Progesterone induces side-branching of the ductal epithelium in the mammary glands of peripubertal mice

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Abstract

Development of the functional secretory epithelium in the mammary gland of the female mouse requires the elongation of the anlage through the mammary fat pad to form the primary/secondary ductal network from which tertiary ductal side-branches and lobuloalveoli develop. In this study we examined the hormonal requirements for the spatial development of the primary/secondary epithelial network and tertiary side-branches by quantifying ductal growth and epithelial cell proliferation in normal and hormone-treated BALB/c mice between 21 and 39 days of age. In normal mice, an allometric increase in ductal length commenced at 31 days of age and resulted in completion of the primary/secondary ductal network by 39 days of age. Concurrent with this allometric growth was a significant increase in cellular proliferation in the terminal end-buds (TEBs) of the ductal epithelium from 29 days of age, as determined by 5-bromo-2′-deoxyuridine (BrdU) incorporation. A level of cellular proliferation similar to that in the TEBs of 33-day-old control mice could be induced in the TEBs of 25-day-old mice following treatment for 1 day with estrogen (E), or progesterone (P) or both (E/P), indicating that both E and P were mitogenic for epithelial cells of the peripubertal TEBs. However, the period of allometric ductal growth in untreated mice did not correspond to an increase in serum E or P (which might have been expected during the estrous cycle). In addition, epithelial growth was not observed in mammary glands from 24-day-old mice that were cultured *in vitro* with E, P or E/P. In contrast to treatment with E, treatment with P promoted a dramatic increase, relative to control mice, in the number of tertiary branch points upon the primary/secondary ductal network. BrdU labeling of mammary glands from 24–33-day-old mice pelleted with cholesterol (C), E, P or E/P confirmed the greater mitogenicity of P on the epithelial cells of the secondary/tertiary ducts as compared with C or E. Concurrent with these changes, localized progesterone receptor (PR) expression in clusters of cells in the ductal epithelium was associated with structures that histologically resembled early branch points from ductules. In conclusion, our results suggest that additional endocrine growth factor(s) other than E and P contribute to the development of the primary/secondary ductal network, and that P is responsible for the formation of tertiary side-branches in the mammary glands of mice during puberty.

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Introduction

The mammary gland undergoes a series of well-coordinated phases of development prior to lactation (Imagawa *et al.* 1990). At birth, the anlage of mammary epithelium in the female mouse is a small, branched structure connected to the nipple. With the onset of puberty at around 4 weeks of age there is rapid proliferation within the terminal end-buds (TEBs) which results in the elongation of the ducts into the fat pad to yield a structure upon which tertiary side-branches then form. Finally, during pregnancy, lobuloalveolar structures develop on this ductal network (Vonderhaar 1984, Vonderhaar & Plaut 1992).

Development of the anlage into a functional mammary gland in female mice represents the upregulation of different endocrine and local factors during each phase of growth. Although a large number of studies have indicated the requirement for estrogen (E) and progesterone (P) in the development of the mammary gland during puberty, the roles of these hormones in the development of each of the structural components of the mammary gland

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remain unclear. Previous studies have indicated that the increased mitotic activity in the TEBs, which leads to ductal elongation, is thought to be mediated by E and either insulin-like growth factor-I (IGF-I) or growth hormone (GH) in most species (Topper & Freeman 1980, Imagawa *et al.* 1990, Kleinberg 1997). Recent evidence suggests that P also may play a role in the development of the mammary gland. Mammary glands only demonstrate hormonally induced lobuloalveolar development in whole-organ culture if pubescent (24–25-day-old) mice are first primed for 9–15 days with estrogen and progesterone (E/P) (Vonderhaar 1984, Vonderhaar & Plaut 1992). Likewise, ovariectomized mice treated with P also show lobuloalveolar development in whole-organ culture (Plaut *et al.* 1999), suggesting an essential role for P in preparing the mammary gland for development during pregnancy. Finally, mice lacking the progesterone receptor (PR) have severely limited mammary gland development (Lydon *et al.* 1995). Whole-mount analysis of ovary-intact and ovariectomized PR-deficient mice treated with E and P revealed a more basic ductal structure with less extensive lateral branching than that in similarly treated wild-type mice. The lack of branching ultimately resulted in a lack of lobuloalveolar development in the hormonally treated mice (Lydon *et al.* 1996). In addition, mammary glands from ovary-intact transgenic mice that overexpress the A form of the PR demonstrate extensive lateral side-branching at 10–14 weeks of age compared with wild-type controls (Shyamala *et al.* 1998).

Using morphometric analysis and immunohistochemical methods, we have conducted a spatio-temporal study of the morphological and proliferative changes in the mammary glands of normal and hormone-treated peripubescent mice in order to clarify the roles of E and P in the development of the mammary ductal network. Our evidence indicates that P is responsible for the formation of tertiary side-branches on the primary/secondary ductal network, and that an unidentified factor(s) other than E may be required for primary/secondary ductal growth in the mammary glands of peripubertal mice.

Materials and Methods

Reagents

Waymouth's medium, penicillin, proteinase K, streptomycin and gentamycin sulfate were purchased from Life Technologies (Gaithersburg, MD, USA). Estradiol-17β, P, insulin, aldosterone, hydrocortisone, thyroxine (T_4), tri-iodothyronine (T_3), HEPES and 5-bromo-2′-deoxyuridine (BrdU) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Bovine serum albumin was purchased from Miles (Kankakee, IL, USA). Ovine prolactin (oPRL, Lot 17), murine leutinizing hormone (mLH), murine follicle-stimulating hormone (mFSH), and relaxin were obtained through the National Hormone and Pituitary Program, the National Institute of Diabetes & Digestive & Kidney Diseases (NIDDK), the National Institutes of Health (NIH) and Dr A F Parlow. Epidermal growth factor (EGF), IGF-I, nerve growth factor (NGF) and transforming growth factor (TGF)-α were purchased from Becton-Dickinson (Franklin Lakes, NJ, USA). Colony-stimulating factor (CSF), fibroblast growth factors (FGF) a and b, and anti-TGF-β neutralizing antibody were obtained from R & D Systems (Minneapolis, MN, USA). Tumor necrosis factor (TNF)-α was obtained from Boehringer Mannheim (Indianapolis, IN, USA). Rat monoclonal anti-BrdU was purchased from the Accurate Chemical and Surgical Corp. (Westbury, NY, USA).

Pellets

Pellets (10 mg) containing either cholesterol (C; control), E (2001:1, C:E), P (2000:1000, C:P) or E/P (2002:1001:1, C:P:E) were implanted subcutaneously as previously described (Plaut *et al.* 1993).

Animals

For all experiments, BALB/c mice were obtained from the Frederick Cancer Research and Development Center (Frederick, MD, USA). Animal experimentation was conducted under the guidelines described in the *NIH Guide for the Care and Use of Laboratory Animals*. Mice were kept with a ratio of 12 h light:12 h darkness photoperiod and were allowed access to standard lab. chow and water *ad libitum*.

Ductal outgrowth study

Female BALB/c mice were either left intact (normal, $n=48$) or implanted subcutaneously with a P ($n=48$) or an E/P pellet ($n=4$) at 24 days of age. The abdominal No. 4 mammary glands were subsequently excised from mice killed at between 24 and 39 days of age. The mammary glands were mounted on slides, fixed overnight, stained with carmine alum, defatted and clarified for morphometric analysis (Banerjee *et al.* 1976). Computer-assisted morphometry was performed using the C-Imaging 1280 image-analysis system (Compix Inc., Cranberry Township, PA, USA) using procedures developed for corneal innervation (Jacot *et al.* 1997). Briefly, photographs (10 in × 12 in) of the mammary gland whole mounts were hand-traced with a fine-point permanent marker. The prints were then bleached using Farmer's Reducing Agent, as specified by the manufacturer (Eastman Kodak Co., Rochester, NY, USA), leaving only the ink tracings of the ducts, which were then digitized and analyzed using the C-Imaging 1280 image-analysis system. Ductal length, the number of branch points, the number of TEBs and the ductal outgrowth area were determined from the binary

images. Ductal length was calculated as the total length of ducts (when placed end to end) in the mammary fat pad. The ductal outgrowth area was the total area of the gland occupied by the ducts and associated stroma.

Cellular proliferation study

Female BALB/c mice at 24 days of age were implanted subcutaneously with a C, E, P or E/P pellet. At 1, 3, 5, 7 and 9 days after pellet implantation, three mice from each treatment group were killed. Two hours prior to being killed, each animal received BrdU intraperitoneally (70 μg/g body weight). The No. 4 abdominal glands were removed, formalin-fixed for 2 h, embedded in paraffin and sectioned (5 μm). Following deparaffinization, the sections were treated with 2 M HCl at 40 °C for 60 min. The endogenous peroxidase activity was blocked with peroxide and, following treatment with normal rabbit serum, the sections were stained immunohistochemically using a rat monoclonal anti-BrdU antibody and the Vector ABC Kit (Vector Laboratories, Inc., Burlingame, CA, USA). Sections were analyzed by determining the percentage of BrdU-positive epithelial cells in the TEBs and primary and secondary/tertiary ducts by using the C-Imaging 1280 image-analysis system.

In vitro *culture*

Female BALB/c mice were killed at 24–25 days of age and their No. 4 mammary glands removed aseptically and placed in whole-organ culture according to the method described previously (Plaut *et al.* 1993, Atwood *et al.* 1995). Mammary glands ($\geq$3 glands/treatment) were cultured in Waymouth's serum-free medium supplemented with insulin and various hormone and growth-factor combinations in an attempt to induce ductal elongation and branching. Glands were subsequently removed from culture at various intervals of up to 14 days and then stained as whole mounts for morphological examination as described above.

Serum hormone analysis

Female BALB/c mice at 24 days of age were implanted subcutaneously with a C pellet or an E/P pellet. Blood was collected at 1, 3, 5, 7 and 9 days post-implantation after cervical dislocation and exsanguination. Serum was pooled from 10 mice for each time-point and duplicate samples were assayed for E and P by radioimmunoassay (Coat-A-Count; Diagnostic Products Corp., Los Angeles, CA, USA).

Progesterone receptor immunohistochemistry

Nulliparous, 25-day-old BALB/c mice were treated with C, E, P or E/P pellets for 9 days. They were killed at 34 days of age, and their mammary glands were excised and fixed in 4% paraformaldehyde buffered with 0·05 M PBS (pH 7·2) at 4 °C for 6 h. The tissue was rinsed four times with 0·05 M PBS (pH 7·2), dehydrated in graded alcohols over a period of 1·5 h at 4 °C, penetrated in a tissue processor at 60 °C under vacuum, embedded in paraffin and sectioned at 5–6 μm. Sections were deparaffinized and rehydrated to 0·15 M PBS (pH 7·4) or 0·05 M Tris–HCl (pH 7·2). Endogenous peroxidase was blocked with peroxide, and non-specific proteins were blocked with DAKO (Carpenteria, CA, USA) Serum-Free Protein Block or normal mouse serum. Monoclonal antibody PR-AT 414·9 (an immunoglobulin G1 (IgG1)) raised against peptide 533–547 of the human PR was a gift from Dr Abdul Traish (Boston University) and recognizes the PR of mouse uterus (Traish & Wotiz 1990). The primary antibody to PR was applied as a 1:500 dilution for 60 min at 37 °C. Biotinylated secondary antibody and ABC reagent from the Vector Elite Mouse Kit (Vector Laboratories, Inc.) were used according to the manufacturer's recommendations. A solution of DAB at twice the dilution recommended for the Vector DAB Kit, or the manufacturer's recommended dilution for the DAKO Animal Research Kit, was used as the chromagen. Secondary antibody alone produced background staining in fat-cell cytoplasm but no nuclear staining in any cells. To control for background and false-positive staining, a control mouse monoclonal IgG1 (B1·1) was substituted for the primary antibody in the reaction. Sections were counterstained with Harris' Hematoxylin. In all cases, uteri from 34-day-old mice primed with E/P for 9 days were used as positive-staining controls.

In situ *hybridization*

Riboprobes were synthesized from a 395 bp cDNA corresponding to the common form of the PR cDNA (Schott *et al.* 1991) subcloned into PCRscript (Stratagene, La Jolla, CA, USA). Sense and antisense probes were transcribed from NotI- and EcoRV-digested templates using T7 and T3 polymerase respectively (MAXIscript Kit; Ambion, Austin, TX, USA). BALB/c mice (24 days of age) were treated with pellets for 13 d. Mammary tissue was fixed with 4% paraformaldehyde in PBS (pH 7·4) for 16 h at 4 °C, dehydrated, embedded in paraffin, and sections (4 μm) mounted onto ProbeOn Plus slides (Fisher Scientific, Pittsburgh, PA, USA). Sections were dewaxed and rehydrated, treated with 0·2 M HCl, digested with proteinase K (15 μg/ml;), post-fixed in 4% paraformaldehyde and acetylated with two changes of 0·25% acetic anhydride in 0·1 M triethanolamine buffer (pH 8·0). After dehydration, the sections were prehybridized for 1 h in hybridization buffer (50% formamide, 5 × standard saline citrate (SSC), 10% dextran sulfate, 2% SDS, 100 μg/ml salmon-sperm DNA, 1 mg/ml yeast soluble tRNA, 100 mM dithiothreitol) at 55 °C. Sections were hybridized

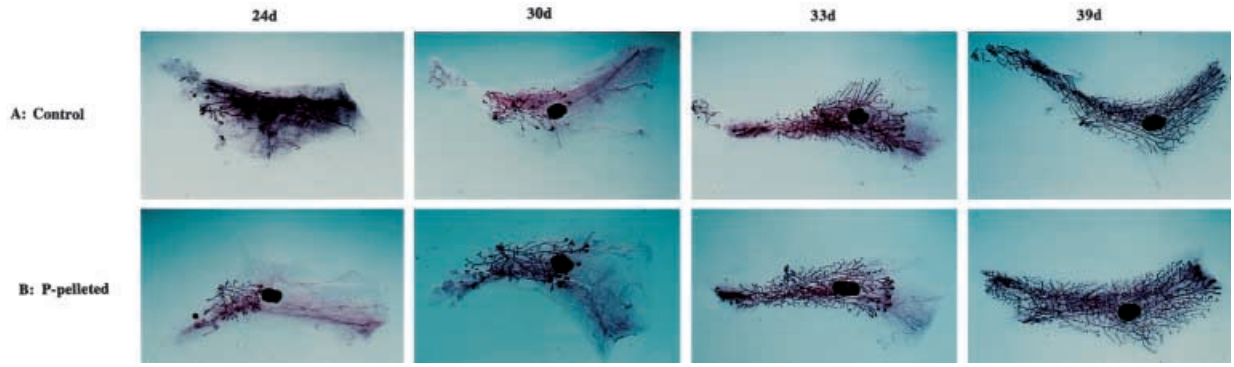

Figure 1 Carmine alum-stained abdominal No. 4 mammary glands at 24, 30, 33 and 39 days of age from (A) control and (B) P-pelleted, female BALB/c mice.

with cRNA probe (5×10^4 c.p.m./μl) overnight at 55 °C under a Parafilm coverslip. Coverslips were removed in 2 × SSC, 50% formamide and the sections washed in 2 × SSC, 50% formamide at 60 °C for 30 min. Digestion with RNase A (20 μg/ml) was performed in 10 mM Tris, 1 mM EDTA, 500 mM NaCl (pH 8·0) for 30 min at 37 °C prior to stringency washes at 2 × and 0·1 × SSC at 37 °C. Sections were exposed to emulsion (NTB-2; Eastman Kodak) for 5 weeks and counterstained with nuclear fast red.

Mathematical analyses

Results are expressed as means ± S.E.M. unless otherwise indicated. Correlation analysis of morphometric parameters was performed using STAT-VIEW Version 4·01 (Abacus Concepts Inc., Berkeley, CA, USA). Comparisons of morphometric parameters between time-points for control, P- and E/P-pelleted mice were performed using two-way analysis of variance with SUPERANOVA Version 1·1 (Abacus Concepts Inc.). Curves were fitted to ductal growth rate (ductal length) versus age for both normal and P-pelleted mice using CRICKET GRAPH Version 1·3·1 (Cricket Software, Malvern, PA, USA). Significance is defined in all cases as $P \leq 0{\cdot}05$.

Results

Morphometric analysis

Morphometric analysis of ductal development indicated a significant and rapid increase in outgrowth area, ductal length and number of branch points for normal mice between 24–39 days of age (Figs 1A, 2A and 3). The growth rate of the mammary ducts with respect to length was allometric to the overall growth rate of the mouse (data not shown) between 31–39 days of age (Figs 1A and 3). In normal mice, there were 6·5-, 10- and 11-fold increases in the outgrowth area, the length of the ducts and the number of branch points respectively, between 24 and 39 days (Figs 1A, 2A and 3).

In P-pelleted mice, the outgrowth area between 24 and 39 days of age generally was not significantly different from that of normal mice, but there was a significant

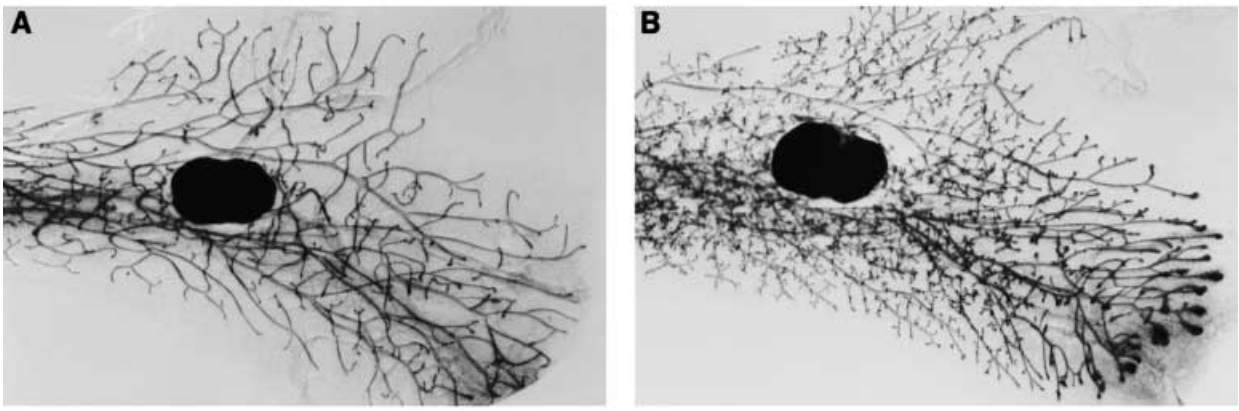

Figure 2 Higher magnification of carmine alum-stained abdominal No. 4 mammary gland from (A) control and (B) P-pelleted female BALB/c mice at 39 days of age, demonstrating increased tertiary ductal branching in the latter.

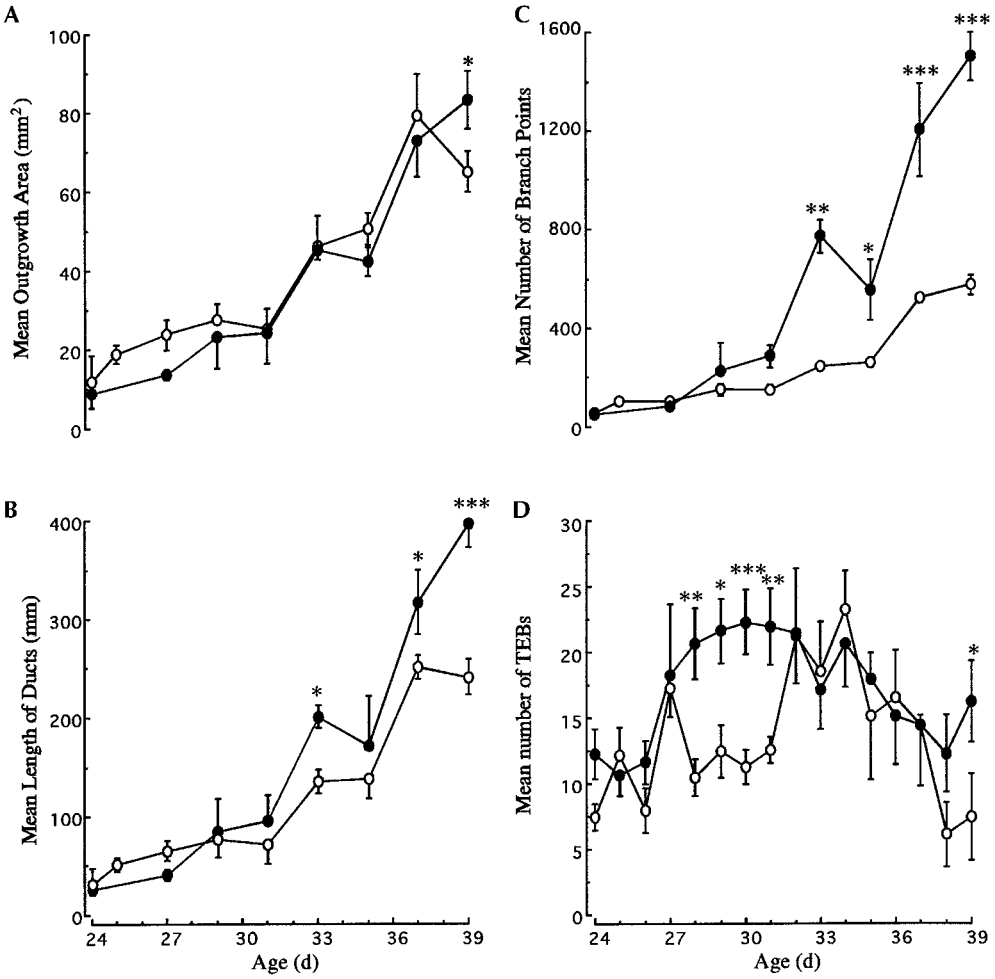

Figure 3 The outgrowth area (A), length of ducts (B), number of branch points (C) and number of TEBs (D) (means ± S.E.M.) in mammary glands (n=6) of control (○) and P-pelleted mice (●) between 24 and 39 days of age. Photographs of whole mounted and stained mammary glands were analyzed for the above parameters using the C-Imaging 1280 image-analysis system. Significant differences for corresponding values between control and P-pelleted mice for the morphological parameters were as follows: *P<0·05; **P<0·01; ***P<0·001. The rate of growth of the ductal epithelium between 24 and 39 days post-partum in normal mice, as determined by the change in the length of the ducts, can be described by the following equation:

$$y = -38\,743 + 5098x - 249x^2 + 5{\cdot}34x^3 - 0{\cdot}0425x^4;\ r^2 = 0{\cdot}96$$

where y=length of the ducts and x=age in days. The rate of growth of the ductal epithelium in P-pelleted mice, as determined by the change in the length of the ducts between 24 and 39 days post-partum, is described by the following equation:

$$y = 11\,750 - 1552x + 76{\cdot}2x^2 - 1{\cdot}66x^3 + 0{\cdot}0136x^4;\ r^2 = 0{\cdot}96$$

where y=length of the ducts and x=age in days. These equations allow the prediction of mammary ductal length in the mammary glands of BALB/c mice at any time between 24 and 39 days of age. The morphometric analyses described in this study may prove useful in future comparisons of ductal growth between mice strains, in the study of agents that either inhibit or promote ductal growth, and in the fractal analysis of mammary gland architecture, particularly comparisons between normal and transgenic mice.

increase in the number of branch points and the length of the ducts between 31 and 39 days of age compared with controls (Figs 1B, 2B and 3). This was reflected in 7·5-, 15- and 34-fold increases in the outgrowth area, the length of the ducts and the number of branch points respectively (Figs 1B, 2B and 3). Between 28 and 31 days of age, the number of TEBs in P-pelleted mice was significantly increased relative to control mice (Fig. 3). This rapid

Table 1 Comparison of outgrowth area, length, branch points and TEBs in normal mice at 24 and 33 days of age, with E/P-pelleted mice at 33 days of age and with mammary glands cultured *in vitro* with E/P for 9 days

	In vivo			***In vitro***
Age of gland	24 days	33 days	33 days	33 days
Treatment	None	None	E/P-pelleted mice for 9 days	E/P media for 9 days
Outgrowth are (mm^2)	$12{\cdot}0 \pm 3{\cdot}3^{a}$	$46{\cdot}4 \pm 4{\cdot}3^{b}$	$55{\cdot}1 \pm 3{\cdot}7^{b}$	$13{\cdot}4 \pm 2{\cdot}9^{a}$
Length of ducts (mm)	$32{\cdot}4 \pm 8{\cdot}2^{a}$	$135{\cdot}9 \pm 12{\cdot}1^{b}$	$193{\cdot}5 \pm 19{\cdot}4^{c}$	$25{\cdot}3 \pm 8{\cdot}9^{a}$
Number of branch points	$42{\cdot}7 \pm 7{\cdot}1^{a}$	$247{\cdot}0 \pm 10{\cdot}8^{b}$	$537{\cdot}3 \pm 94{\cdot}9^{c}$	$39{\cdot}4 \pm 4{\cdot}5^{a}$
Number of TEBs	$7{\cdot}5 \pm 1{\cdot}0^{a}$	$20{\cdot}3 \pm 1{\cdot}9^{b,c}$	$45{\cdot}7 \pm 9{\cdot}9^{c}$	$14{\cdot}7 \pm 1{\cdot}7^{a,b}$

[a–c]Different letters indicate significant differences ($P<0{\cdot}05$) between treatments within a parameter.

increase in the number of TEBs immediately precedes the increase in the ductal length and the number of branch points compared with control mice at 33 days of age. Mice pelleted with E/P also showed a significant increase in ductal length and number of branch points compared with normal mice at 33 days of age (Table 1).

Significant correlations were found between the left and right glands for outgrowth area, length of ducts and the number of branch points and TEBs in both the normal and P-pelleted mice between 24 and 39 days of age (Table 2). For control and P-pelleted mice respectively, there also were significant correlations between ductal outgrowth area and length ($r^2=0{\cdot}98$, $n=52$, $P<0{\cdot}001$; $r^2=0{\cdot}99$, $n=44$, $P<0{\cdot}001$), outgrowth area and number of branch points ($r^2=0{\cdot}91$, $n=52$, $P<0{\cdot}001$; $r^2=0{\cdot}96$, $n=44$, $P<0{\cdot}001$), and ductal length and number of branch points ($r^2=0{\cdot}97$, $n=52$, $P<0{\cdot}001$; $r^2=0{\cdot}99$, $n=44$, $P<0{\cdot}001$) between 24 and 39 days of age. There was no correlation between the number of TEBs and outgrowth area, ductal length or the number of branch points for either control or P-pelleted animals during this time.

Cell proliferation

Ductal epithelial cells were the predominant cell type labeled with BrdU, along with occasional fibroblasts (Figs 4 and 5). As shown in Fig. 4 and quantified in Fig. 6, there was significant labeling in TEBs of control mice at 25 days, but even more in P-pelleted mice. The quantification of the labeling indicated a gradual increase in cellular proliferation in the TEBs of control mice, from $8{\cdot}8 \pm 1{\cdot}3\%$ at 25 days to $21 \pm 2{\cdot}9\%$ at 33 days (Fig. 6A), consistent with the rapid increase in ductal growth during this time (Figs 1 and 3). In contrast, E-pelleted mice showed a gradual decrease in cellular proliferation in the TEBs, from $15{\cdot}3 \pm 2{\cdot}9\%$ at 25 days to $6{\cdot}4 \pm 0{\cdot}5\%$ at 33 days (Fig. 6B). Cellular proliferation in the TEBs of P-pelleted mice also was initially elevated ($20{\cdot}6 \pm 3{\cdot}6\%$) at 25 days but remained at this level to 33 days (Fig. 6C). Cellular proliferation in the TEBs of E/P-pelleted mice also was initially high but showed a downward trend to 33 days (Fig. 6D). Cellular proliferation in the secondary/tertiary ducts was low in control mice ($3{\cdot}1 \pm 1{\cdot}3\%$) and was significantly reduced in E-pelleted mice ($0{\cdot}8 \pm 0{\cdot}5\%$) at 25 days; values remained at these levels to 33 days (Figs 5B and 6A and B). In contrast, cellular proliferation in the secondary/tertiary ducts in P-pelleted mice ($16{\cdot}7 \pm 2{\cdot}6\%$) and E/P-pelleted mice ($10{\cdot}8 \pm 2{\cdot}8\%$) was initially higher at 25 days (Figs 4C and D and 6C and D), but fell to control levels by 29 days (Fig. 6C and D). A pattern of cellular proliferation similar to that observed for the secondary and tertiary ducts was observed for the primary ducts (Fig. 6A–D). Overall total proliferation in TEBs and primary and secondary/tertiary ducts of P-pelleted mice was significantly greater than that in control mice.

Serum hormone analysis

The concentrations of E and P in control mice remained constant at a very low level between 25 to 33 days of age (Fig. 7). The concentration of both E and P in E/P-pelleted animals was 17 times higher than that in control

Table 2 Correlation between the left and right glands for outgrowth area, length, branch points and TEBs in normal and P-pelleted mice between 24 and 39 days

	Control mice			**P-pelleted mice**		
Parameter	r	*P*	*n*	r	*P*	*n*
Outgrowth area	0·95	<0·001	29	0·98	<0·001	29
Length	0·98	<0·001	29	0·98	<0·001	22
Branch points	0·98	<0·001	29	0·98	<0·001	22
TEBs	0·69	<0·001	47	0·60	<0·001	47

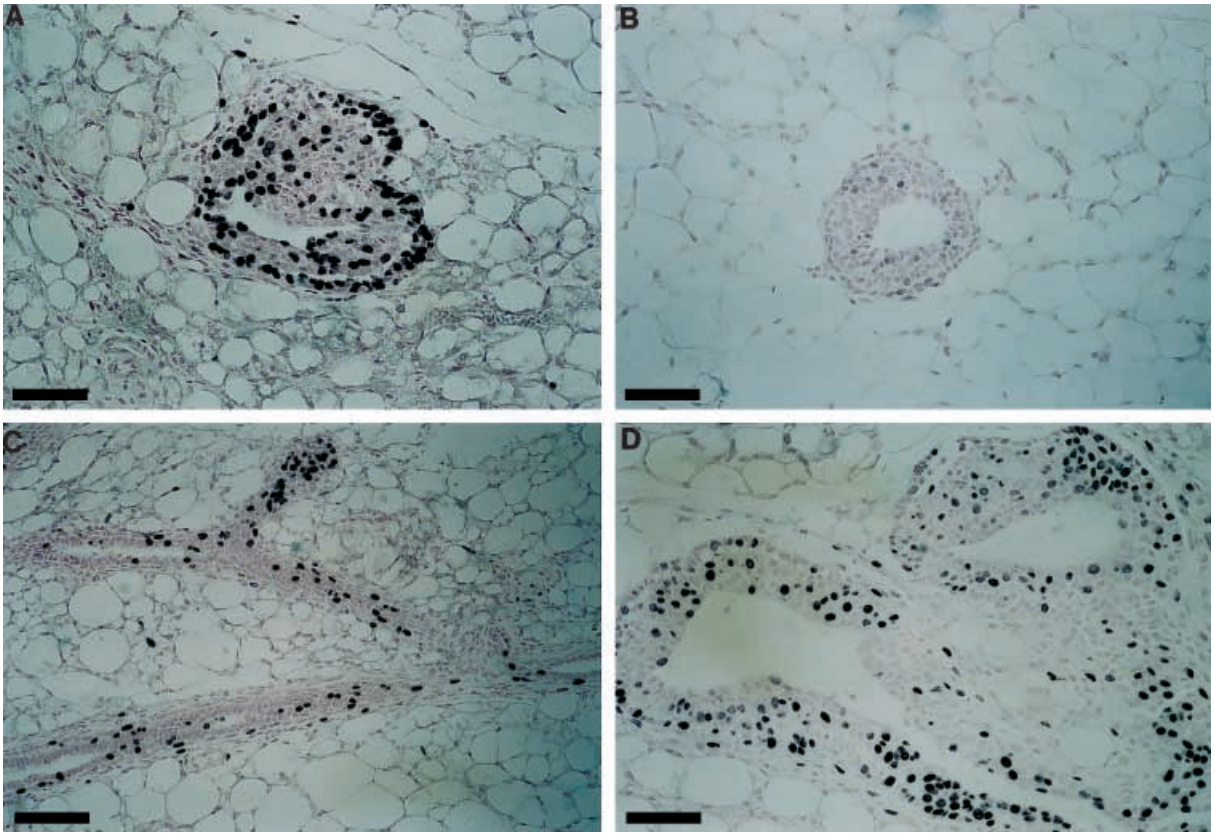

Figure 4 BrdU-labeled epithelial cells in No. 4 mammary glands from female BALB/c mice on day 25 (day 1 of pellet) pelleted with either cholesterol (A), estradiol (B), progesterone (C) or estradiol and progesterone (D). Mammary glands were removed, formalin-fixed for 2 h, sectioned and stained immunohistochemically using a rat monoclonal anti-BrdU to detect incorporation into cellular DNA. Sections were counterstained with nuclear fast red (scale bar represents 75 μm).

mice at 25 days, and progressively decreased to approximately four times that in control mice by 33 days. The E/P ratio in control and E/P-pelleted mice was similar between 25 and 33 days of age (1:235–1:438 for control mice and 1:262–1:542 for E/P-pelleted mice).

Progesterone receptor expression

Positive reactions for the PR ranged from lightly stained nuclei to very deeply stained nuclei in the same structure, suggesting that PR is not as strongly expressed in some cells as in others. Sections through TEBs showed that up to 50% of the epithelial cells in the TEBs and TEB necks were PR-positive in the hormone-treated mice. These data are consistent with our observations in C57BL/6 mice (Seagroves *et al.* 2000) as well those of other workers (Shyamala *et al.* 1998, Zeps *et al.* 1999). In all treatment groups, the ducts contained a mixture of PR-positive and PR-negative cells. All identifiable myoepithelial cells were negative for PR. Many of the division-competent, undifferentiated, large light cells (ULLCs) in all treatment groups were positive for PR; some of the PR-positive cells also were the division-competent small light cells (SLCs) (Chepko & Smith 1997). Although most of the PR-positive epithelial cells in the mammary glands were randomly scattered in the ducts, there were numerous cases of clusters of PR-positive cells in both the large and the small (Fig. 8) mammary ducts. Early branch points, identified by serial sectioning and through-focus micrography, always contained clustered PR-positive ULLCs. These clusters were occasionally associated with PR-positive or PR-negative SLCs.

In situ *hybridization for progesterone receptor mRNA*

The results obtained by immunohistochemistry were supported by the observations obtained by *in situ* hybridization. PR mRNA was found in epithelial cells from all treatment groups. In cholesterol (C)-treated tissue (Fig. 9A), it can be seen that the TEBs are PR-positive, in agreement with Shyamala *et al.* (1997) and Shyamala (1999). Although the level of PR mRNA was relatively low, there was a scattered pattern of expression of PR mRNA along ducts (data not shown), as reported

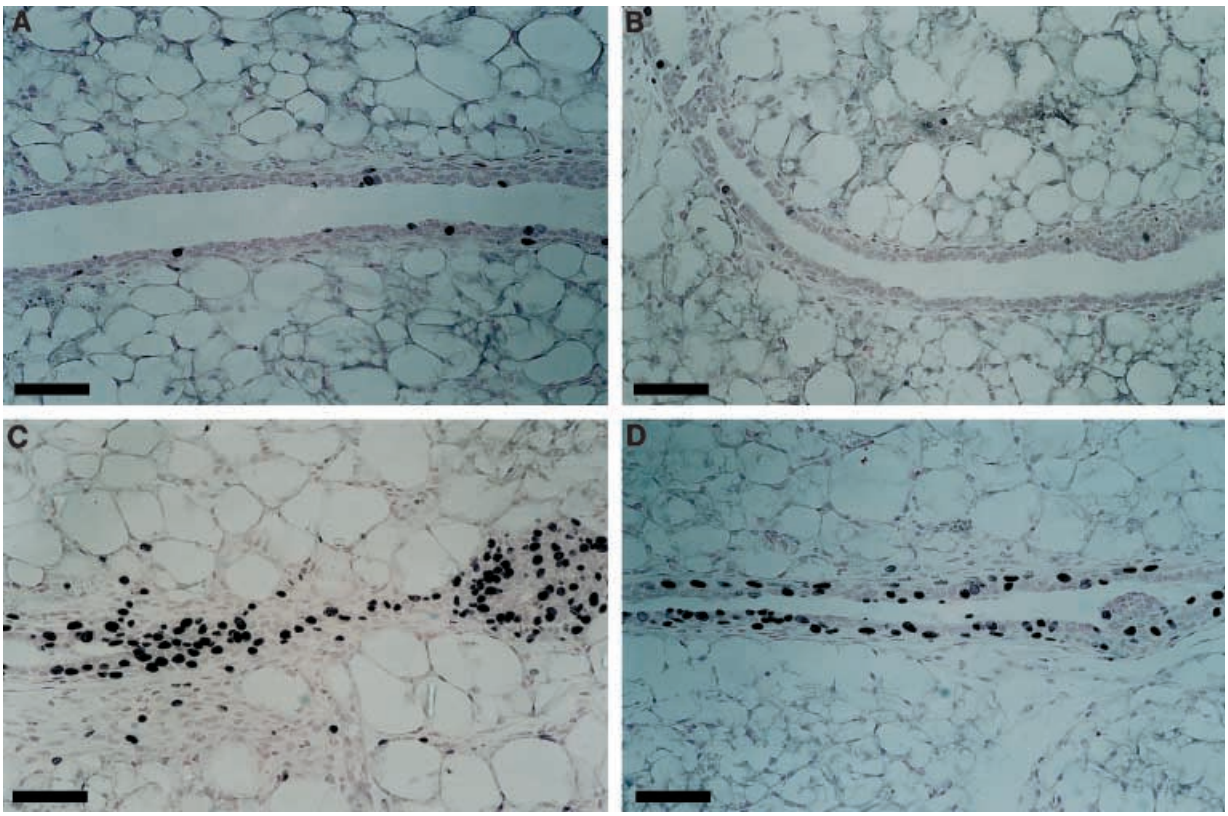

Figure 5 BrdU-labeled epithelial cells in No. 4 mammary glands from female BALB/c mice on day 31 (day 7 of pellet) pelleted with cholesterol (A) and, on day 33 (day 9 of pellet), estradiol (B), progesterone (C) and estradiol and progesterone (D). Mammary glands were removed, formalin-fixed for 2 h, sectioned and stained immunohistochemically using a rat monoclonal anti-BrdU to detect incorporation into cellular DNA. Sections were counterstained with nuclear fast red (scale bar represents 75 μm).

previously (Seagroves *et al.* 2000). No signal was observed with the sense probe (Fig. 9B). In glands from both P- and E/P-treated mice there appeared to be some concentration of signal around ductal branch points (Fig. 9C and D). Although the experiments were not designed to test this directly, no obvious induction of the PR mRNA by E was observed.

In vitro *culture*

The profound effects of E and P on the epithelial growth of the mammary glands *in vivo* prompted us to attempt to replicate these effects in whole-organ culture, using mammary glands from non-primed female mice. In contrast to the mitogenic effects of these hormones *in vivo*, no similar responses in ductal development were recorded *in vitro* (Table 1). There was no significant difference between the outgrowth area, length of ducts or the number of branch points in mammary glands cultured in media for 9 days with E (0·1–1 nM) and/or P (10–100 nM) compared with those of mammary glands prior to culture at 24 to 25 days of age. The addition of a variety of hormones and growth factors along with E and/or P also was without effect (not shown).

Discussion

Ductal development in the normal peripubertal female mouse

Using a novel morphometric analysis technique that has enabled us to quantify ductal length, ductal outgrowth area and the number of ductal side-branches, as well as the number of TEBs, we have investigated the influence of E and P on the development of ductal structures in the mammary glands of peripubertal female BALB/c mice. Our analyses suggest that the first phase of near-isometric growth of the ductal epithelium to 31 days of age was probably independent of E and P, as the concentrations of serum E and P up to 33 days of age are unchanged.

The second phase of mammary gland development was defined by an allometric increase in the size of the ductal tree from 31 to 37 days of age, resulting from a significant increase in cellular proliferation in the TEBs during this time. The allometric increase in ductal development

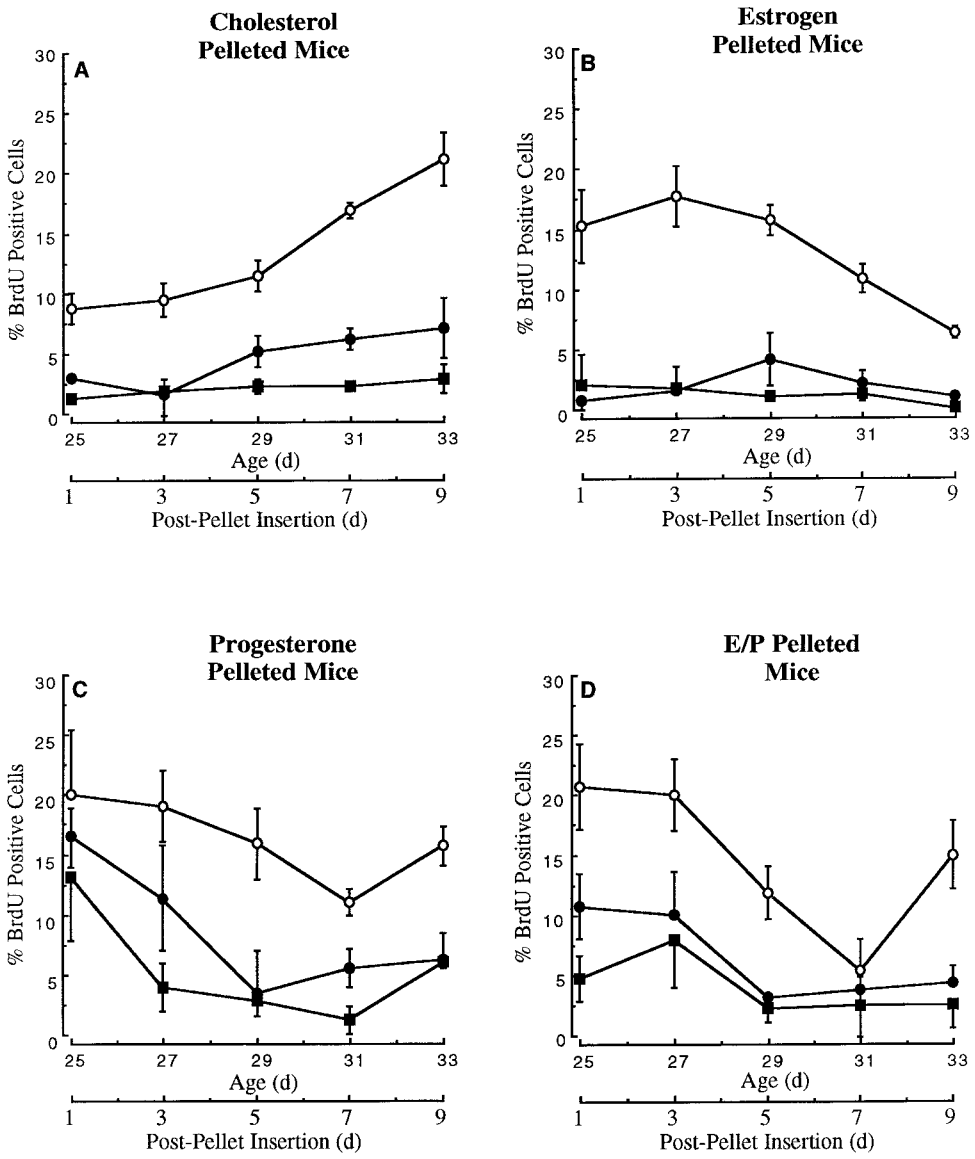

Figure 6 The percentage (± S.D.) of BrdU-labeled epithelial cells in the TEBs (○), secondary/tertiary ducts (●) and primary (■) ducts of mice ($n=3$ per point) pelleted with cholesterol (A), estradiol (B), progesterone (C) or estradiol and progesterone (D) pellets for 0–9 days from 24 days of age. Two hours prior to being killed, each animal received BrdU intraperitoneally (70 µg/g body weight). The No. 4 abdominal glands were then removed, formalin-fixed, sectioned and stained immunohistochemically using a rat monoclonal anti-BrdU to detect BrdU incorporation into cellular DNA. Sections were analyzed using the C-Imaging 1280 image-analysis system.

during this short time-period, together with the symmetrical development of both left and right glands between 31–39 days of age, implicates an endocrine factor, sharply upregulated around 31 days, as responsible for the outgrowth of the primary/secondary ducts through the mammary fat pad. Thereafter, as the outgrowth area approached 90% of the total available area, the rate of growth declined. This slowing of ductal development probably reflects the ductal epithelium reaching the bounds of the mammary fat pad, possibly in conjunction with an increased gradient of growth-inhibiting compounds that determine the spatial arrangement of the ductal epithelium (Silberstein & Daniel 1987, Daniel & Robinson 1992, Silberstein *et al.* 1992). The primary/secondary ductal network was complete by 37 days of age. It should be noted that it is unlikely that the withdrawal of

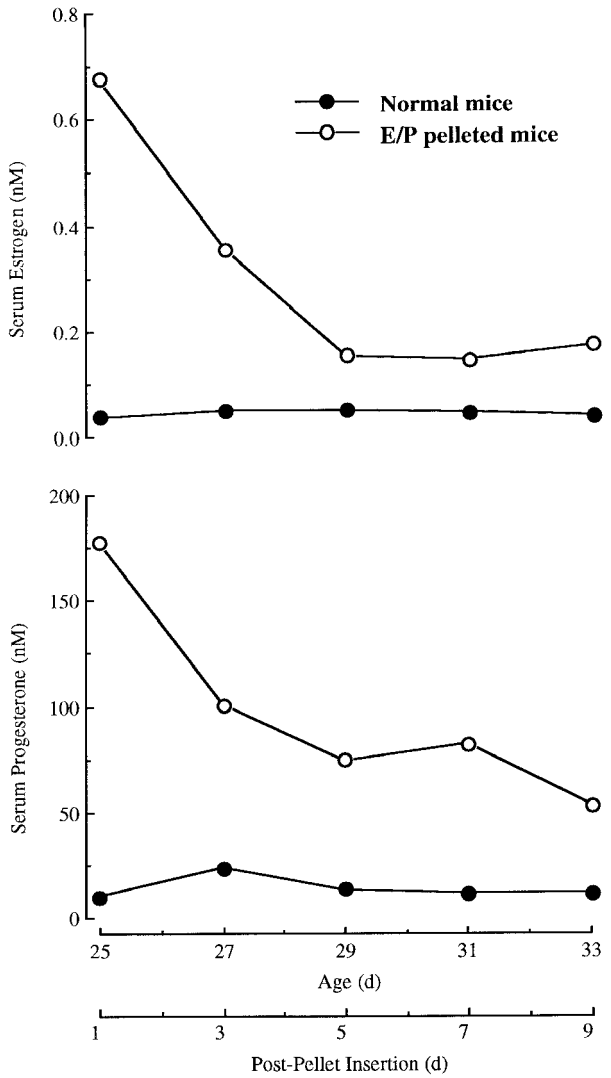

Figure 7 Female BALB/c mice were implanted with a pellet containing either cholesterol (●) or E/P (○) at 24 days of age. Blood was collected from mice at 1, 3, 5, 7 and 9 days post-implantation, after cervical dislocation and exsanguination. Serum was pooled from 10 mice for each time-point and analyzed by radioimmunoassay (Coat-A-Count) for E and P.

an inhibitory substance in the dam's milk is responsible for the initiation of allometric growth as increased ductal growth occurs 10 days post-weaning, and early weaning has no apparent effect on mammary growth (Flux 1954).

During the third phase of mammary gland development, there is a uniform increase in the number of tertiary ducts arising from the primary/secondary ductal network over a 2–3-month period (Vonderhaar 1988, Imagawa *et al.* 1990). Our analyses indicate that there was little tertiary side-branching on the primary/secondary ductal network to 39 days of age. However, as discussed below, mice pelleted with P at 24 days of age showed a dramatic increase in tertiary ductal side-branching from 33 to 39 days of age, indicating that P promotes ductal side-branching. The symmetry of tertiary side-branching between left and right glands in normal and P-pelleted mice during this later phase of development indicates that the development of tertiary side-branching during normal mammary development is probably a result of increased serum P concentration during each estrous cycle. Synergistic action of P with PRL (Imagawa *et al.* 1990) derived from either the pituitary or the mammary glands (Ginsburg & Vonderhaar 1995) was not investigated.

As conception in the mouse can occur as early as the 5th postnatal week, well before complete tertiary branching development of the mammary gland has occurred, the elevated level of serum P as a result of pregnancy would promote an effect similar to that seen in P-pelleted mice, resulting in a rapid increase in the growth of tertiary ducts. Therefore, from a physiological point of view, P may be responsible not only for maintaining pregnancy, but also for priming the mammary gland to readiness, allowing for the completion of the ductal structure upon which the lobuloalveolar structures are to develop as PRL levels increase. Similarly, the absolute requirement for P in priming mammary glands from peripubescent mice for lobuloalveolar growth in whole-organ culture (Plaut *et al.* 1999) may reflect its role in initiating the capacity of ducts to form tertiary branches on which the lobuloalveoli can develop *in vitro*.

Progesterone promotes ductal side-branching

The present data indicate that P promotes ductal side-branching on the primary/secondary ductal network in the mammary glands of intact peripubertal mice. The primary role of P in promoting the dramatic increase in tertiary side-branching of the mammary ducts was confirmed by the high initial level of cellular proliferation in the medial ducts of mice treated with P or E/P compared with mice treated with C or E. Although the serum concentration of P in P-pelleted mice fell following pellet insertion, it is clear that P differentially promotes cellular proliferation leading to ductal side-branching. Conversely, since the serum levels of both P and E are low in normal animals at this time, and as this strain of mouse does not enter estrous until after 33 days of age (as judged by the presence of corpora lutea) it is unlikely that endogenous E and/or P affected ductal outgrowth prior to this time.

Our data are consistent with studies showing that E and P administered to intact (Freeman & Topper 1978) and castrated male (Daane & Lyons 1954, Blair *et al.* 1957) and female (Haslam 1988*a*, *b*) mice results in extensive ductal branching compared with mice given E alone (Daane & Lyons 1954, Freeman & Topper 1978). Our data are also consistent with observations of mice lacking PRs, which have been shown to have severely limited tertiary

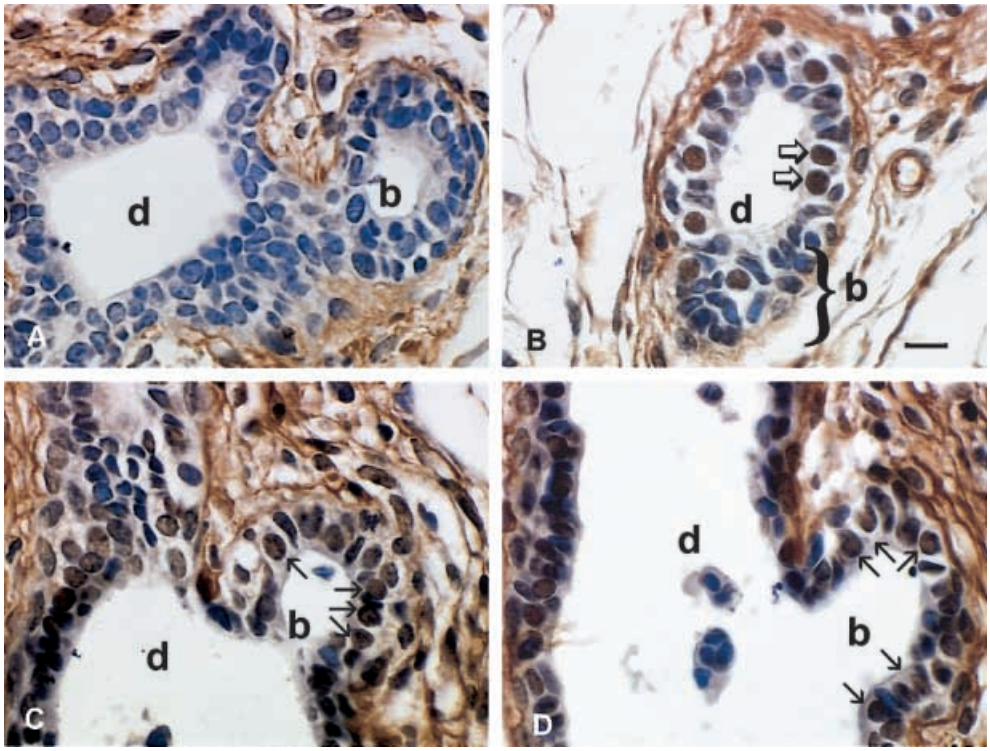

Figure 8 Immunohistochemistry for PRs in No. 4 mammary glands from 37-day-old female BALB/c mice. (A) Cholesterol-treated; stained with control monoclonal antibody B1·1 as the primary antibody. (B–D) Stained with αPR as the primary antibody. (B) Treated with estradiol and progesterone. (C, D) Progesterone-treated: solid arrows indicate clusters of PR-positive nuclei at putative branch points (b); open arrows indicate ULLCs; 'd' indicates the ductal lumen. Scale bar = 10 μm.

ductal side-branching (Lydon *et al.* 1995). Furthermore, the requirement for PRs, in both epithelial and stromal cells, for normal ductal development has been demonstrated by reciprocal transplantation studies with PR knockout mice (Humphreys *et al.* 1997). Progesterone-induced cellular proliferation, as well as multilobular and lobuloductal branching morphogenesis, has been detected in cultured mammary organoids from mice (Hamamoto *et al.* 1987) and rats (Darcy *et al.* 1995). Using a whole-organ-culture approach, we could not stimulate an increase in ductal branching in mammary glands from 33-day-old mice treated with P *in vitro*; this suggests that the effect of P on ductal side-branching in the intact mouse mammary gland requires other synergistic factors. No other hormones or growth factors tested (PRL, FGF, EGF, IGF-I, GH, relaxin, CSF, αTGF, TNF-α, LH, FSH, T_3, T_4 and anti-TGF-β neutralizing antibodies) stimulated epithelial outgrowth *in vitro*. It will be of interest to determine, in future studies, if other ovarian endocrine factors are required for E-induced primary/secondary ductal formation and P-induced ductal side-branching.

Our present data indicate that P does not play a major role in the elongation of the primary/secondary ductal network. This is supported by the observation, in PR knockout mice, that the mammary glands appear to have normal primary/secondary ductal outgrowth (Lydon *et al.* 1995). Although the increase in systemic P induced an immediate and significant doubling of TEB cell proliferation in P-pelleted mice, and a sevenfold increase in ductal cell proliferation above that of C-pelleted mice, the cellular proliferation was not translated into an increase in ductal length until 33 days of age. The fact that ductal length was greater after 33 days in P-pelleted mice than in control mice corresponded to the development of numerous tertiary side-branches. The structural and functional consequences of P-induced cell division within the TEBs remain to be determined.

Our findings suggest that as the mouse matures, P, rather than E, has a major role in promoting epithelial cell proliferation for the formation of ductal side-branches. However, our results contrast with the small amount of DNA synthesis induced in ductal epithelium and TEBs in 5-week ovariectomized mice treated with P (Haslam 1988*b*). The small effect of P on cellular proliferation in ovariectomized mice may be explained by the fact that PR expression is upregulated by E (Haslam & Shyamala 1981). This is supported by the synergistic effect of E and P on ductal DNA synthesis observed in 10-week

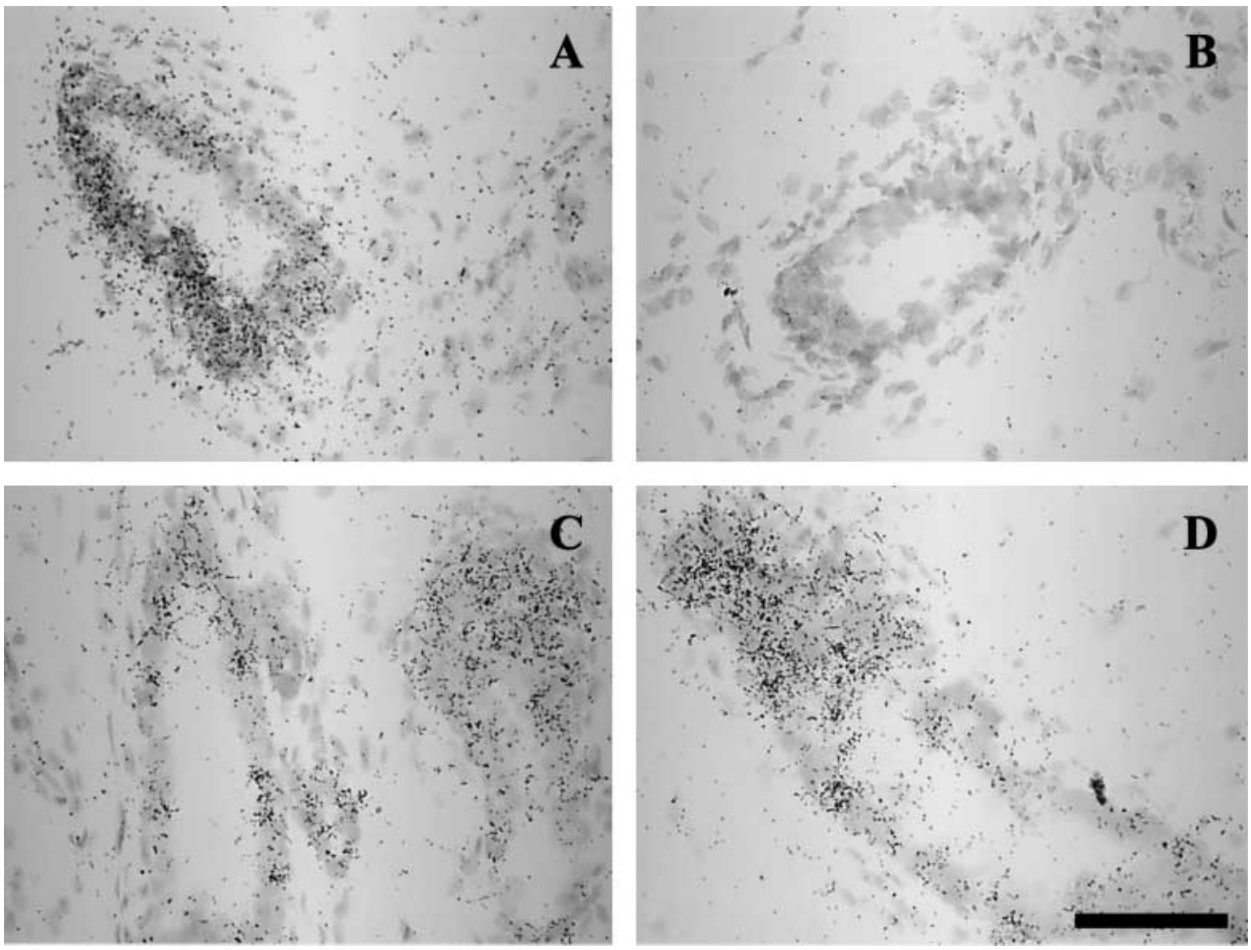

Figure 9 *In situ* hybridization analysis of PR gene expression in mammary glands of BALB/c mice primed for 13 days with cholesterol (A), progesterone (C) or estradiol and progesterone (D). (B) Sense control for (A). The nuclear fast red counterstain was used. Scale bar = 50 μm.

ovariectomized mice (Haslam 1988*a*, *b*). The mitogenic effect of P on both ducts and TEBs at 24 days of age in the present study implies the presence of epithelial cells containing active PRs and may reflect the emerging secretion of a low level of E by the ovaries. Indeed, expression of PR protein and mRNA was localized in clusters of epithelial cells that histologically resemble branch points. Our recent results (Seagroves *et al.* 2000) indicate that the spatial distribution of PR changes in the mammary gland during the course of morphogenesis, suggesting that ductal side-branching is initiated by a subset of cells. Silberstein *et al.* (1996) also noted high levels of PR mRNA and protein in some epithelial cells of actively growing end-buds, ductal branches and mature ducts of prepubertal mice.

The role of E in the elongation of the epithelium

The acknowledged mammogenic effect of E was indicated by a significant increase in TEB cell proliferation to 27 days (3 days post-pelleting) in E-pelleted mice relative to controls. This systemic effect of E on ductal elongation is in accordance with studies showing that E administered to prepubertal intact or ovariectomized female mice (Flux 1954, Munford 1957, Traurig & Morgan 1964) and intact (Freeman & Topper 1978) or castrated (Daane & Lyons 1954) male mice results in increased cell proliferation and ductal growth. The significant decrease in TEB cell proliferation to control levels by 31 days (7 days post-pelleting) paralleled the decrease in serum E, consistent with the fact that serum E largely stimulates ductal growth. These results support the idea that E acting locally is required for end-bud development (Daniel *et al.* 1987, Haslam 1988*a*, Silberstein *et al.* 1994). Implants of estradiol placed directly into the mammary glands of young ovariectomized mice stimulated end-bud development only in the vicinity of the implants (Haslam 1988*a*). These effects of E on the TEBs are mediated through estrogen receptors (ERs) located in the stromal compartment (Cunha *et al.* 1997). The lower rate of proliferation in the

mammary glands from E-treated mice by 33 days of age probably signaled the completion of the primary/secondary ductal network and the end of phase 2 growth.

In contrast to the TEB scenario, the lower rate of cell proliferation in the primary and medial ducts indicates a disparity in the mitogenic action of E that may reflect the differentiative state of the epithelial cells. ERs are found in more differentiated cells (luminal cells of the end-bud, ductal epithelium and stroma adjacent to the ducts), but not in the rapidly proliferating cap cells of the TEBs (Daniel *et al.* 1987). These observations imply a role for E in the maintenance of ductal structure (Silberstein *et al.* 1994) and suggest that the mitogenic action of E on cap cells is conveyed by another intermediate factor. Indeed, no change in the concentration of serum E in control mice was observed between 31 and 33 days, when mammary epithelial growth was maximal, although this does not rule out local action by E on the epithelial cells and, thus, its importance in ductal growth. Silberstein *et al.* (1994) have shown from localized ER blockade studies that E acts directly on the mammary gland and not primarily through the stimulation of pituitary mammogens during ductal mammogenesis. We did not observe an increase in serum PRL during the allometric phase of ductal growth (B Terry-Koroma and B K Vonderhaar, unpublished observations). Growth hormone levels were not tested. Interestingly, mammary glands of 33-day-old nulliparous mice placed into whole-organ culture did not respond to E or P alone or in combination at low or high concentrations with respect to ductal outgrowth and/or branching. Given that these glands are composed of epithelium within a stromal framework (required for E responsiveness *in vivo*), and that E has been shown to upregulate PR in mixed epithelial/fibroblast cultures (Haslam & Levely 1985), it is tenable that another growth factor, sharply upregulated at around 31 days, is also involved in stimulating the development of the primary/secondary ductal network. This is further supported by our finding that mice treated with both E and P for 9 days did not show increased ductal length, outgrowth area or number of branch points compared with P-treated animals at the same time. It remains to be determined as to whether this factor(s) mediates its effects through epithelial cells or through the surrounding stroma.

In conclusion, our results indicate that the mammotrophic functions of E and P are distinctive. Progesterone plays a definitive role in the formation of ductal side-branches on the already-formed primary/secondary ductal network, whereas E probably regulates the expression and location of PRs and promotes the development of the primary/secondary ductal network. Although E and P are clearly involved in stimulating the proliferation of epithelial cells in the TEBs, other (as yet unidentified) factors may also be required for both primary/secondary ductal formation and tertiary side-branching between 31 and 37 days of age in the mouse mammary gland.

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