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Association of increased estrogen receptor β2 expression with parity-induced alterations in the rat mammary gland

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Abstract

In this study, we investigated the cellular and molecular events involved in parity-related alterations in mammary gland (MG) proliferation and differentiation. Rat MGs were removed on day 9 of either first (nulliparous), second (primiparous) or third (multiparous) pregnancy. Expression of steroid hormone receptors along with cellular biomarkers of proliferation and differentiation were quantified in all MG tissue compartments by immunohistochemistry. Wnt-4 (a *Wingless*-like morphogenic gene involved in MG development), ERβ and ERβ2 mRNA were evaluated by RT-PCR analysis. Serum levels of mammotrophic hormones were measured. In comparison to nulliparous and primiparous rats, multiparous animals exhibited decreased luminal cell proliferation and PR levels, whereas α-lactalbumin, ERα, ERβ and ERβ2 expression were increased. In myoepithelial cells, while parity induced a decrease in proliferative activity, subsequent pregnancies and lactations lead to an increased state of differentiation. Our results showed that at least two periods of pregnancy and lactation were necessary to modify the studied parameters. The lower proliferative activity and higher differentiation state of the multiparous MG are associated with both a decreased PR expression and increased ERα and ERβ expression. Since ERβ and/or ERβ2 isoform expression was related to parity history, results suggest that the decreased proliferative activity and PR expression observed in the MG of multiparous animals may be associated with overexpression of ERβ and/or the ERβ2 isoform, thereby antagonizing the proliferative effects associated with ERα.

Keywords: Mammary gland; Parity; Progesterone receptor; Proliferation; Estrogen receptor α; Estrogen receptor β; α-Lactalbumin

1. Introduction

The mammary gland (MG) undergoes proliferation and completes its differentiation during pregnancy and lactation. The luminal and myoepithelial cells that comprise the parenchyma undergo remarkable changes contributing to mammary development [1]. A vast majority of the studies examining MG development to date have focused on the mechanisms controlling luminal cell growth and differentiation, whereas myoepithelial cells have been largely neglected. Fortunately, these two cell types can be easily distinguished today employing newly available monoclonal antibodies [1,2].

In rodents, alveolar proliferation and functional differentiation of mammary epithelial cells are modulated by estrogen, progesterone and peptide hormones in combination with stromal signals [3]. The normal MG expresses estrogen (ERα and ERβ) and progesterone (PR) receptors and several studies suggest that hormonal effects may be mediated through a paracrine mechanism [4–6]. Recently, it has been shown that progesterone induces the expression of Wnt-4 during pregnancy [7]. The Wnts are a group of secreted glycoproteins that act as short-range signaling molecules. Therefore, it has been suggested that Wnt-4 may be the downstream mediator of progesterone signaling during mammary development [7].

Progesterone has been found to be essential for side branching [8], alveolar growth [9,10], and suppression of lactogenic onset during pregnancy [11]. The last-mentioned action of progesterone is, at least partly, a consequence of inhibiting α-lactalbumin synthesis and secretion [11]. The role of estradiol following ductal morphogenesis is not clear, although the hormone is believed to be responsible

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for PR induction in luminal epithelial cells [12]. During lactation, the MG has been described as estrogen-insensitive since estrogen does not elicit PR induction [13]. The explanation for this insensitivity is unclear. Previous studies by Saji and co-workers [14,15] regarding ERβ presence and cellular distribution suggested that one function of ERβ is to antagonize the effects of ERα in the epithelium. This assumption is consistent with the observation that during lactation, co-localization of the two ERs (formation of ERα/ERβ heterodimers) is highest [14], and the dominant repressor isoform (ERβ2) is up-regulated [15].

Parity results in multiple changes within the MG. These changes include a persistent increase in the differentiated state [16], a lower proliferative index in rodents [17], and a decrease in the incidence of breast cancer in both humans and rodents [18]. Moreover, in terms of hormone responsiveness in rodent mammary explants, the epithelium from parous females is different to that from nulliparous animals [19]. In accordance with previous studies, we have shown that MGs of nulliparous rats attained their highest level of bromodeoxyuridine (BrdU) incorporation on day 9 (D9) of pregnancy, whereas α-lactalbumin expression was minimal at this time [20]. Therefore, in this study we have investigated the cellular and molecular events involved in parity-associated changes within the rat MG by evaluating proliferation index, differentiation status, steroid hormone receptor profiles, and hormonal milieu in nulliparous, primiparous and multiparous rats on D9 of pregnancy.

2. Materials and methods

2.1. Animals

Female adult rats (>200 g body weight) of a Wistar-derived strain bred at the Department of Human Physiology (Santa Fe, Argentina) were used. Animals were maintained under a controlled environment (22 ± 2 °C; lights on from 06:00–20:00 h) and had free access to pellet laboratory chow (Constantino, Córdoba, Argentina) and tap water. Female rats were caged overnight with males of proven fertility. The day that sperm were found in the vagina was designated as D1 of pregnancy. Animal studies were conducted in accordance with the Guide for the Care and Use of Laboratory Animals issued by the National Academy of Sciences (USA).

2.2. Experimental protocol

Two hours prior to sacrifice, rats were injected ip with 6 mg/100 g body weight BrdU (Sigma Chemical Co, St Louis, MO). Age matched female rats (11–12 months old) were sacrificed by decapitation on D9 of first (nulliparous), second (primiparous) or third (multiparous) pregnancy. Trunk blood was collected, the serum was stored at −20 °C until used for hormone assays, and the MGs were removed.

Experimental groups were designated as follows: (a) nulliparous (primigravid animals with eight or more implantation sites), (b) primiparous (rats that underwent one successful pregnancy and a complete period of lactation), (c) multiparous (animals that underwent two successful pregnancies and complete periods of lactation).

In primiparous and multiparous animals, eight pups were left with their mothers until weaning on day 21. A period of 2 weeks after weaning was permitted before the next mating to allow for MG involution.

2.3. Tissue samples

The inguinal MGs (fourth pair) were excised. One gland was frozen immediately in liquid nitrogen and stored at −80 °C until RT-PCR analysis. The other was either processed for whole-mount according to Thompson et al. [21] or fixed in 10% buffered formalin for 6 h at 4 °C and embedded in paraffin. Serial 5 μm sections were mounted on 3-aminopropyl triethoxysilane (Sigma)-coated slides and stained by either hematoxylin–eosin or immunohistochemistry.

2.4. RNA isolation, RT-PCR analysis and control selection for RNA quantification

An optimized RT-PCR protocol was employed to analyze the relative expression levels of Wnt-4, ERβ, and ERβ2 mRNAs. Total RNA was isolated using the single-step, acid guanidinium thiocyanate-phenol-chloroform extraction method [22], and RNA reverse transcription was performed according to Ramos et al. [23].

Primer pairs used for amplification of the target genes are shown in Table 1. The primers were selected based upon Saji et al. [15] for ERβ and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and Chan et al. [24] for L19 ribosomal protein (GAPDH and L19 were used to normalize RT-PCR reactions). The primers for Wnt-4 were selected based upon a previous reference on the mouse sequence [7] and compared with the published sequence of the rat (GenBank accession no AF188608). The rat ERβ2 isoform has an additional 54-bp in-frame sequence between exons 5 and 6 of the wild type rat ERβ [25]. Therefore, according to the published cDNA sequence (GenBank accession no AF042059), the ERβ2 antisense was created to recognize 20-bp of this insertion.

To perform comparative PCR analysis, aliquots of cDNA samples equivalent to 650 ng of total RNA input were used in each PCR amplification. Each reaction mixture contained 2.5 U *Taq* DNA polymerase (Promega), 2.5 mM $MgCl_2$ (Promega), 0.1 mM of each of the four dNTP's (Promega), and 10 pmol of each primer (Invitrogen Argentina) in a final volume of 25 μl of 1× PCR *Taq* buffer.

Following initial denaturation at 97 °C for 5 min, a touch-down program was employed for Wnt-4, ERβ and

Table 1
Sequence for PCR primers

Primer name	Sequence	Product size (bp)
Wnt-4 sense	5′-AGG AGT GCC AAT ACC AGT TCC-3′	278
Wnt-4 antisense	5′-TGT GAG AAG GCT ACG CCA TA-3′	
ERβ sense	5′-TTC CCG GCA GCA CCA GTA ACC-3′	262
ERβ antisense	5′-TCC CTC TTT GCG TTT GGA CTA-3′	
ERβ2 sense	5′-GAG CTC AGC CTG TTG GAC C-3′	174
ERβ2 antisense	5′-ACT CTT CAT CTG CGC AAC GT-3′	
GAPDH sense	5′-CAG CCG CAT CTT CTT GTG-3′	466
GAPDH antisense	5′-AGT TGT CAT ATT TCT CGT GGT TCA-3′	
L19 sense	5′-GAA ATC GCC AAT GCC AAC TC-3′	290
L19 antisense	5′-ACC TTC AGG TAC AGG CTG TG-3′	

ERβ2 mRNA detection: the annealing temperature in the first cycle was set at 65 °C and was decreased by 1 °C every two cycles over the next 10 cycles. The remaining cycles (23 for Wnt-4 and 26 for ERβ and ERβ2) were performed at 58 °C (Wnt-4) or 56 °C (ERβ and ERβ2) annealing temperature. The reaction mixture was subjected to successive cycles of denaturation at 95 °C for 30 s, annealing at different temperatures for 1 min, and extension at 72 °C for 1 min. A final extension cycle at 72 °C for 5 min was included. The optimal number of cycles for each reaction was determined experimentally to yield linear relationships between signal intensity and cycle number. The optimal number of cycles was 33 for Wnt-4, 36 for ERβ and ERβ2, 27 for GAPDH (55 °C annealing temperature), and 26 for L19 (58 °C annealing temperature) using separate reactions for each target gene. In all assays, negative controls using RNA without reverse transcription and *Taq* polymerase-negative tubes were performed in order to minimize introduction of potential artifacts. PCR products were cloned using the TA cloning kit (Invitrogen Argentina) and specificities were confirmed by DNA sequencing (data not shown). These products were then used as positive controls.

PCR products were resolved on 2% agarose gels containing ethidium bromide (Sigma) in 0.5× Tris-borate EDTA running buffer. Molecular weights were determined by comparison to molecular weight standards (Cien Marker, Biodynamics; Buenos Aires, Argentina). Agarose gel images were digitized using a Sony ExwaveHAD color video camera (Sony Electronics Inc., Sony Drive, Park Ridge, NJ) and the Image Pro-Plus 4.1.0.1® image system analyzer (Media Cybernetics, Silver Spring, MD). Band densities from three independent experiments were determined by densitometry. In order to select an internal control, the expression levels of GAPDH and L19 in all experimental conditions were evaluated. As shown in Fig. 1, GAPDH mRNA expression was consistently higher in the MG of multiparous animals compared with nulliparous while L19 maintained a consistent expression level. Therefore, L19 mRNA was used to normalize RT-PCR reactions. Relative levels of the specific mRNAs analyzed were expressed in arbitrary units.

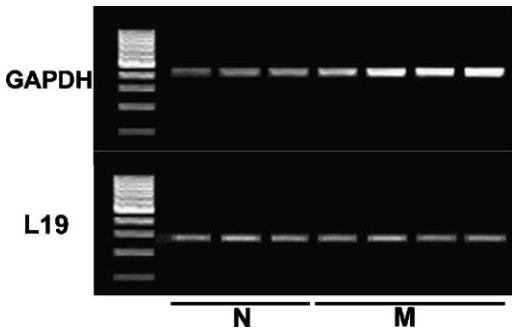

Fig. 1. Control selection for RNA quantification. GAPDH and L19 representative ethidium bromide-stained gels. GAPDH mRNA expression was consistently higher in the mammary gland of multiparous animals (M) compared with nulliparous rats (N), while L19 mRNA maintained a constant expression. Therefore, L19 was selected to normalize RT-PCR reactions.

2.5. Immunohistochemistry

The following primary antibodies were employed: anti-BrdU (clone 85-2C8), anti-ERα (clone 6F11), and anti-α-smooth muscle actin (α-SMA, clone αsm-1) from Novocastra Laboratories Ltd. (Newcastle upon Tyne, UK); rabbit anti-ERβ from Zymed Laboratories (San Francisco, CA, USA); anti-PR (clone AT) from Affinity Bioreagents (Golden, CO, USA); anti-p63 (clone C-12) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and anti-α-lactalbumin from Dr. L. Bussmann (IBYME, Bs As, Argentina).

When not otherwise specified, routine immunostaining protocols previously described were followed [20,26]. Primary antibodies were identified by streptavidin-biotin peroxidase labeling employing diaminobenzidine (DAB) (Sigma) as a chromogen substrate. Samples were counterstained with Mayer's hematoxylin and mounted with permanent mounting medium (PMyR, Buenos Aires, Argentina).

For ERβ immunodetection, following dewaxing, the antigens were retrieved by boiling in 10 mM citrate buffer in a microwave oven for 10 min at 700 W. Endogenous peroxidase was blocked by incubating sections with PBS containing 0.5% Triton X-100 for 1 h, followed by 1.5% non-fat milk containing 5% normal goat serum for 30 min. The incubation with anti-ERβ (10 μg/ml) was performed overnight at 4 °C in PBS buffer containing 3% BSA and 0.3% Triton X-100. This antibody was revealed employing

the streptavidin-biotin peroxidase method followed by DAB staining (Sigma).

Immunostaining of BrdU labeled cells was performed following microwave pre-treatment for antigen retrieval and acid hydrolysis for DNA denaturation [20].

A sequential double immunoassay technique was employed [26]. The initial immunohistochemical reaction (BrdU, ERα, ERβ, PR, p63 or α-lactalbumin) was completed without counterstaining. Elution of the first set of reagents was accomplished by microwave treatment. Visualization of the second antigen (α-SMA) was achieved by the nickel-intensified DAB technique. Slides were counterstained with Nuclear Fast Red and mounted with permanent mounting medium.

Each immunohistochemical assay included both positive and negative controls. For negative controls, the primary antibody was replaced with non-immune serum (Sigma).

2.6. Morphometry and image analysis

Immunostained tissue sections were evaluated using an Olympus BH-2 microscope (Olympus Optical Co. Ltd., Tokyo, Japan). Expression of the different markers examined was assessed in both the alveolar structure (distinguishing between luminal, myoepithelial and intra-alveolar stromal cells), and in the inter-alveolar stroma (fat pad).

Percentages of cells staining for BrdU, ER (α and β), PR, p63, and α-lactalbumin was determined using random fields, counting a total of 1000 cells per tissue section. Each cellular compartment (luminal, myoepithelial and stromal) was evaluated separately. The volume fraction (Vv) of α-lactalbumin expression was measured in two sections per animal as previously described [20].

Images of α-SMA stained sections covering the alveolar structure were recorded employing a Sony Exwave-HAD color video camera (Sony Electronics Inc.) attached to an Olympus microscope. The microscope was prepared for Koehler illumination. This was achieved by recording a reference image of an empty field for the correction of unequal illumination (shading correction), and by calibration of the measurement system with a reference slide to determine background threshold values. The reference slide contained a series of tissue sections stained in absence of primary antibody. The resolution of the images was set to 640 × 480 pixels and the final screen resolution was 0.103 μm/pixel. Two sections were evaluated for each specimen and thirty representative fields in each section were scored using a Dplan 40× objective. Myoepithelial differentiation includes the development of the myofilament system and the spreading of the cellular processes in a stellate shape [27]. Therefore, the proportion of the alveolar perimeter occupied by cytoplasmic projections of the myoepithelial cells (α-SMA positive) was measured using the Image Pro-Plus 4.1.0.1® system (Media Cybernetics) and was designated as lineal density.

2.7. Hormone assays

PRL, GH, estradiol, progesterone and IGF-I were measured by radioimmunoassay (RIA). PRL and GH were assayed, as previously described by Díaz-Torga et al. [28]. For IGF-I, serum samples and IGF-I standards were subjected to the acid–alcohol cryoprecipitation method [29]. The IGF-I antibody (UB2-495), provided by Drs. L. Underwood and J.J. Van Wyk and distributed by the Hormone Distribution Program of the NIDDK, was used.

Estradiol and progesterone concentrations were measured after ethyl ether (Merck, Buenos Aires, Argentina) and hexane (Cicarelli, Rosario, Argentina) extraction, respectively [28]. The antibodies were provided by Dr. G.D. Niswender, and labeled hormones were purchased from Dupont NEN (Boston, MA, USA).

Assay sensitivities were 1.6, 3.2, and 32 ng/ml, 1.7 pg/ml and 1.2 ng/ml for PRL, GH, IGF-I, estradiol and progesterone, respectively. Intra- and interassay coefficients of variation were 7.2 and 12.8% for PRL, 8.3 and 13.2% for GH, 8.2 and 14.1% for IGF-I, 9.2 and 13.3% for estradiol, and 7.5 and 11.9% for progesterone, respectively.

2.8. Statistics

Statistical analyses were performed using the Kruskal–Wallis one-way ANOVA, and significance between groups was determined using the Dunn's post-test. $P < 0.05$ was accepted as indicating a significant difference between groups.

3. Results

3.1. Gradual changes in mammary gland histo-morphological features from nulliparous to multiparous rats

Mammary glands on D9 of the first gestation (nulliparous rats) were primarily comprised of alveoli arranged in clusters and inter-alveolar ducts. The alveolar lumina at this stage were small and inconspicuous owing to epithelial cell swelling, whereas myoepithelial cells were scarcely detectable around the alveolar edges. In contrast, MGs on D9 of the third pregnancy were primarily comprised of alveoli exhibiting distended lumens. The luminal cells of multiparous rats appeared to exhibit greater maturity due to the presence of abundant lipid droplets and vacuoles. As shown in Fig. 2, gradual differences observed between groups are evident in MG whole-mounts. Alveoli of greater size are exhibited by multiparous MGs.

3.2. Phenotypic and functional features of the parenchyma compartment

Luminal and myoepithelial cells were individually identified in the MG parenchyma. In order to quantify the

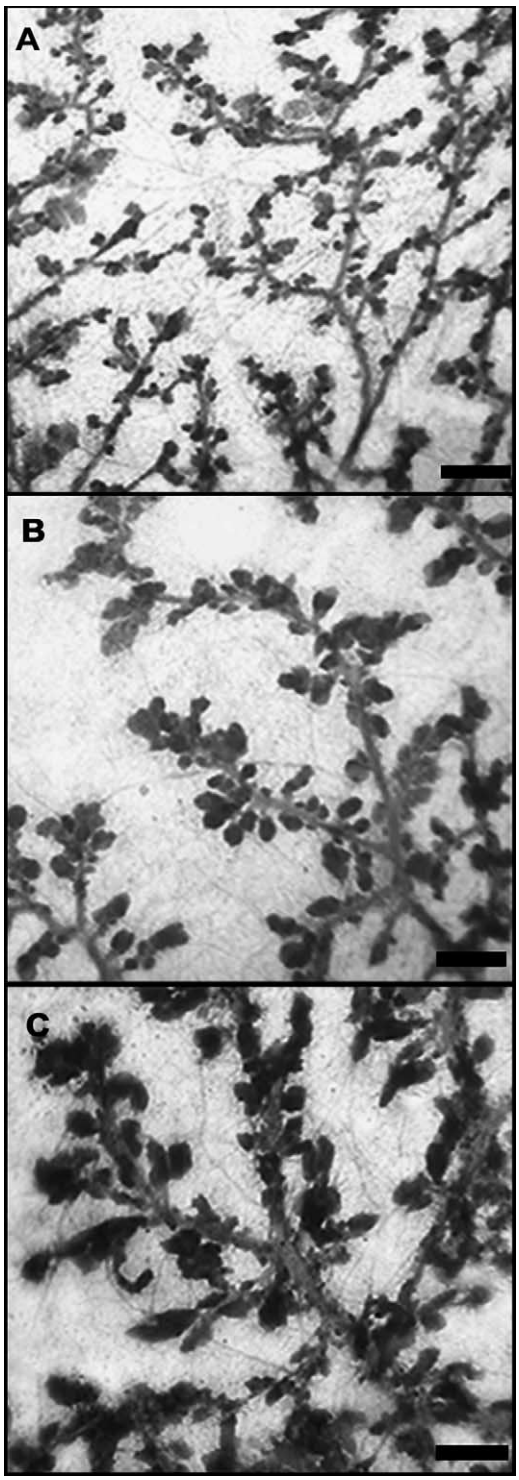

Fig. 2. Parity associated changes in morphological features of the rat mammary gland. Mammary gland whole-mounts from nulliparous (A), primiparous (B) and multiparous (C) rats on D9 of pregnancy. Gradual changes from nulliparous to multiparous animals were observed. Multiparous animals exhibit alveoli of greater size than other groups. The scale bar represents 500 μm in all figures.

percentage of myoepithelial cells in the alveolar structure, co-expression of α-SMA and p63 (a selective myoepithelial cells nuclear marker [2]) was assessed by sequential double immunostaining (Fig. 3A). All p63 positive cells co-expressed α-SMA demonstrating that p63 immunoreactivity was restricted to myoepithelial cells. Our results showed that these cells represent approximately 20% of the total epithelial compartment; this percentage did not change in any of the groups examined (Fig. 3B). To assess myoepithelial maturation and differentiation, we measured the lineal density of their cytoplasmic processes. Myoepithelial cell differentiation increased gradually with subsequent periods of pregnancy and lactation, acquiring in multiparous animals the most differentiated state (Fig. 3C–F).

Luminal and myoepithelial proliferative activities within the alveolar structure were evaluated by incorporation of BrdU. No differences in luminal or myoepithelial cell proliferation were observed between nulliparous and primiparous groups. A significant ($P < 0.05$) decrease in proliferative activity was observed in the parenchyma of multiparous rats compared to nulliparous and primiparous animals (Fig. 4A). The percentage of luminal cells that incorporated BrdU in nulliparous and primiparous rats was two-fold higher than in multiparous animals. Analogous changes were observed in the proliferative rate of myoepithelial cells; however, the absolute values of BrdU incorporation were consistently lower in myoepithelial cells when compared to those obtained for luminal cells (Fig. 4A).

Expression of α-lactalbumin, which was only observed in the luminal compartment of the parenchyma, was assessed as a marker of luminal differentiation. The multiparous animals exhibited a significant ($P < 0.05$) increase in α-lactalbumin expression when compared with other groups (Fig. 4B).

In the alveolar structure, the percentage expression of PR, ERα and ERβ was evaluated in both luminal and myoepithelial cells. A significant ($P < 0.05$) decrease in the number of luminal PR positive cells was observed in multiparous animals (Fig. 4C). In contrast, the number of luminal ERα positive cells were significantly ($P < 0.05$) greater in multiparous rats in comparison to the other groups (Fig. 4D). In addition, the level of ERβ protein was significantly ($P < 0.05$) increased in multiparous rats (Fig. 4E). The myoepithelium did not express PR (Fig. 4C), whereas very low levels of ERα and ERβ were observed in these cells. No differences were observed between experimental groups in the expression of either ERα or ERβ in the myoepithelium (Fig. 4D and E).

3.3. Phenotypic features of the stromal compartment

Proliferation values of stromal cells were always less than 1% and no significant differences were observed between any groups examined (data not shown). The percentage of intra-alveolar stromal cells (mainly fibroblasts)

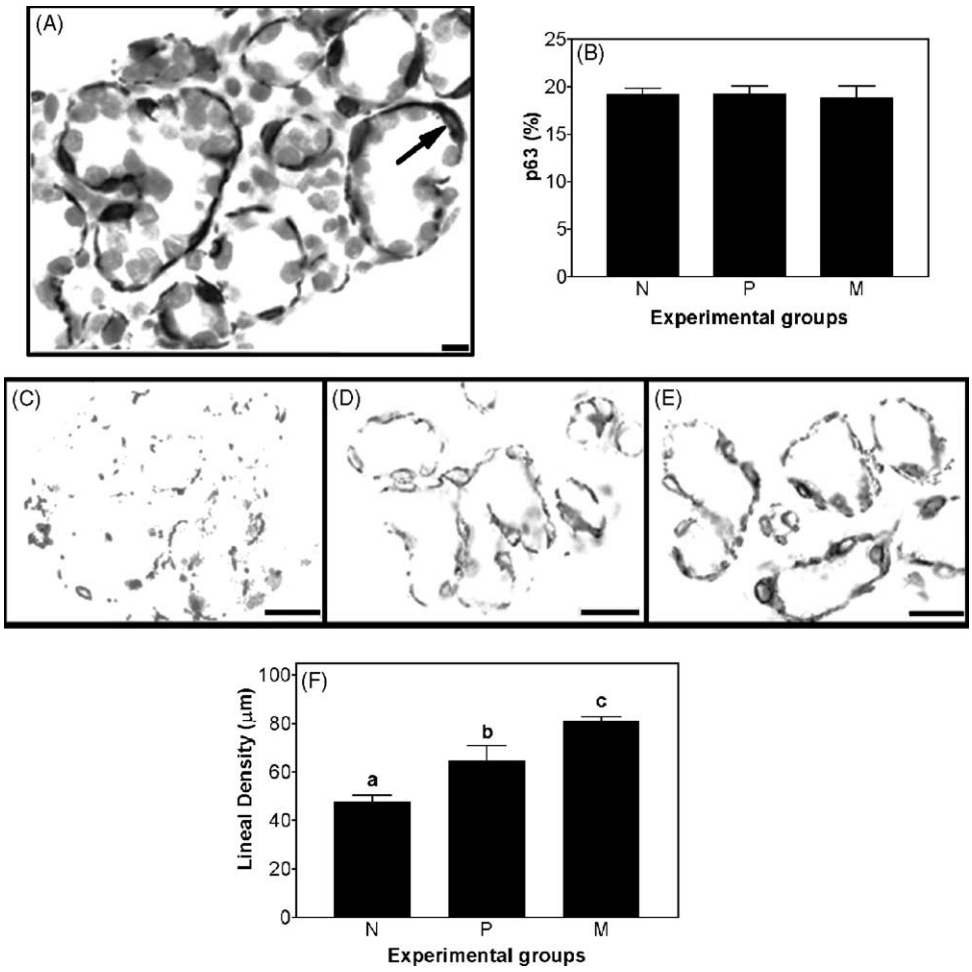

Fig. 3. Expression of myoepithelial biomarkers in the mammary alveoli of nulliparous (N), primiparous (P) and multiparous (M) animals on D9 of pregnancy. (A) p63/α-SMA double immunohistochemistry of a multiparous animal. All p63 positive cells (dark nuclei pointed by arrow) also expressed α-SMA (dark cytoplasm). Neither p63 nor α-SMA expression was detected in any other cell type. (B) Percentage of p63 expression. (C–E) α-SMA immunostaining in N, P and M animals, respectively. Note the continuous ring around the luminal cells in M rats. (F) Myoepithelial lineal density. This parameter was increased gradually with subsequent periods of pregnancy and lactation. In all graphs, bars represent mean values (±S.E.M.) of five or more animals per group. Means with different letters differ significantly ($P < 0.05$, Kruskal–Wallis followed by Dunn). The scale bar represents 20 μm in all figures.

that expressed ERα and ERβ was similar in all groups examined (Table 2). However, the percentage of ERα and ERβ positive cells within the inter-alveolar stroma (mainly adipocytes) was significantly ($P < 0.05$) increased in multiparous rats compared with nulliparous and primiparous animals (Table 2). No stromal cells stained positive for PR in any group studied.

3.4. Are parity-related changes in proliferative activity and steroid receptor profiles associated with modifications in hormonal milieu, Wnt-4 and/or ERβ mRNA expression?

Three possible mechanisms were considered to account for the low rate of proliferation and decreased PR expression observed in the presence of increased levels of ERα in

Table 2
ER (α and β) expression in the mammary stroma

Experimental groups*	Intra-alveolar stroma				Inter-alveolar stroma			
	ERα (%)	*n*	ERβ (%)	*n*	ERα (%)	*n*	ERβ (%)	*n*
N	3.1 ± 0.2	9	13.1 ± 1.3	6	17.9 ± 0.9[a]	9	11.7 ± 1.8[a]	6
P	3.5 ± 0.3	5	9.4 ± 1.2	5	11.2 ± 2.4[a]	5	7.5 ± 1.2[a]	5
M	2.3 ± 0.4	9	13.3 ± 1.2	5	26.9 ± 1.0[b]	9	16.6 ± 1.4[b]	5

The values are means ± S.E.M. Means with different letters differ significantly ($P < 0.05$, Kruskal–Wallis followed by Dunn).
* N: nulliparous animals; P: primiparous animals; M: multiparous animals.

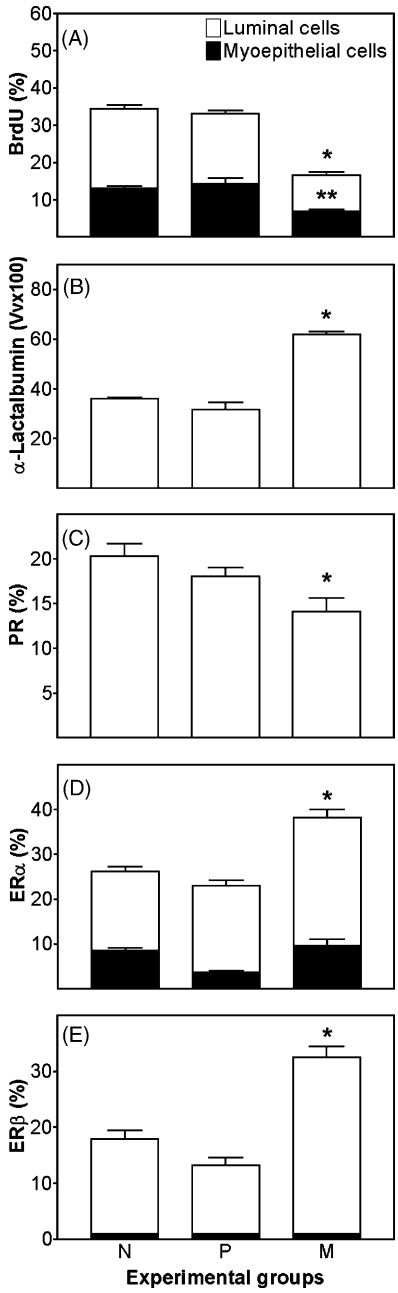

Fig. 4. Parenchyma parameters evaluated in rat mammary alveoli on D9 of pregnancy in nulliparous (N), primiparous (P) and multiparous (M) animals. (A) BrdU incorporation, (B) α-lactalbumin expression, (C) PR expression, (D) ERα expression, and (E) ERβ expression. In all graphs, bars represent mean values (±S.E.M.) for luminal (white bars) and myoepithelial (black bars) cells of five or more animals per group, with significant ($P < 0.05$) differences between groups being indicated by the asterisk (Kruskal–Wallis followed by Dunn).

multiparous rats: (1) modification of the hormonal environment, (2) involvement of the Wnt-4/PR dependent signaling pathway and, (3) expression of ERβ/different isoforms of ERβ.

As shown in Table 3, no significant differences in blood levels of the different hormones assayed were observed. Moreover, the relative amount of Wnt-4 mRNA did not change between groups (Fig. 5).

Representative ethidium bromide-stained images of ERβ and ERβ2 mRNA by RT-PCR analysis are shown in Fig. 6. On D9 of pregnancy, all groups expressed both ERβ and ERβ2 mRNA (Fig. 6A and C, respectively). However, the relative amount of total ERβ and ERβ2 mRNA was significantly ($P < 0.05$) increased in multiparous animals in comparison to nulliparous and primiparous groups (Fig. 6B and D).

4. Discussion

In the present study we show that at least two periods of pregnancy and lactation were necessary to modify the studied parameters. The decreased proliferative activity of epithelial cells and high α-lactalbumin expression observed in the MG of multiparous rats were associated with a down-regulation of PR expression and up-regulation of both ERα and ERβ. Moreover, the enhanced expression of α-lactalbumin, ERα and ERβ/ERβ2 in these animals is indicative of a different state of differentiation of the luminal compartment. Furthermore, a different hormonal milieu was not responsible for the observed changes, since no differences were observed in any of the hormones assayed. Even though the number of myoepithelial cells did not change, this particular cell type appears to require pregnancies and lactations to acquire a fully differentiated state. The proliferative rate and α-lactalbumin expression in 1-year-old nulliparous pregnant rats employed in the present study did not differ from results previously reported for younger animals [20].

It is generally agreed that differentiated alveolar cells undergo apoptosis, and that the luminal compartment is reconstituted in subsequent pregnancies from undifferentiated mammary stem cells [30]. However, it has been demonstrated that some differentiated alveolar cells bypass apoptosis and remain in the parous gland, where they can give rise to a clonal population of alveolar cells during subsequent pregnancies [19]. The persistence of luminal cells that undergo cellular differentiation in former pregnancies may explain, in part, our findings that alveolar luminal cells of multiparous animals on D9 of pregnancy exhibited a lower proliferation index and higher α-lactalbumin expression than the other groups.

Direct evidence for the importance of PR in MG development is revealed in both PR-null mutant mice [10] and in transgenic mice that have a disruption of the PR-A/B ratio [31,32]. Both alterations in PR expression result in a marked impairment of MG development. Our results demonstrate that PR is localized exclusively in the luminal epithelium as previously reported [6,33]. We also observed that the percentage of luminal cells that express PR is higher in pregnant nulliparous and primiparous animals where the epithelium exhibited the greatest proliferation index and

Table 3
Serum concentrations of hormones on D9 of pregnancy

Groups[a]	*n*	PRL (ng/ml)	GH (ng/ml)	IGF-I (ng/ml)	Estradiol (pg/ml)	Progesterone (ng/ml)
N	6	6.5 ± 1.6	50.6 ± 14.5	892.2 ± 38.0	7.1 ± 1.5	50.5 ± 10.9
P	6	6.3 ± 3.2	51.1 ± 8.5	856.3 ± 33.8	6.0 ± 1.1	49.1 ± 10.0
M	6	7.3 ± 0.7	45.9 ± 6.2	886.7 ± 59.6	6.6 ± 1.2	50.9 ± 5.0

The values represent mean ± S.E.M.
[a] N: nulliparous animals; P: primiparous animals; M: multiparous animals.

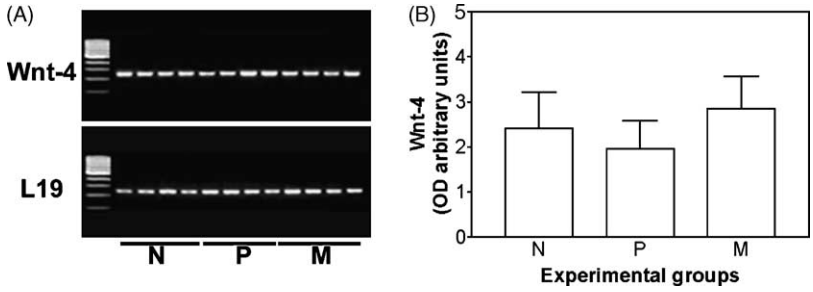

Fig. 5. Wnt-4 mRNA expression. Wnt-4 mRNA was measured by RT-PCR analysis in mammary tissue of nulliparous (N), primiparous (P) and multiparous (M) rats. Representative ethidium bromide-stained agarose gels are shown in (A). Levels of Wnt-4 mRNA were expressed in OD arbitrary units relative to L19 mRNA values (B). No significant changes associated with parity were observed in the Wnt-4/L19 ratio. Bars represent mean values (+S.E.M.) of five or more animals per group.

the lowest α-lactalbumin expression compared with multiparous rats. Previous studies have shown that in primiparous non-pregnant animals (which were cycling again), PR expression was lower than in their age-matched virgin controls [33,34]. The use of different animal models might explain the apparent discrepancies with our results regarding PR expression in primiparous rats. On the other hand, our results revealed that down-regulation of PR in the parous MG is not associated with a decrease in Wnt-4 mRNA expression, which remains unchanged between groups. On D9 of

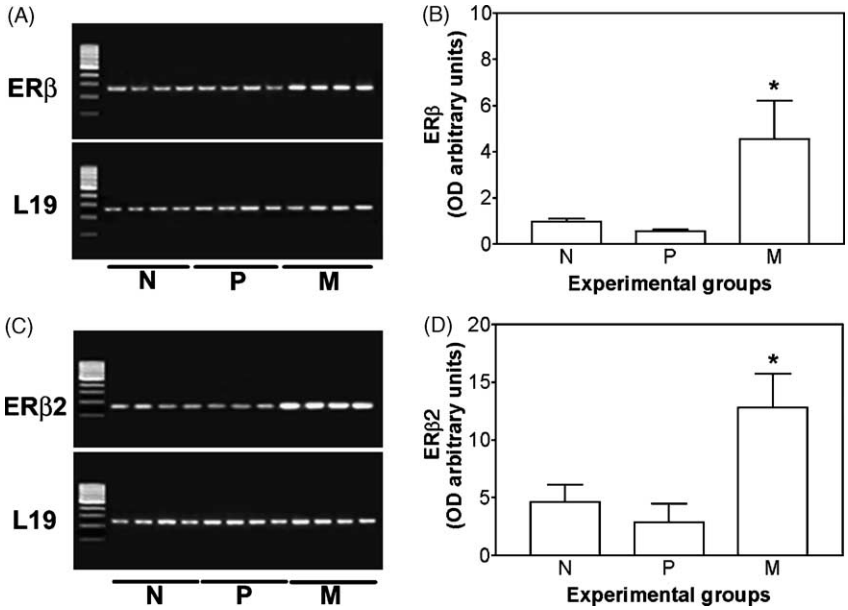

Fig. 6. Expression of ERβ and ERβ2 mRNA in the rat mammary gland was related to parity history. Representative RT-PCR ethidium bromide-stained agarose gels are shown in (A) and (C). Densitometric values of ERβ and ERβ2 mRNA, expressed in OD arbitrary units relative to L19 mRNA levels, are shown in (B) and (D), respectively. In multiparous animals (M), the ERβ and ERβ2 mRNA expression was increased compared to nulliparous (N) and primiparous rats (P). Bars represent mean values (±S.E.M.) of five or more animals per group, with significant ($P < 0.05$) differences between groups being indicated by the asterisk (Kruskal–Wallis followed by Dunn).

pregnancy, the MG is in a high rate of development, which could explain our observations regarding Wnt-4 expression, as this expression has been correlated with lobuloalveolar development both during pregnancy and in older virgin animals [35].

ERα and ERβ are both present in the rat MG, although they exhibit a different pattern of expression [14]. We observed that expression of both types of ER was greater in luminal cells of multiparous animals on D9 of pregnancy in comparison to the other groups. In addition, ERβ and ERβ2 mRNA expression was up-regulated in multiparous rats. In contrast, previous reports have shown that ERα expression in primiparous cycling rats was lower compared with virgin controls [34,36]. Thordarson et al. [36] suggested that this decreased expression of ERα was associated with a decline in GH levels; however, in a later report these authors did not observe decreased GH levels in primiparous rats [37]. Our results revealed that serum levels of GH, as well as PRL, IGF-I, estradiol and progesterone remain unchanged with subsequent gestations and lactation. Therefore, the increased ER expression observed in multiparous rats is not a consequence of an altered hormonal milieu; it may be the result of a biochemical modification of the mammary tissue itself.

An important question that needs to be addressed is why the MGs of multiparous animals during pregnancy (proliferative phase according with Saji et al. [15]) exhibit decreased PR levels and proliferative activity in the presence of increased levels of ERα. Even though these observations do not correlate with the widely accepted paradigm that PR expression is associated with ER activation, this finding is consistent with previous reports that estrogen does not induce PR expression or proliferation in the lactating MG [13,38]. The basis for the apparent estrogen-insensitivity of the lactating MG is unclear. During lactation, the highest level of ERα and ERβ co-expression was observed [14], as well as up-regulation of ERβ2 mRNA expression [15]. ERβ2 is an alternative splicing product of the rat ERβ that has been suggested to be a dominant negative regulator of both ERα and ERβ function [39,40]. These findings lead to the suggestion that one role of ERβ/ERβ2 is to suppress ERα actions in the MG epithelium [14]. They also hypothesized that formation of ERα/ERβ heterodimers may inhibit ERα stimulated PR induction. Even though we have not evaluated ERα and ERβ co-expression, this hypothesis would be in accordance with our results and the previous observation of very low PR levels within the MG during lactation in mice reported by Haslam and Shyamala [13]. Similar results were observed in the luminal epithelium of the mouse uterus suggesting ERβ was associated with a decrease in PR expression [41]. In our model, one possible explanation may implicate ERβ/ERβ2 as a suppressor/regulator of the proliferative mechanisms associated with formation of ERα homodimers. Formation of ERα homodimers has been shown to stimulate gene transcription [42–44], whereas, formation of ERα/ERβ heterodimers has been shown to exert a negative regulatory effect on ERα transcriptional activity by decreasing estrogen-sensitivity of ERα positive cells [45]. Therefore, ERβ may play a role as a modulator of estrogen stimulated cell proliferation and the relative expression levels of the different ER isoforms in various cells may affect responses to estrogens.

The mammary stroma of multiparous animals revealed a different expression pattern of several markers in comparison to nulliparous and primiparous rats. In the intralobular stroma, neither ERα nor ERβ expression differed between groups; however, in the interlobular stroma, we observed that expression of both ERα and ERβ was increased in multiparous animals. Adipose deposition was dependent upon estrogen, acting specifically through ERα stimulating lipoprotein lipase activity; however, when the hormone acts through ERβ its effects are lipogenic [46]. D'Cruz et al. [16] have demonstrated that the adipocyte-differentiation related protein is increased in parous animals and have suggested that the mammary stroma of parous animals exhibits a different grade of differentiation. The increased expression of both types of ER in the interlobular stroma (mainly adipose) of multiparous animals may indicate a higher responsiveness to estrogen.

The incidence of mammary carcinoma was significantly reduced when rats completed a full-term pregnancy (reviewed in Sivaraman and Medina [18]) and nurse their pups [34]. The most widely accepted explanation for how parity confers refractoriness to the development of breast cancer is offered by Russo and Russo [47], who postulate that protection is given following pregnancy-induced differentiation of the target structures for carcinogenesis. We observed an increase in lineal density of myoepithelial cells in multiparous animals, suggesting an increased state of myoepithelial cell differentiation. This differentiated cell type could act as a natural barrier to MG cell proliferation and tumorigenesis, avoiding invasion. Several lines of evidence have shown that differentiated myoepithelial cells may act as a natural tumor suppressor in humans [48], therefore, we suggest that myoepithelial differentiation could be one of the cellular events involved in the protection against breast cancer associated with pregnancy and lactation by acting as a barrier to cell proliferation and tumorigenesis. Increased mRNA and protein expression of ERβ observed in multiparous animals may also serve a protective function in regulating proliferation of epithelial cells within the MG since recent studies have implied that ERβ and/or ERβ2 may exert a protective role in inhibiting breast tumorigenesis [49–51].

In summary, our results reveal that to acquire a fully differentiated state in the MG at least two periods of pregnancy and lactation were necessary. Multiple MG changes induced by parity include increased myoepithelial cell cytoplasmic processes, decreased PR expression and proliferation index, as well as increased α-lactalbumin, ERα and ERβ expression in luminal cells. A different hormonal milieu was not responsible for the observed changes, since no differences were found in any of the hormones assayed. A more plausible explanation may implicate a role for either ERβ and/or

ERβ2 in contributing to the apparent estrogen-insensitivity of the MG observed in multiparous animals.

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