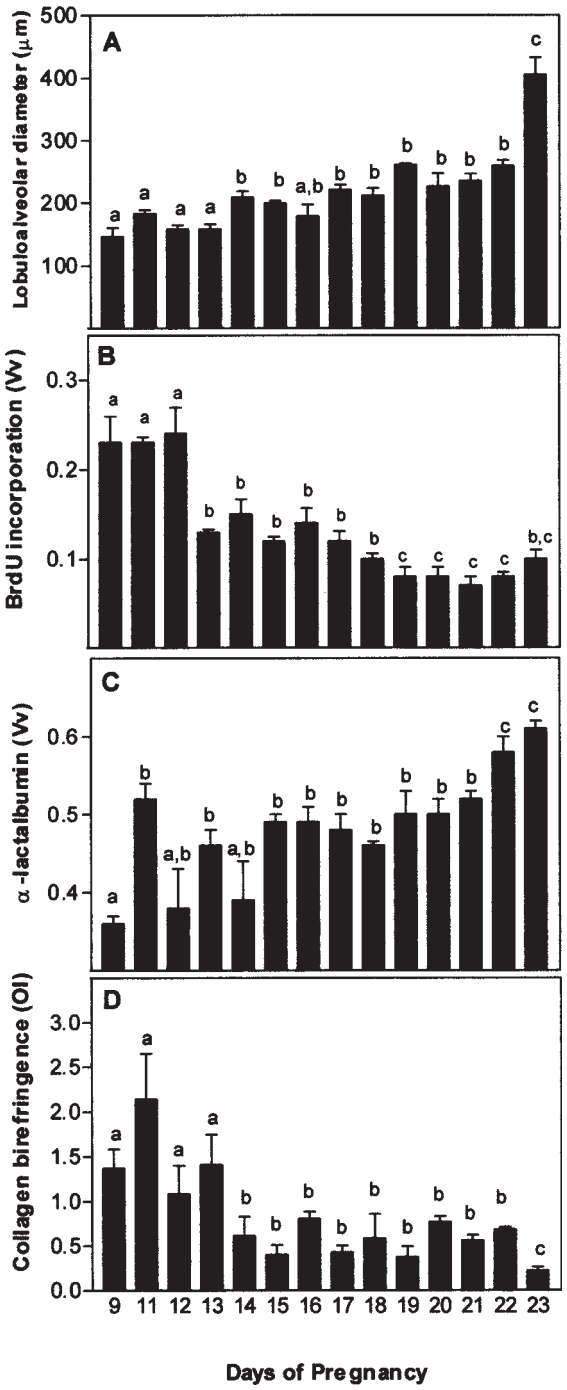

Fig. 1. Temporal changes in parameters related to mammary gland growth and differentiation throughout the second half of pregnancy. (**A**) Lobuloalveolar diameter; (**B**) epithelial BrdU incorporation; (**C**) α-lactalbumin expression; (**D**) intensity of collagen birefringence. Symbols and bars represent mean values (±SEM) of three to four animals. Different letters represent significant differences between days of pregnancy ($p < 0.05$, Kruskal-Wallis and Dunn post-test).

Effect of Absence of Rlx

Considering Rlx secretion pattern *(12)*, we chose two different periods of rat pregnancy to study the role of Rlx in the growth and development of mammary gland: d 11 through

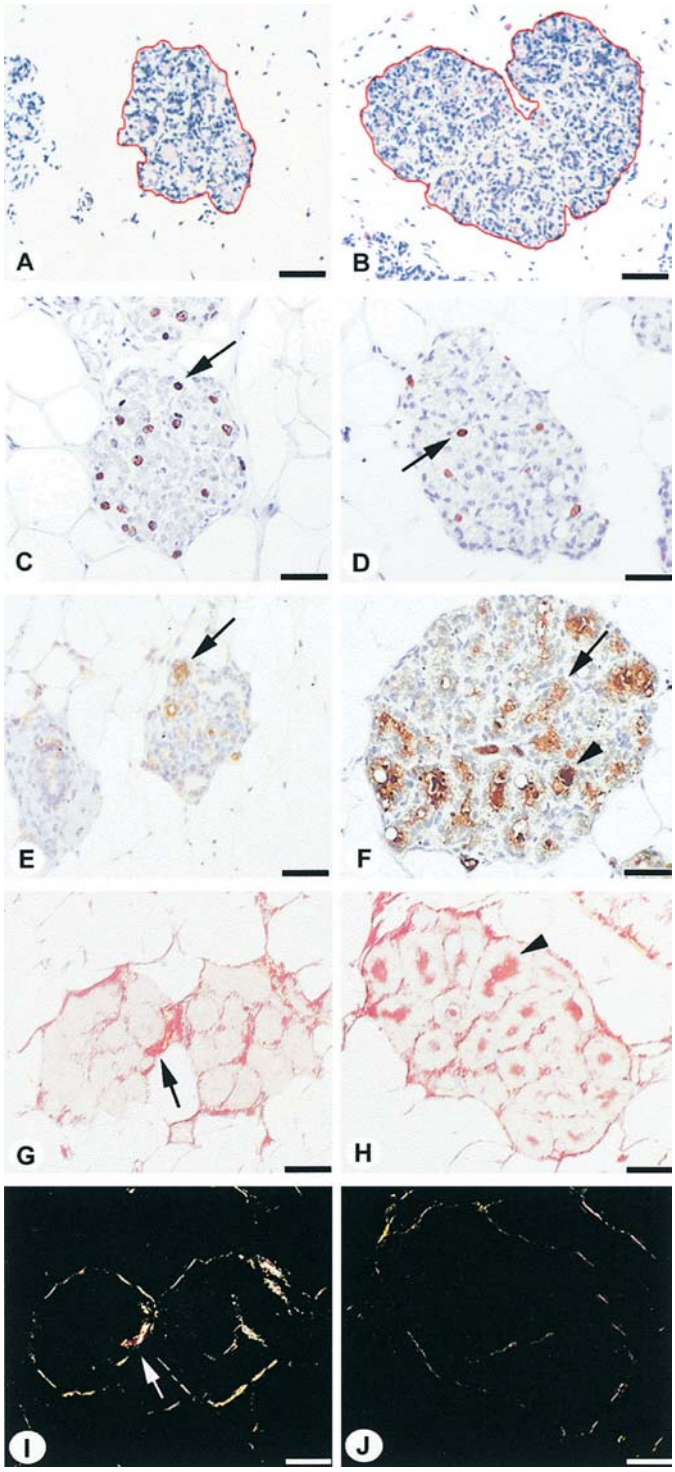

Fig. 2. Histologic features of mammary glands at d 9 (**left**) and at d 22 (**right**) of pregnancy. (**A,B**) Hematoxylin and eosin (H&E)-stained sections showing outlines drawn to obtain lobuloalveolar diameter. (**C,D**) BrdU incorporation, with arrows identifying BrdU positively stained nuclei. Note the absence of positive nuclei in the stroma. (**E,F**) α-Lactalbumin expression. At d 9 positive staining is limited to the cytoplasma of a few epithelial cells (arrow); at d 22 besides the strong staining in the cytoplasm of numerous epithelial cells (arrow), luminal spaces are filled with positively stained secretion products (arrowhead). (**G,J**) Picrosirius-stained sections. Note that while in samples evaluated with conventional illumination, not only are collagen fibers observed positively stained (arrow), but secretion products are also acidophilic (arrowhead) (G,H); under polarized light microscopy, only birefringent collagen fibers shine against a dark background (arrow) (I,J). A regular arrangement illustrates the absence of collagen degradation at d 9 (**I**), while at term (d 22), fragmentary and irregularly disposed collagen fibers (displaying some loss of birefringence) are found (J). Bar = 50 μm.

d 13 (period with low Rlx serum levels) and d 20 through d 23 (high Rlx levels). In our study, lack of Rlx was evidenced by less nipple development, as was reported by Kuenzi et al. *(13)* and Zhao et al. *(14)*.

d 11 to d 13

As shown in Fig. 3, few of the studied parameters were significantly modified in the absence of Rlx (OVX + P_4 + 17β-estradiol [E_2] group). In the stroma, while collagen birefringence was significantly increased on d 12 (Fig. 3D), BrdU incorporation by the cellular compartment remained unchanged (data not shown). Parenchymal structures were even less sensitive to the absence of Rlx. While percentage of the area occupied by the lobuloalveolar structure (data not shown), the lobuloalveolar diameter, and α-lactalbumin expression were not modified (Fig 3A,C), BrdU incorporation by epithelial cells decreased significantly only at d 12 (Fig. 3B).

d 20 to d 23 (Delivery)

In the absence of Rlx (Fig. 3), collagen remodeling measured as collagen birefringence was significantly modified following a pattern similar to that shown from d 11 to d 13. The percentage of area occupied by lobuloalveolar structures (control vs OVX + P_4 + E_2) was modified neither at d 22 (33.0 ± 6.6 vs 44.4 ± 7.5) nor at d 23 (53.8 ± 5.8 vs 49.7 ± 6.9) ($p > 0.05$). No differences were found in BrdU incorporation by either stromal or epithelial cells. α-Lactalbumin expression was modified in the absence of Rlx, showing a significant decrease at d 21 and d 22. No differences were found in any of the studied parameters on d 23.

Discussion

This study focused on mammary gland growth and differentiation throughout the second half of pregnancy and addressed the role of Rlx on these processes. Temporal patterns and spatial distributions (when applicable) of parameters useful to evaluate proliferation, secretion, and collagen remodeling were established in intact pregnant rats. In agreement with Joshi et al. *(15)*, epithelial cell proliferation was greatest between d 9 and d 12 and dropped precipitously at d 13. From d 13 to term, proliferation rate remained at low levels. On the other hand, lobuloalveolar diameters and percentage of area occupied by lobuloalveolar structures increased progressively. Changes in proliferative/apoptotic ratio and/or secretion accumulation into the lumen may account for this progressive increase *(16,17)*.

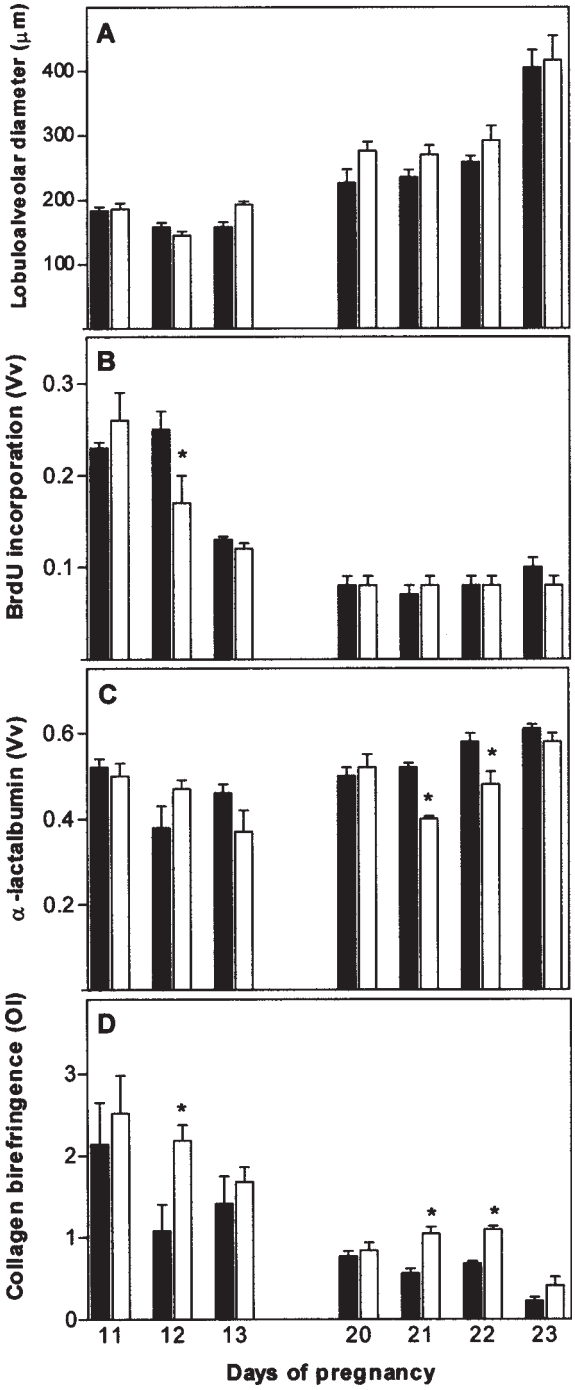

Fig. 3. Paranchymal and stromal parameters, as modified by the absence of Rlx in control (■) vs OVX+P_4+E_2 (□) animals. (**A**) Lobuloalveolar diameter; (**B**) epithelial BrdU incorporation; (**C**) α-lactoalbumin expression; (**D**) intensity of collagen birefringence. Bars represent mean values (±SEM) of four animals. Mann-Whitney *U* test was used. Asterisks represent significant differences between groups; $p < 0.05$.

Synthesis of milk proteins by luminal mammary cells is hormonally regulated and could be considered synonymous with secretory cell differentiation *(18–21)*. In rat mammary gland, α-lactalbumin mRNA increased continuously but not uniformly from d 12 of pregnancy until parturition *(22)*. The dramatic fall in circulating P_4 at the end of pregnancy permits maximum stimulation of α-lactalbumin synthesis by PRL *(18)*. In concordance with these results, we found detectable levels of α-lactalbumin by immunohistochemistry on all days studied, with the highest values near parturition (d 22 and d 23 of pregnancy).

Hormonal control of rat mammary gland proliferation and collagen remodeling is not fully understood. While high levels of P_4, PRL or placental lactogen, and E have been correlated with periods of maximum proliferation of the rat mammary tissue during pregnancy *(1)*, the role of Rlx remains unclear *(3–7,12,23)*. In the rat, serum Rlx levels become detectable by d 10 of pregnancy, increase rapidly by d 14, and remain relatively constant until d 20. During the antepartum period, there is an increase in serum Rlx to maximal levels, followed by a rapid decline before birth *(12)*. Our results showed that lobuloalveolar diameter and percentage of area occupied by lobuloalveolar structure were not affected by the lack of Rlx. BrdU incorporation in epithelial cells was only modified at d 12 in the absence of Rlx. Stromal cells proliferated at a very low rate throughout pregnancy, and no differences were found between control and Rlx-deficient animals. Previous studies reported an increase in total DNA content in response to Rlx using mammary glands from OVX and/or hypophysectomized rats *(3–5)*. The use of different animal models and methodologies might explain the apparent discrepancies with our results regarding BrdU incorporation. The lower proliferation index that we found at d 12 in the absence of Rlx was not enough to modify mammary gland development at term.

The connective tissue environment in the adult mammary gland profoundly influences epithelial growth, ductal branching, epithelial differentiation, and the ability of epithelium to produce milk *(2)*. In the present study, a marked decrease in collagen birefringence (evidence of intense remodeling) was observed at term in control animals. In this stage, collagen remodeling might allow the elongation and growth observed in the lobuloalveolar structures. In pregnant rats with the absence of Rlx, collagen remodeling within the lobuloalveolar structure decreased at d 12, d 21, and d 22. Our findings were consistent with previous results using different animal models *(7,23,24)*. The absence of Rlx modified collagen fiber organization; however, this collagen remodeling is not enough to induce either changes in lobuloalveolar epithelial cell proliferation or in fat pad penetration during the studied periods. If Rlx is indeed stimulating connective tissue remodeling in the mammary gland, it seems reasonable that it might modify proliferation rate in fibroblasts. In the uterine cervix, a well-established target organ for Rlx action, Rlx promotes epithelial and stromal cell proliferation *(25)* and induces collagen remodeling *(7,12,26,27)*. Using BrdU incorporation, we have demonstrated that Rlx does not modify fibroblasts' proliferation in the mammary gland.

α-Lactalbumin expression, a mammary gland differentiation marker *(22)*, was shown to be decreased by the absence

of Rlx. The present work provides no indication about the level of Rlx action. It remains equally possible that changes in α-lactalbumin expression were owing to a direct action of Rlx on epithelial cell function *(6)* or an indirect action through either stromal-epithelial interactions or through modulation of PRL secretion *(28)*. Nevertheless, studies in which whole mammary glands were cultured in vitro, they achieved a complete differentiation and milk protein synthesis even in the absence of Rlx *(9)*, suggesting a minor role, if any, of Rlx.

While in the absence of Rlx there were significant differences at scattered days of pregnancy, these do not make clear trends. Furthermore, no differences were found on d 23 in any of the studied parameters, suggesting that compensatory mechanisms might be activated to overcome the effects of the absence of Rlx. According to our observations, unlike the critical role that Rlx plays either on uterine cervix dilation at parturition or in nipple development during rat pregnancy, Rlx has a minor role in the growth and development of mammary gland during pregnancy.

Materials and Methods

Animals

Animal studies were conducted in accordance with the *Guide for Care and Use of Laboratory Animals* issued by the USA National Academy of Science (Bethesda, MD, 1996).

Primiparous rats (200–250 g of body wt) of a Wistar-derived strain bred at the Department of Human Physiology (Santa Fe, Argentina) were used. Animals were maintained under controlled environment (22 ± 2ºC; lights on from 6:00 AM to 8:00 PM) with free access to pellet laboratory chow (Alimentos Balanceados Costantino, Córdoba, Argentina) and tap water. When applicable, at d 9 of pregnancy (d 1 = day when sperm was found in vaginal smears) bilateral ovariectomy was performed under ether anesthesia. In our colony, delivery in untreated control rats occurs at d 23 between 12:30 AM and 2:00 PM.

In pregnant OVX rats, ovarian steroid replacement therapy was administered continuously from d 9 to d 23 using silicon-tubing implants (Specialty Manufacturing, Midland, MI). P_4 and E_2 (Sigma, St. Louis, MO) were administered according to protocols previously described *(11)*. Pregnant rats OVX at d 9 and treated with $P_4 + E_2$ is a useful model to study the effects of the absence of Rlx, since previous results have shown that pregnancy is not interrupted and normal viable fetuses are obtained at term *(26,29)*.

Tissue Handling

Inguinal mammary glands from the fourth pair were removed, fixed in 10% buffered-formalin for 6 h at room temperature, and embedded in paraffin. To determine the proliferative index, 2 h before sacrifice, rats were injected intraperitoneally with 6 mg/100 g of body wt BrdU (Sigma) dissolved in phosphate-buffered saline.

Serial 5-µm sections were mounted on 3-aminopropyl triethoxysilane-coated slides; following dewaxing and rehydration, they were assigned to any of the following staining procedures: H&E, Picrosirius, or immunohistochemistry.

Lobuloalveolar Morphometry

Lobuloalveolar diameters and the percentage of area occupied by lobuloalveolar structures on the whole mammary gland were measured on H&E-stained sections. Microscopic fields covering the entire slides from one section of each specimen were digitized using a 4X D-plan objective lens. Image analysis was carried out using an Image Pro-Plus 4.1.0.1® system (Media Cybernetics, Silver Spring, MD) as previously described *(30)*. Images were recorded by a Sony ExwaveHAD color video camera attached to an Olympus BH2 microscope (illumination: 12-V halogen lamp, 100 W, equipped with a stabilized light source). The microscope was set up properly for Koehler illumination; a reference image of an empty field for the correction of unequal illumination (shading correction) was recorded, and calibration of spatial measurements with reference rulers was done before any measurement started. Lobuloalveolar structures were defined manually on screen to exclude other structures present in the mammary gland (Fig. 2A,B).

Purification and Generation of Specific Antibody Against Rat α-Lactalbumin

Purification of rat α-lactalbumin was carried out following the method described by Brodreck et al. *(31)* with minor modifications. Whole rat milk (200 mL) was diluted with 2 vol of distilled water and centrifuged at 23,000*g* for 20 min. The skimmed milk was adjusted to pH 4.6 and then centrifuged at 105,000*g* for 60 min to pellet casein. Lactose synthetase was fractionally precipitated at 42–75% saturation of ammonium sulfate, and the pellet was suspended in 20 m*M* HEPES and 150 m*M* KCl, pH 7.0, and dialyzed against the same buffer. The protein solution was passed through a Bio-Gel P60 (Bio-Rad, Hercules CA) column (100 × 5 cm) equilibrated and eluted with 20 m*M* HEPES and 150 m*M* KCl, pH 7.0. Fractions containing α-lactalbumin activity were pooled and dialyzed against 10 m*M* imidazole and 100 m*M* NaCl, pH 7.0. The dialyzed solution containing α-lactalbumin was loaded on a DEAE cellulose (Cellex D; Bio-Rad) column (50 × 5 cm) equilibrated with 100 m*M* NaCl, pH 7.0. After being rinsed with the same buffer, protein was eluted with a linear gradient of 0.1–0.4 *M* NaCl. Three activity peaks corresponding to α1-, α2-, and α3-lactalbumin were recovered. Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the peaks showed a single protein band.

Antiserum against α-lactalbumin was raised in rabbits by intradermal injections of α1-lactalbumin according to Vaitukaitis *(32)*. Specific antibodies were isolated by means of an affinity column of α-lactalbumin coupled to cross-linked agarose (Affi-Gel 10; Bio-Rad). Western blot analy-

sis revealed that antibody reacts equally with the three purified α-lactalbumin isoforms and that milk or homogenate from lactating mammary gland showed three bands with an M_r corresponding to α1-, α2-, and α3-lactalbumin.

Immunohistochemistry

BrdU incorporation and α-lactalbumin expression were measured by immunohistochemistry as previously described *(33,34)*. An overnight incubation at 4°C with 1:400 monoclonal anti-BrdU (clone 85-2C8; Novocastra, UK) or 1:400 polyclonal anti-α-lactalbumin was performed. Biotinylated secondary antibodies (Sigma) were used, and the biotin was detected with streptavidin-peroxidase complex (Sigma). Diaminobenzidine was used as chromogen substrate and hematoxylin as counterstaining solution.

Each immunohistochemical run included positive and negative controls. Specificity of primary antiserum was tested by suppliers (or by us) using Western blot assays of tissues containing the proteins under investigation.

To quantify the proliferative index and α-lactalbumin expression, the point-counting procedure *(35)* was used. All immunostained epithelial cells, regardless of intensity, were estimated in the chosen field using a glass disk with a squared grid inserted in a focusing eyepiece and an immersion objective lens of ×100. α-Lactalbumin expression in luminal secretion was not included in morphometric measures. The fraction of points hitting immunostained cells (P_i) was divided by the total number of points occurring within the epithelial cell (P). The volume fraction was calculated by applying the formula given by Weibel *(36)*: $V_v = P_i/P$.

For both markers, two sections per rat were evaluated and at least 30 ×100 fields were recorded in each section.

Organization of Collagen Fibers

Sections were stained in Sirius Red (Direct Red 80, Aldrich, Milwaukee, WI) in picric acid solution, and collagen birefringence was quantified by polarization microscopy following the protocols previously described *(27,29, 37)*. For each animal, 30 images captured from one section were evaluated. Images were captured using a 40X D-plan objective lens. Using Auto-Pro macrolanguage, an automated standard sequence operation was created to measure average optical intensity (OI) and the area occupied by organized collagen. Since both parameters measured to evaluate collagen organization (OI and area of birefringence) showed a high correlation index (Spearman correlation coefficient [rs]: 0.96; $p < 0.01$), OI was chosen to express the results.

Statistical Analyses

Differences in the studied parameters (i.e., proliferative index, lobuloalveolar morphometry, α-lactalbumin expression, and organization of collagen fiber bundles) were obtained by Kruskal-Wallis test and Dunn post-test. To evaluate differences between the intact control group throughout pregnancy and hormone-treated group, the Mann-Whitney *U* test was used. Spearman correlation test was performed to determine the association between OI and area of collagen birefringence *(38)*.

Acknowledgments

We are very grateful to Mr. Juan C. Villarreal and Mr. Juan Grant for indispensable assistance in animal care. This study was supported by grants from the Argentine National Council for Science and Technology (CONICET) (PIP 528/98), the Argentine National Agency for the Promotion of Science and Technology (ANPCyT) (PICT 99 5-7001), and the agreements "CONICET/Brazilian National Council for Scientific and Technological Development (CNPq)" (1786) and "SECYT-CAPES" (BR04/99/OG). LK is a fellow of the ANPCyT, GSM is career investigator of the CNPq, JGR is a postdoctoral fellow, and LEB and EHL are career investigators of the CONICET.

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