The Breast as a Developing Organ

2.1 Introduction

The breast is a bilateral organ that in the female undergoes dramatic changes in size, shape, and function in association with infantile growth, puberty, pregnancy, lactation, and post-menopausal regression [1–4]. The fact that the breast is the source of the most frequently diagnosed malignancy in the female population [5, 6] requires to fully understand how the various phases of development are influenced by endocrinological and reproductive events, because they will ultimately determine the risk of developing breast cancer [3, 4, 7].

The development of the human breast is a lifelong process initiated during embryonic life. While the main growth spurt occurs with lobule formation at puberty, the development and differentiation of the breast are completed only by the end of the first full-term pregnancy [3]. It has long been known that the risk of breast cancer shows an inverse relationship with early parity [3, 4, 7–12]. Case control studies have demonstrated that breast cancer risk increases with the age at which a woman bears her first child. The important factor in this protection seems to be related to the interval of time between menarche and the first pregnancy, since increased risk has been reported when this interval is lengthened over 14 years [12]. In order to be protective, however, pregnancy has to occur before age 30. In fact, evidence indicates that women who first become pregnant after that age appear to have a risk above that of nulliparous women [12]. Although multiparity appears to confer additional protection, the protective effect remains largely limited to the first birth. The protection conveyed by an early reproductive event persists at all subsequent ages, even in women older than 75 years of age [7, 12]. Although the ultimate mechanisms through which an early first full-term pregnancy protects the breast from cancer development are not known, a likely explanation has been provided by studies performed in experimental animal models: that completion of full-term pregnancy prior to the administration of a chemical carcinogen inhibits rat mammary cancer initiation through the induction of differentiation. The fact that mammary gland differentiation activates specific genes such as inhibin [13], mammary derived growth factor inhibitor [14], a serpin-like gene [15] and others whose function remains to be determined [15, 16], led us to postulate that these genomic changes prime the breast epithelium to subsequent hormonal milieu, and that this is also responsible for the protection that an early full-term pregnancy confers to women. Currently there is no explanation for the higher risk to develop malignancies exhibited by nulliparous and late parous women. The fact that experimentally induced rat mammary carcinomas develop only when the carcinogen interacts with the undifferentiated and highly proliferating mammary epithelium of young nulliparous rats [3, 4, 17–21], suggests that the breast of late parous and of nulliparous women might exhibit some of the undifferentiated and/or cell proliferative characteristics that predispose the tissue to undergo neoplastic transformation [3, 19–22]
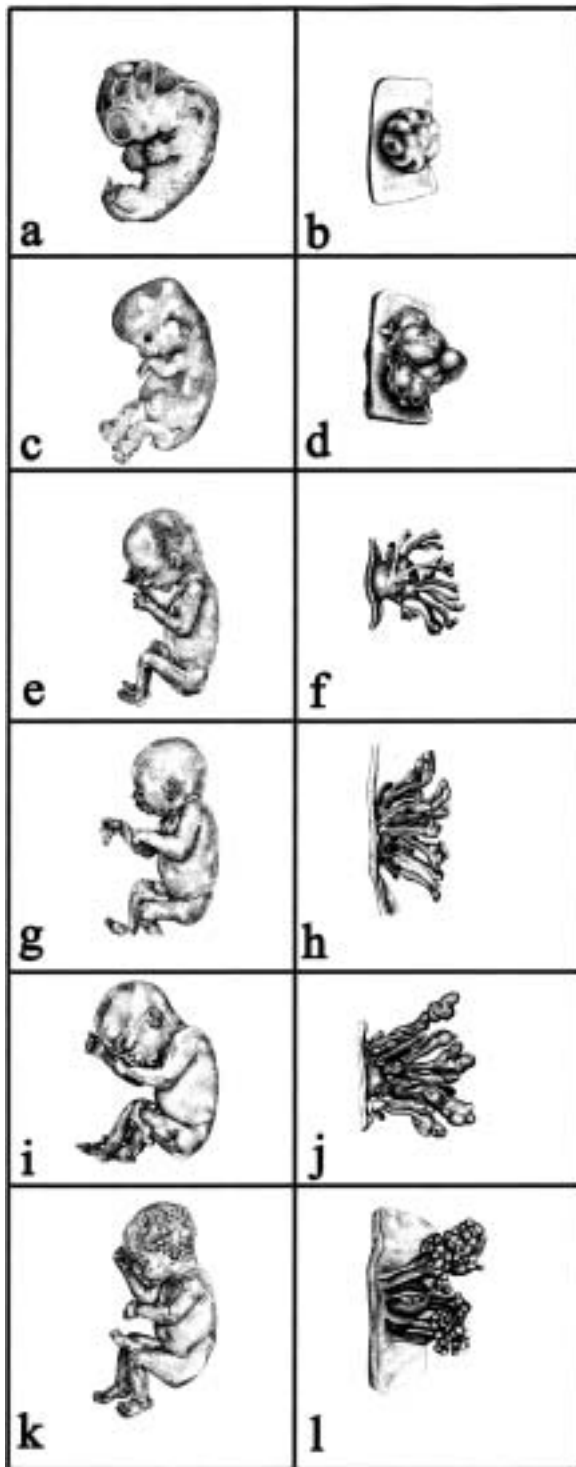
2.2 Prenatal and Perinatal Development

The mammary gland parenchyma arises from a single epithelial ectodermal bud. Most authors agree on the successive stages of development of the mammary gland during the embryonic and fetal stages. However, there are variations in nomenclature, and in the exact time of appearance of each structure, depending upon whether the authors choose to express the age of the embryo based on the estimated time of conception, the last missed menstrual period, or the length of the embryo. Because of difficulties in precisely establishing the day of conception, we consider that embryonic or fetal length is more accurate for assessing the intrauterine phases of mammary gland development. These phases have been divided into the following stages: Ridge, milk hill, mammary disc, G lobule type, cone, budding, indentation, branching, canalization, and end-vesicle stages (Fig. 2.1, Table 2.1). It is important to emphasize that the last stage or end vesicle stage (Fig. 2.1), which characterized by the production of colostrums, does not represent a fully differentiated stage, because it lacks of fully developed lobules, which only become apparent in the postnatal life at the end of pregnancy.

In the newborn the breast consists of very primitive structures (Fig. 2.2 a–e), composed of ducts ending in short ductules lined by one to two layers of epithelial and one of myoepithelial cells (Fig. 2.2 d, e). Epithelial cells have a finely vacuolated eosinophilic cytoplasm, with typical apocrine secretion. The fine vacuolization is caused by the presence of lipid droplets (Fig. 2.2 e), as confirmed by electron microscopy. However, secretory activity does not seem to be confined to the primitive alveolar structures, since the entire ductal system appears dilated, secretion filled, and lined by a secretory-type epithelium (Fig. 2.2 f–h). These observations suggest that secretory activity is a generalized response of all the mammary epithelium to maternal hormonal levels. The secretory activity of the newborn gland subsides after 3 to 4 weeks of birth [3, 23].

Table 2.1. Stages of prenatal development of the human breast. CR crown rump

Stage	Mammary gland developmental stage	Developmental period/ gestational age		CR length
1	Ridge stage	Embryonic period	Week 4 (day 27–28)	4–5 mm
2	Milk hill stage		Week 4–5 (day 28–30)	6–7 mm
3	Mammary disc stage		Week 5 (day 29–35)	5–12 mm
4	Globule stage		Week 6 (day 36–42)	14–22 mm
5	Cone stage		Week 7 (day 43–49)	28–30 mm
6	Budding stage		Week 8 (day 50–56)	31–42 mm
7	Indentation stage	Fetal period	Week 10–13 (day 64–91)	60–98 mm
8	Branching stage		Week 14 (day 92–98)	105–120 mm
9	Canalization stage		Week 20–32 (day 134–224)	185–300 mm
10	End-vesicle stage – ductal end vesicles lined by a monolayered epithelium contain colostrum	Newborn	Week 40 (day 274–280)	>360 mm
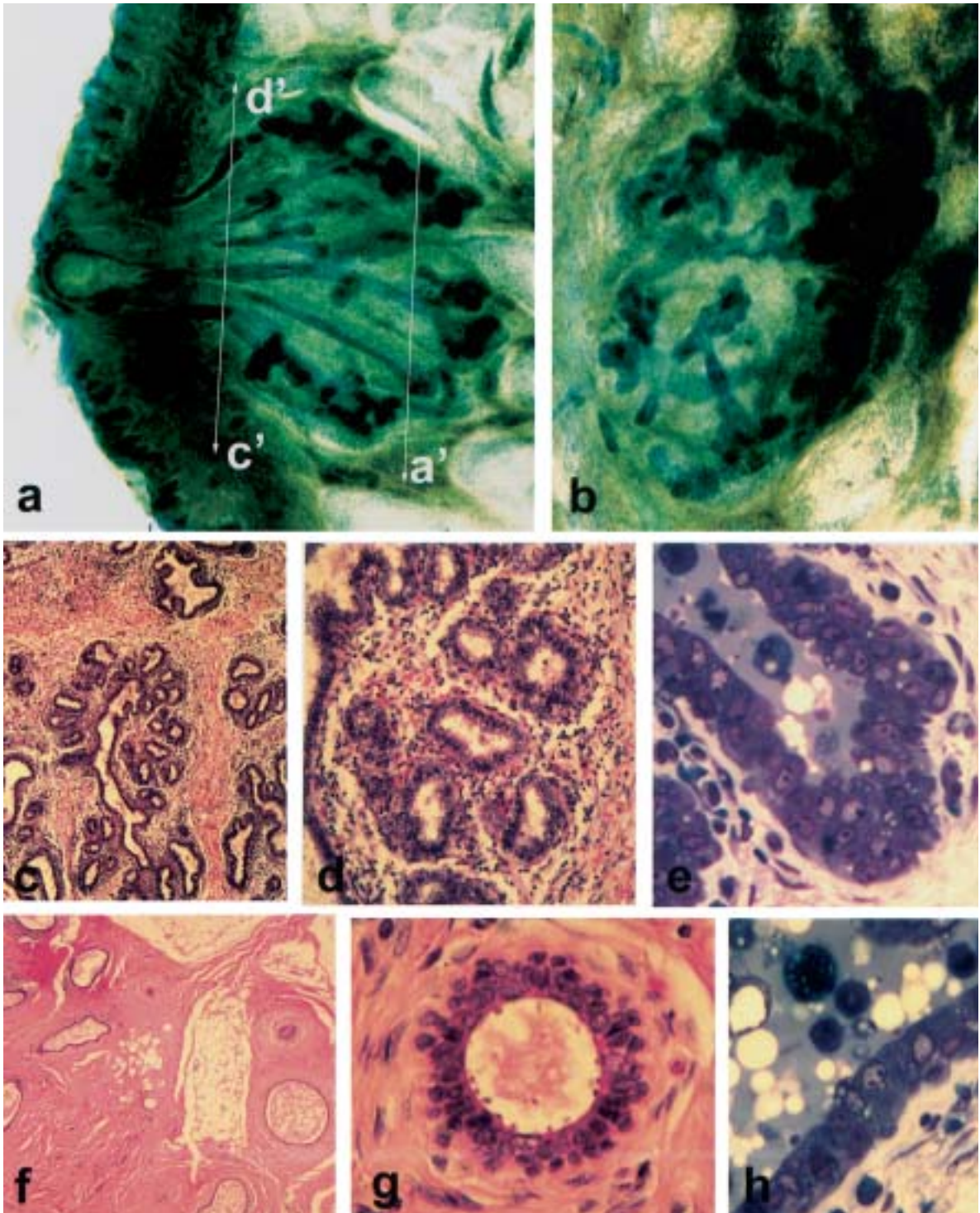
◄ Figure 2.2 a–h

a Mammary gland of a 2-week-old girl is composed of ductal structures that from the galactophorous sinus under the nipple branch and end in primitive alveolar bud (AB). b Cross-section taken at the *a'–b'* level shown in a (a and b, whole mount stained with toluidine blue, × 2.5). c Cross section taken from a at the *a'–b'* level. The lumen of all ducts and AB is dilated and filled with proteinaceous fluid (hematoxylin and eosin [H & E], × 4.0). d The ducts are lined by two layers of epithelial cells. Proliferation takes place chiefly in the basal cells. The inner or luminal cells have secretory properties from which the 'witches milk' is formed (H & E, × 10. e Terminal duct in the breast of a 2-week-old girl. The lumen contains proteinaceous and lipidic secretion. (One-micron section of plastic embedded tissue; toluidine blue, × 40). f Cross section of major lactiferous ducts taken at level *c'–d'* of a (H & E, × 2.5). g The cross section of a duct reveals a luminal layer of epithelial cells projecting snouts into the protein-containing lumen (H & E, × 40). h A thin section of plastic embedded breast tissue shows a pseudostratified epithelium lining the secretion filled lumen (toluidine blue, × 40)

2.3 Postnatal Development

Mammary gland development during childhood does little more than keep pace with the general growth of the body until the approach of puberty. Although the main changes occurring in the mammary gland are initiated at puberty, ulterior development of the gland varies greatly from woman to woman. Mammary gland development can be assessed from the external appearance of the breast [1] or by evaluation of mammary gland area, volume, degree of branching, or degree of structures whose appearance indicates the level of differentiation of the gland, such as the formation of lobules in various stages of development [2, 3, 21].

The adolescent period begins with the first signs of sexual change at puberty and terminates with sexual maturity [1, 2]. Puberty in the female sets in between the ages of 10 and 12 years. With the approach of puberty, the rudimentary *mammae* begin to show growth activity both in the glandular tissue and in the s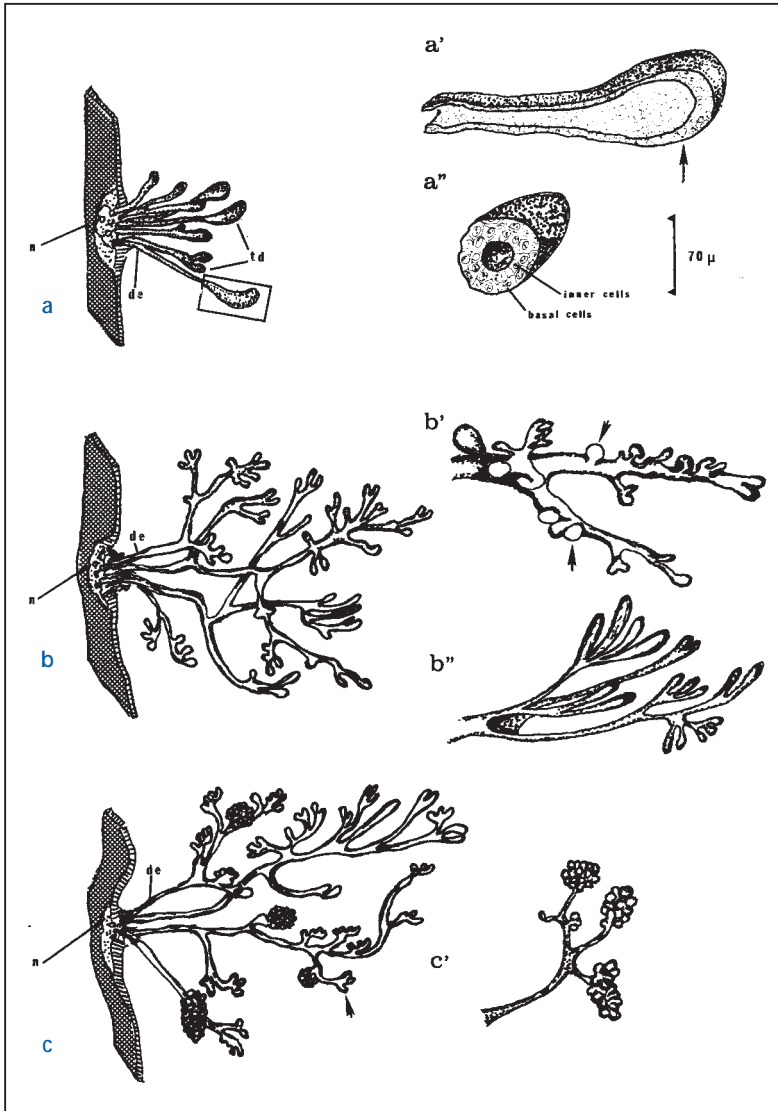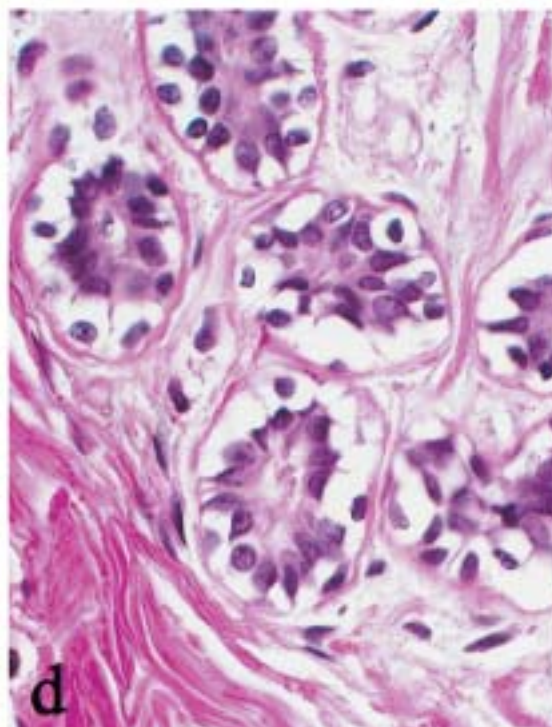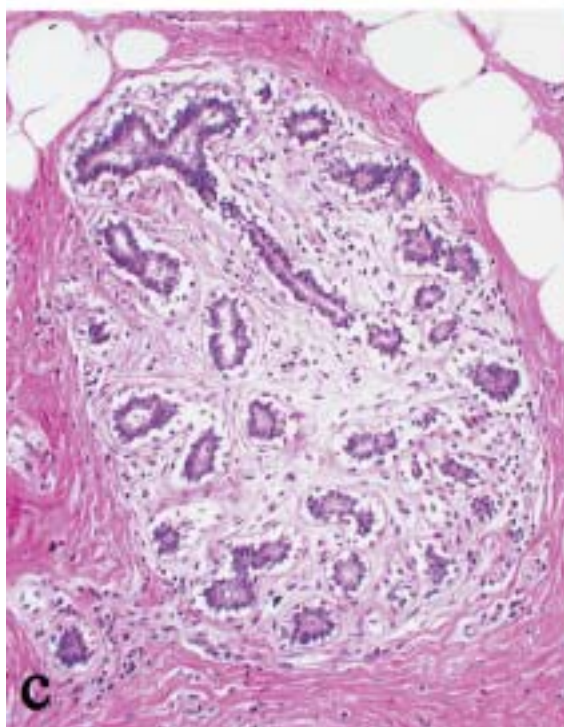urrounding st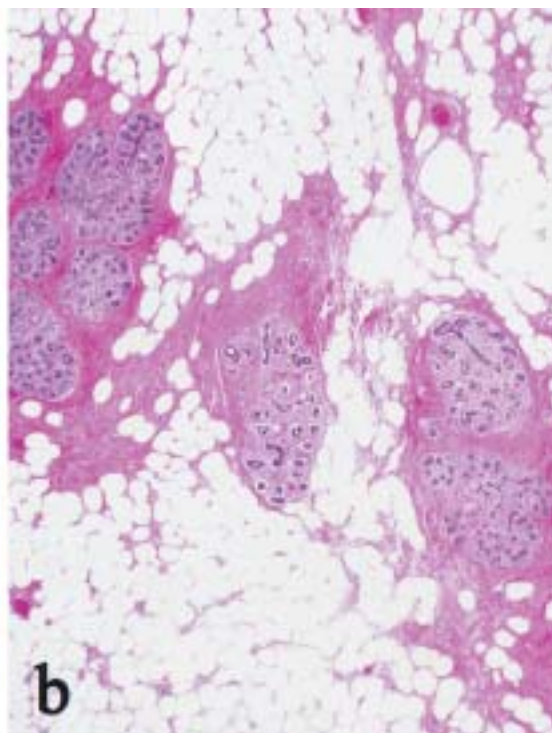roma. Glandular increase is due to the growth and division of small bundles of primary and secondary ducts (Fig. 2.3). They grow and divide partly dichotomously (from the Greek word *dichotomos*, or repeated bifurcation) and partly sympodially (from Greek *syn + podion* base, involving the formation of an apparent main axis from successive secondary axes), on a dichotomous basis. The ducts grow and divide, ending in club-shaped bulbous structures, the terminal end buds (TEBs). Each TEB cleaves into two smaller structures or alveolar buds (AB) (Fig. 2.3 c). We have coined the term AB for identifying that transitional structure that morphologically appears more developed than the TEB and will either progress to form new branches or will further sprout into ductules, which are smaller terminal structures that cluster around a terminal duct. The structural unit composed by the terminal duct and the 4 to 11 ductules that sprouted from it represent the first identifiable lobule, the lobule type 1 (Lob 1), or terminal ductal lobular unit (TDLU), or virginal lobule (Fig. 2.4). Lobule formation in the female breast, which is the hallmark of differentiation, usually starts 1 to 2 years after the first menstrual period. Ovarian hormones play a significant role in breast development. However, the effect of estrogen and progesterone fluctuations during the menstrual cycle on parenchymal cell proliferation has not been definitively elucidated. The normal breast epithelium undergoes cyclic variations in DNA synthesis, as determined in normal breast samples cultured in the presence of ^{3}H-thymidine. Even though cell proliferation and cell death seem balanced to maintain the equilibrium of the resting breast, mammary development induced by ovarian hormones during a menstrual cycle never fully returns to the starting point of the preceding cycle. Accordingly, each ovulatory cycle fosters slightly more mammary development with new budding of structures that continues until about age 35 [3].

The breast tissue of normally cycling non-pregnant adult women contains three identifiable types of lobules, the already described Lob 1 and the more developed lobules type 2 (Lob 2) (Fig. 2.5) and type 3 (Lob 3) (Fig. 2.6). The gradual sprouting of new ductules determines the transition from Lob 1 to Lob 2 when their number reaches an average of 47 per lob-

a Breast tissue of an 18-year-old nulliparous woman composed of lobules type 1 (Lob 1). (Whole mount, toluidine blue, ×2.5). **b**, **c**, **d** Histological sections of the Lob 1 shown in **a** stained with H & E. Photographs taken at ×2.5, ×10 and ×40, respectively
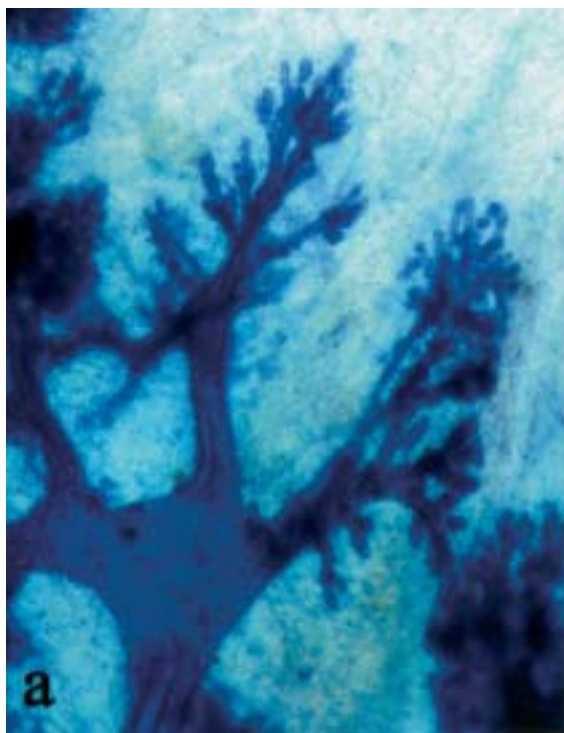
Figure 2.3 a–c

a Mammary gland at birth formed by several excretory ducts (*de*) ending in terminal ducts (*td*). **a'** detail of the inset showing the club-shaped terminal end buds from which lengthening and further divisions of the virginal duct originate; **a"** cross section at the level shown in a'; the duct is lined by the two layers of cells. Proliferation takes place chiefly in the basal cells, whereas the inner (luminal) cells have secretory properties from which the 'witches milk' is formed (*n* nipple). **b** Before the onset of puberty, the ducts grow and divide in a dichotomous or sympodial manner. **b'** Ball-shaped lateral buds sprout from the duct; **b"** new branches and twigs develop from the terminal and lateral buds. **c** Mammary gland at puberty; lobule formation occurs after menarche. The number of lobules progressively increases with age. Some portions of the gland remain as undifferentiated terminal ducts or alveolar buds and do not undergo further development if pregnancy does not supervene (*arrow*); **c'** virginal lobule or lobule type 1 (Lob 1). (Reprinted with permission from: Russo J. et al. Breast Cancer Res. Treat. 2:2, 1982)

Table 2.2. Characteristics of the lobular structures of the human breast. *Lob 1* lobule type 1, *Lob 2* lobule type 2, *Lob 3* lobule type 3

Structure	Lobular area* (μm^2)	Number of ductules/lobules**	Number of cells/cross section***
Lob 1	48 ± 44	11.2 ± 6.3	32.4 ± 14.1
Lob 2	60 ± 26	47.0 ± 11.7	13.1 ± 4.8
Lob 3	129 ± 49	81.0 ± 16.6	11.0 ± 2.0

  * Student's *t*-tests were done for all possible comparisons. Lobular areas showed significant differences between Lob 1 vs. Lob 3 and Lob 2 vs. Lob 3 ($p < 0.005$)

  ** The number of ductules per lobule was different ($p < 0.0l$) in all the comparisons

 *** The number of cells per cross section was significantly different in ductules of Lob 1 vs. Lob 2 and Lob 3 ($p < 0.0l$)

ule, and to Lob 3 when a minimum of 80 ductules are identified around the terminal duct (Table 2.2). Although with progressive branching individual ductules become smaller, the increase in their number results in a concomitant increase in overall size of the more developed lobules (Figs. 2.4–2.6). In Lob 1 each ductule is composed of approximately 32 epithelial cells per cross section and measures an average of $0.232 \times 10^{-2} mm^2$, practically twice the size of the ductules composing the Lob 2. Although individual ductules are further reduced in size in Lob 3, this reduction is less dramatic, although still significant (Table 2.2).

The lobular composition of the breast of sexually mature women is determined by numerous endogenous and exogenous factors. Principal among them are age, and hence, number and regularity of menstrual cycles, endocrine imbalances, use of exogenous hormones, environmental exposures that could act as endocrine disruptors, and pregnancy. In nulliparous women the breast contains a moderate number of undifferentiated structures such as terminal ducts and Lob 1 (Fig. 2.7), although occasional Lob 2 and Lob 3 are also present. Lob 3The percentage of Lob 1 remains almost constant throughout the lifespan of nulliparous women. The facts that Lob 2 are present in moderate numbers during the early reproductive years, and sharply decrease after age 23, while the number of Lob 1 remains significantly higher (Fig. 2.7), and Lob 3 are almost totally absent, suggest that a certain percentage of Lob 1 might have progressed to Lob 2, but very few or no Lob 2 have pro-

Figure 2.5 a–d ▶

a Breast tissue of a 24-year-old nulliparous woman composed of lobules type 2 (Lob 2). (Whole mount preparation, toluidine blue, ×2.5). **b**, **c**, **d** Histological sections of the Lob 2 shown in **a** stained with H & E, and photographed at ×2.5, ×10, and ×40, respectively

gressed to Lob 3. In parous women, on the other hand, a history of one or more full-term pregnancies between the ages of 14 to 20 years correlates with a significant increase in the number of Lob 3. This type of lobules remains present as the predominant structure until a woman reaches the age of 40 (Fig. 2.8). Their percentage decreases after the fourth decade of life, the time at which a decrease in the number of Lob 3 occurs, probably due to their involution to predominantly Lob 1 (Fig. 2.8) [3].
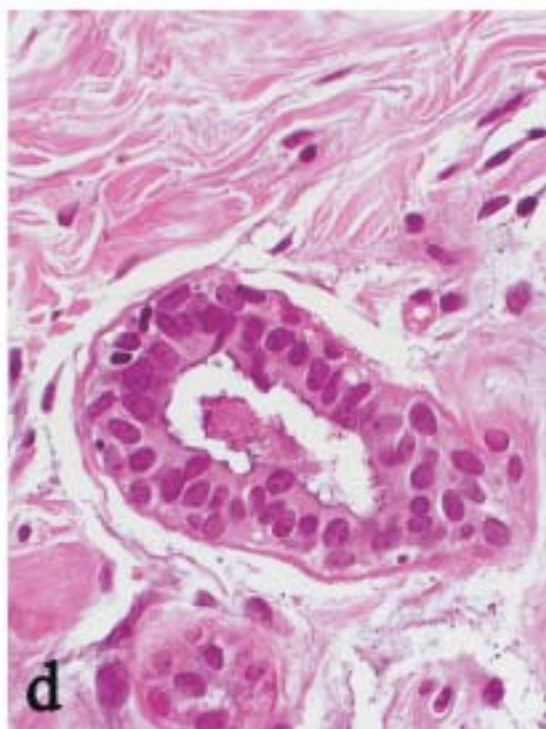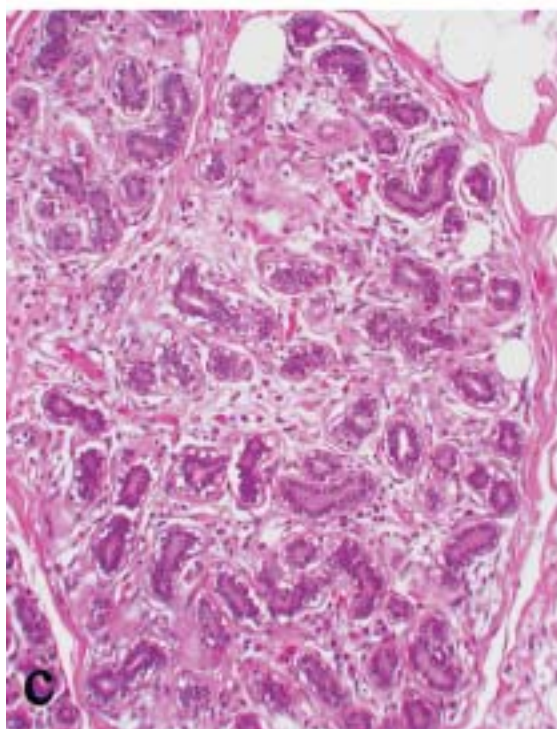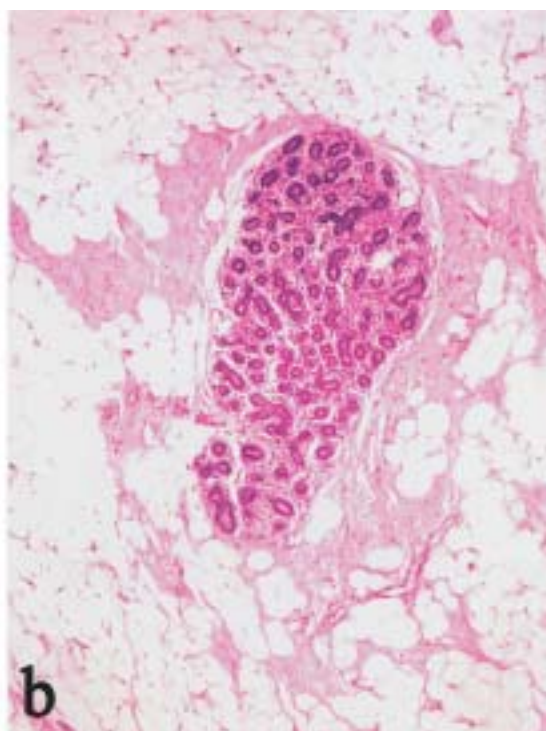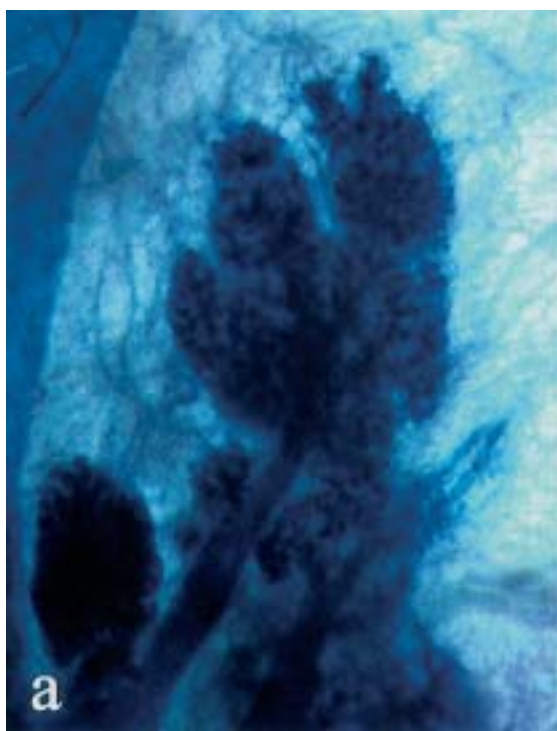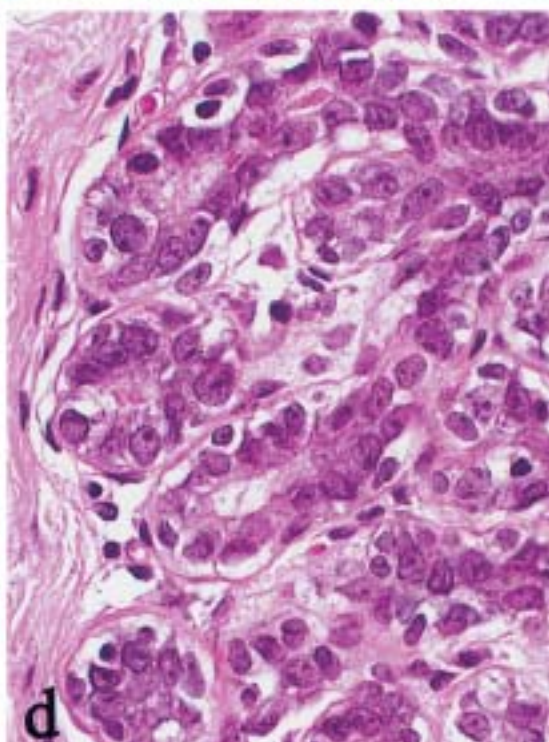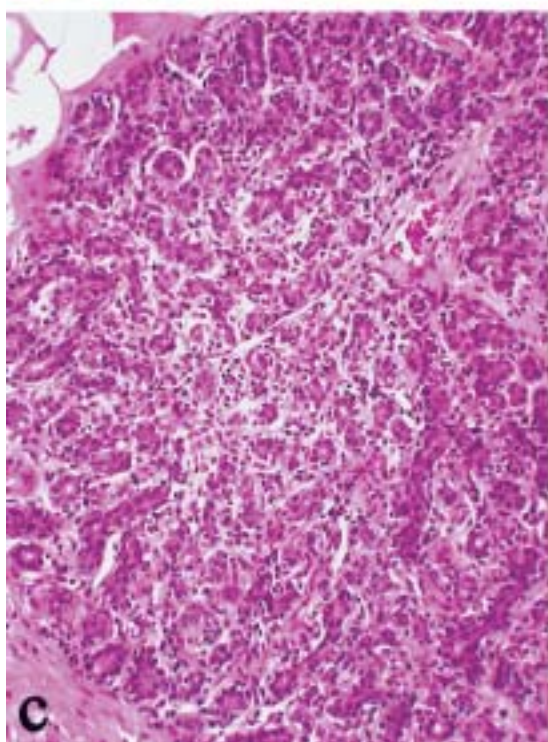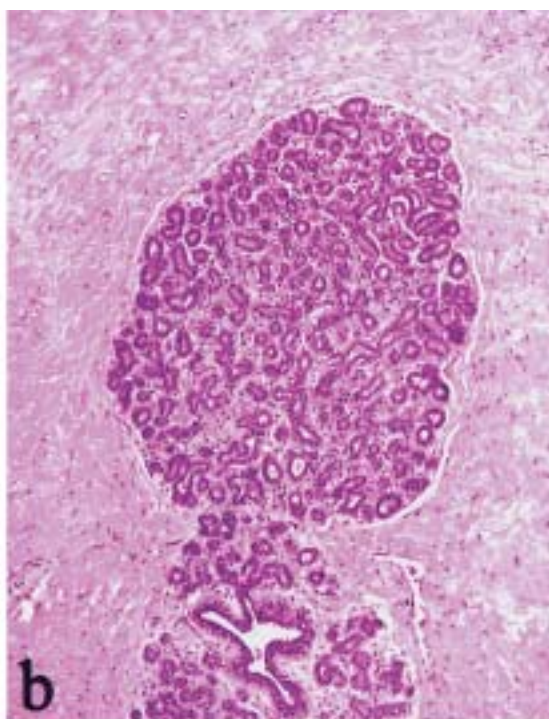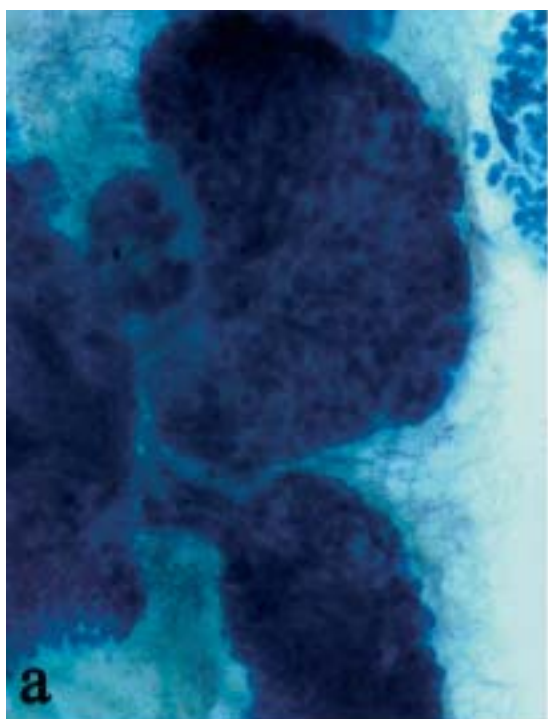
Figure 2.7

Percentage of terminal ductal structures (*TS*), lobules type 1 (Lob 1), 2 (Lob 2) and 3 (Lob 3) in the breasts of nulliparous women between the ages of 14–58 years. Values represent the mean ± SE of 3 samples (14–18); 4 samples (19–23); 1 sample (24–28); 2 samples (34, 38), and 1 sample (54–58). No samples were available for the groups 29–33, 39–43, 44–48, and 49–53. The curves were therefore traced connecting the points available. (Reprinted with permission from: Russo J. et al, Breast Cancer Res. Treat. 23:211–218, 1992)
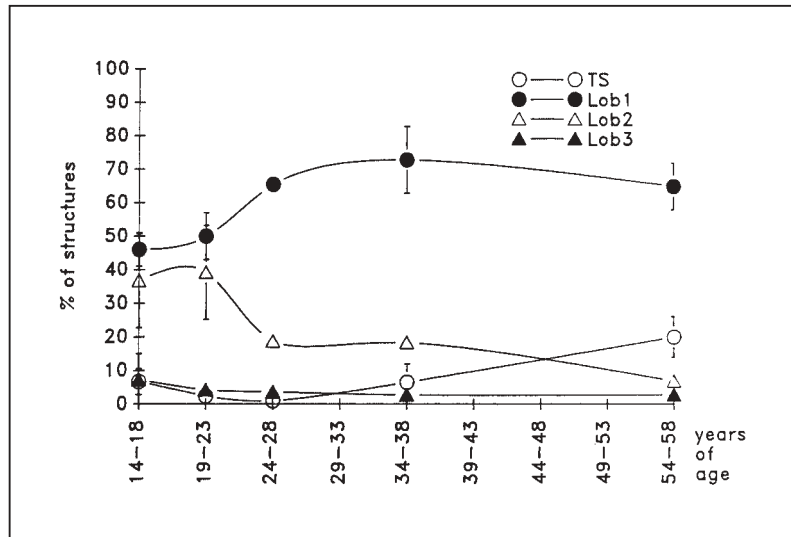
Figure 2.8

Percentage of TS. Lobules type I (Lob 1), 2 (Lob 2) and 3 (Lob 3) in the breasts of parous women between the ages of 14–58 years. Values represent the mean ± SE of 1 sample (14–18); 5 samples (19–23); 6 samples (24–28); 6 samples (29–33); 5 samples (34–38); 7 samples (44–48); 2 samples 49–53); and 3 samples (54–58). (Reprinted with permission from: Russo J. et al, Breast Cancer Res. Treat. 23:211–218, 1992)
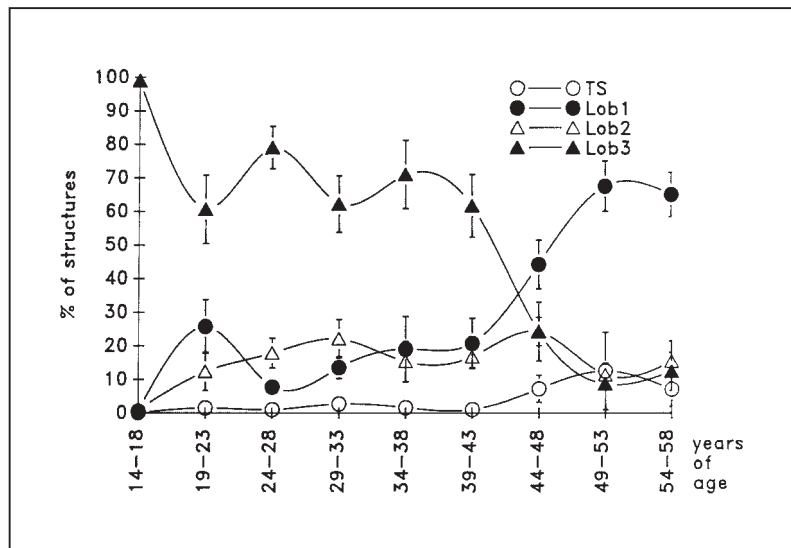
◀ Figure 2.6 a–d

a Breast tissue of a 35-year-old parous woman composed of lobules type 3 (Lob 3). (Whole mount preparation, toluidine blue, × 2.5). b, c, d Histological sections of the Lob 3, shown in a stained with H & E and photographed at × 2.5, × 10, and × 40, respectively
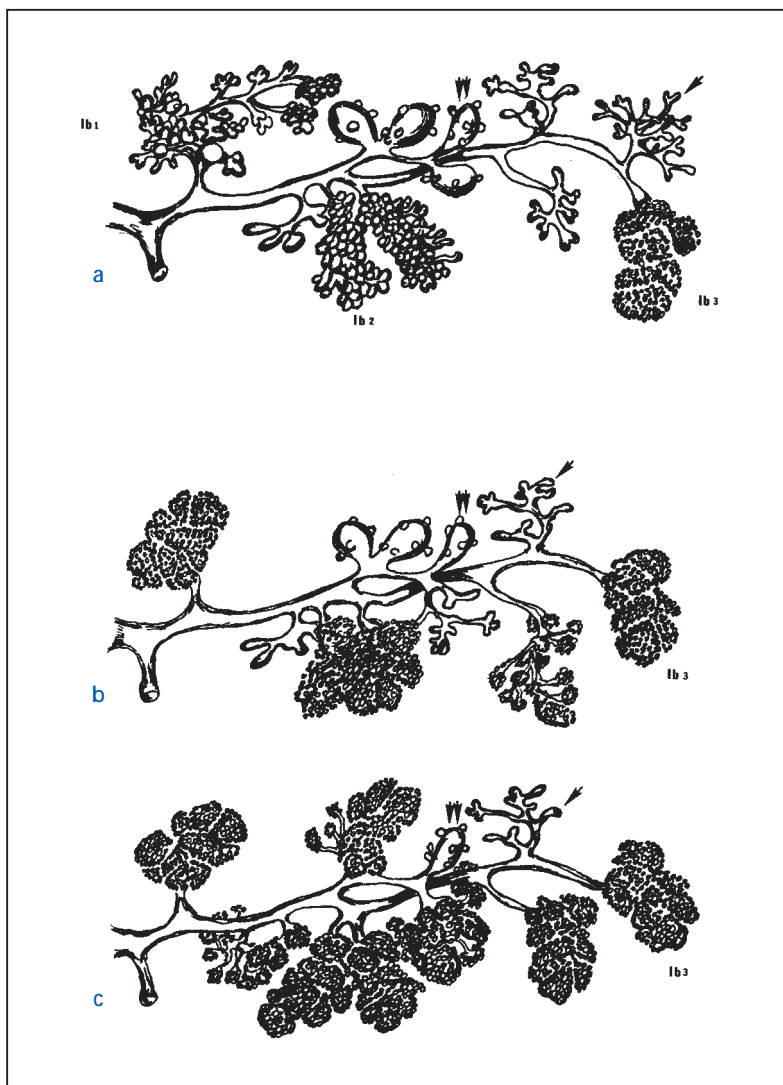
2.4 Pregnancy

The breast attains its maximum development during pregnancy; it occurs in two distinctly dominant phases (Fig. 2.9): an early stage, characterized by ductal lengthening and profuse branching, sustained by active cell proliferation at the distal end of the ductal tree; the rapid increase in number of newly formed ductules results in the progression of Lob 2 to Lob 3 (Fig. 2.6). The intensity of budding and degree of lobule formation goes beyond what has been observed in the virginal breast. By the third month of pregnancy the number of well-formed lobules exceeds the number of primitive budding stages; however, TEBs are still found (Fig. 2.9c). The beginning of secretory activity is indicative of the progression from ductules to secretory acini, which are characteristics of the fully differentiated Lob 4 (Fig. 2.10). In newly formed lobules, the epithelial cells composing each acinus in-

a Lactating breast of a 25-year-old woman showing lobules type 4 (Lob 4). (Whole mount preparation, toluidine blue, × 2.5). b, c, d Histological sections of the Lob 4 shown in a stained with H & E and photographed at × 2.5, × 10, and × 40, respectively
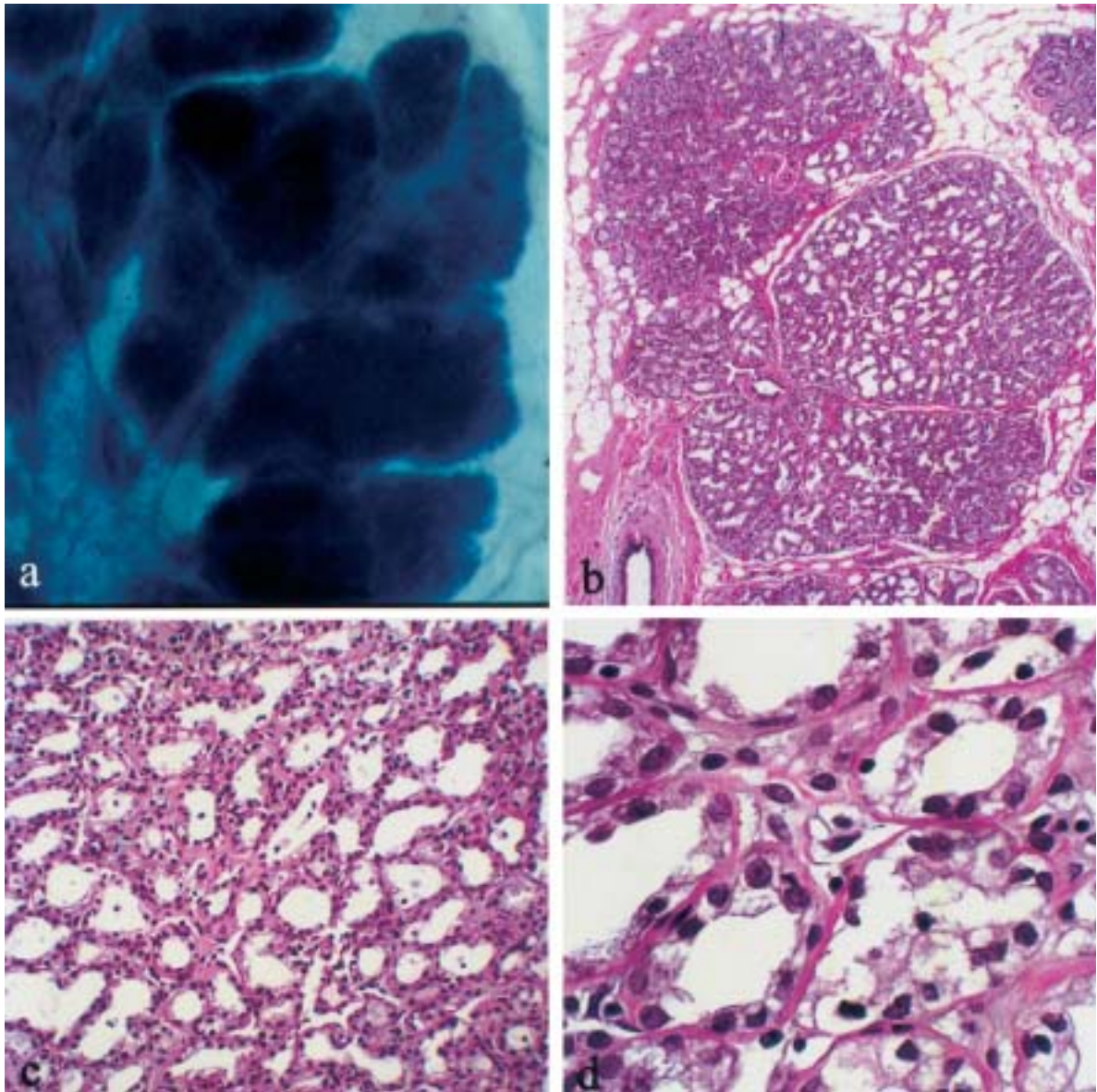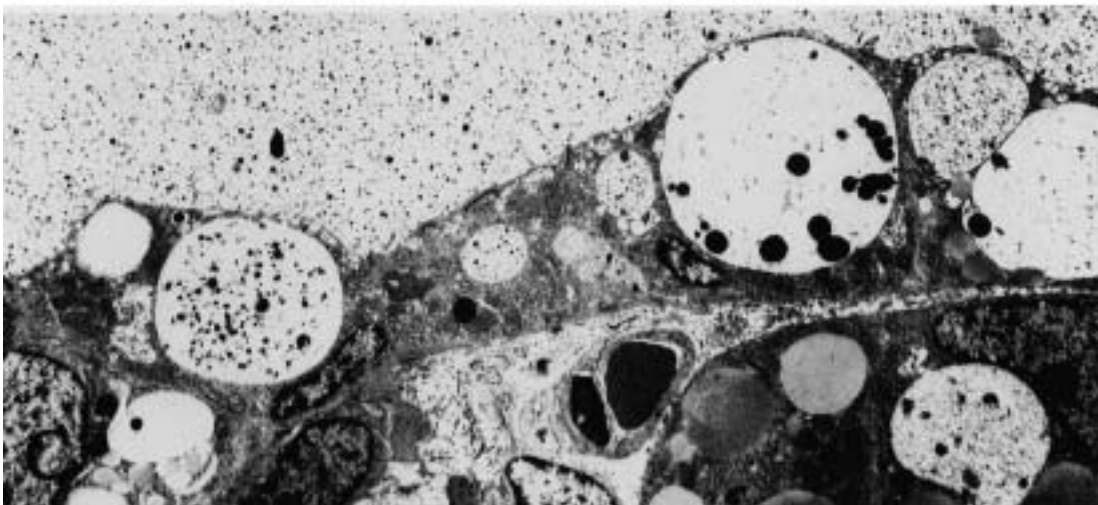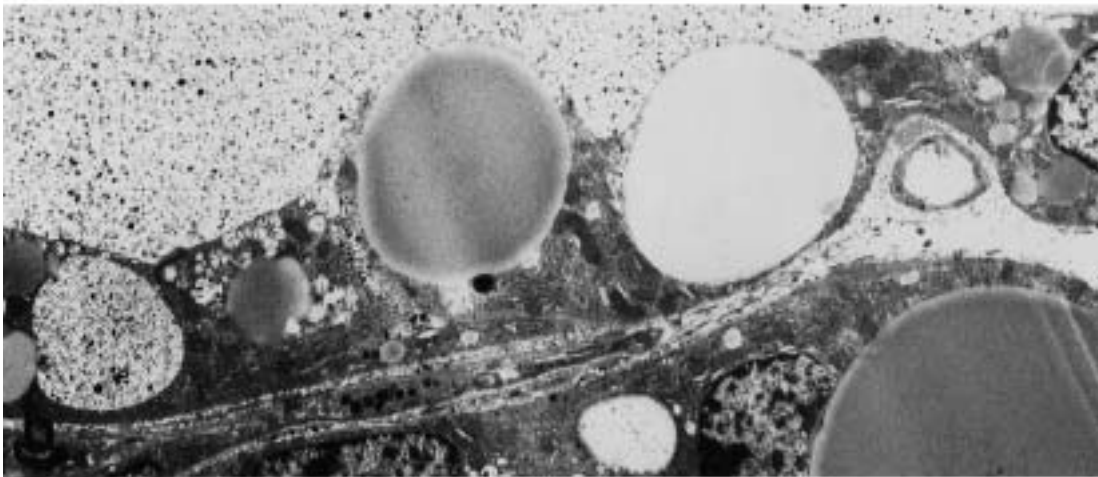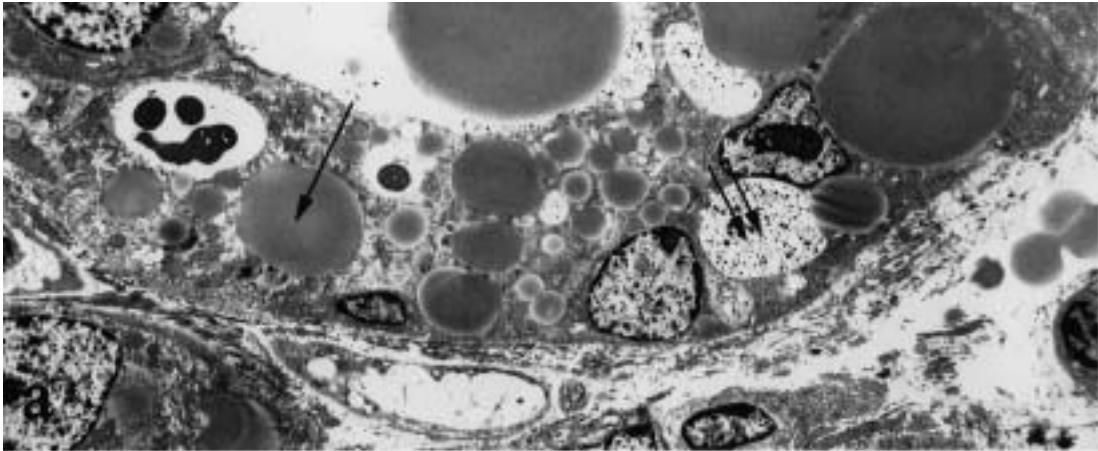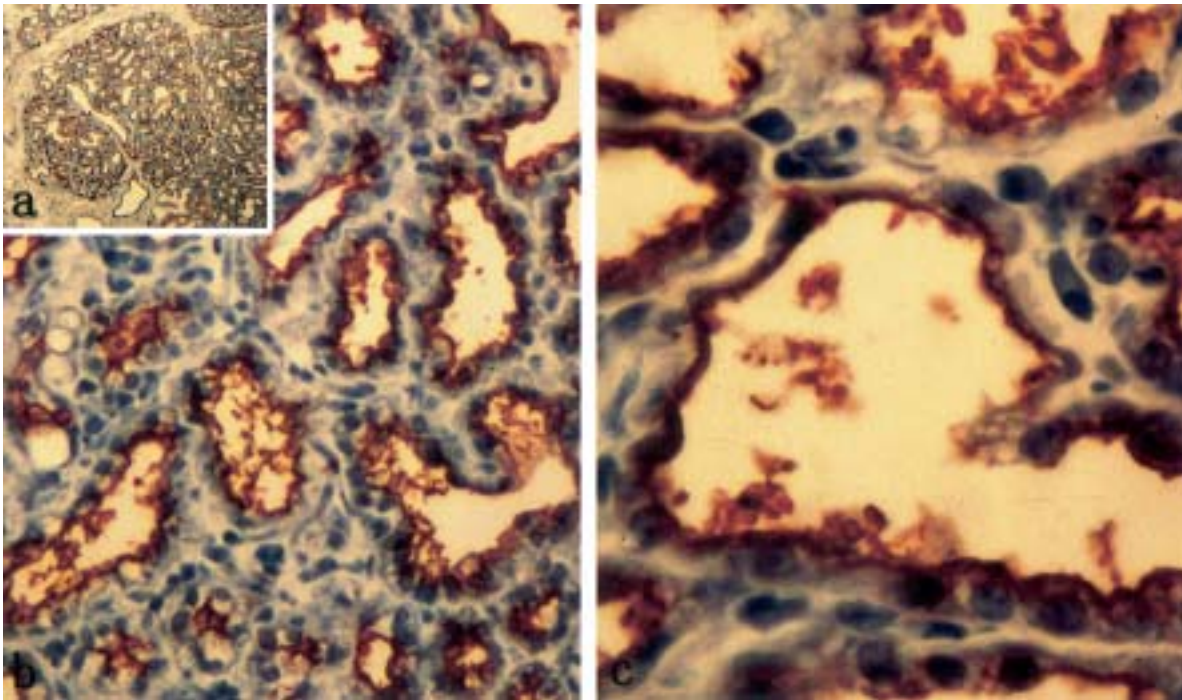
Figure 2.12 a–c ▲

a Immunohistochemical reaction with milk fat globule membrane antigen antibody in Lobule type 4 (Lob 4) (× 2.5). **b** Lumen of Lob 4 alveolar structures decorated with a positive reaction against milk fat globule membrane protein (× 10). **c** Milk fat globule membrane is a mucoprotein that covers the apical border of the luminal epithelial cells (× 40) (**a–c**, 3′, 5′ diaminobenzidine [DAB] counterstained with hematoxylin)

◄ **Figure 2.11 a–c**

Electron micrographs of the normal lactating breast epithelium. **a** A single layer of epithelial cells containing lipid droplets in the cytoplasm (*one arrow*) and small granular material with dense core structures (*two arrows*) (× 2,500). **b** Lipid droplets being released into the lumen (× 3,500). **c** Dense core of proteinaceous material being released into the lumen (× 4,000). (**a–c** stained with uranyl acetate and lead nitrate)

crease in number due to active cell division and in addition their size becomes larger mainly due to cytoplasmic enlargement [3]. By mid-pregnancy, the acini composing each lobule are further enlarged and more numerous. They surround the duct from which their central branch proceeds so thickly that the chief duct, the terminal or intra lobular terminal duct, cannot longer be recognized. The transition between the terminal ducts and the budding acini is gradual, making the histological distinction between the two of them difficult, since both show evidence of early secretory activity. The definitive structure of the ductal tree is essentially settled by the end of the first half of pregnancy; the mammary changes that characterize the second half of pregnancy are chiefly continuation and accentuation of secretory activity. Further progressive branching continues with less prominent bud formation. At this time, the formation of true secreting units or acini, the differentiated structures, becomes increasingly evident. Proliferation of new acini is reduced to a minimum, and the lumina of those already formed become distended by accumu-

lation of secretory material or colostrum (Figs. 2.10–2.12). The epithelium is vacuolated due to the accumulation of lipids (Fig. 2.10d). Under the electron microscope the mammary epithelium shows numerous lipid droplets and proteinaceous material (Fig. 2.11). In Lob 4 reactivity against milk fat globule protein is highly expressed (Fig. 2.12). The secretory acinus formed during pregnancy is a terminal outgrowth that marks the end of glandular differentiation. However, just before and during parturition, there is a new wave of mitotic activity with an increase in the total DNA of the gland. During lactation, the process of growth and differentiation may be observed in the same lobule type, side by side with the process of milk secretion [3, 4, 13, 23].

2.5 Postlactational Changes

From mid-pregnancy onwards, a yellowish fluid containing a high concentration of protein is secreted into the mammary alveoli and may be expelled from the nipple. Lactation starts after postpartum withdrawal of placental lactogen and sex steroids, which appear to prevent the action of prolactin on the mammary epithelium. Colostrum is secreted during the first week postpartum, followed by a 2- to 3-week period of transitional milk secretion, leading to the secretion of mature milk [3, 21].

No major morphological changes of the mammary gland are observed during lactation. The mammary lobules are enlarged and the acini have a dilated lumen filled with granular, slightly basophilic material admixed with fat. There is a significant variation in lobule size throughout the gland, suggestive of a variation in lactogenic activity from lobule to lobule. Milk is continuously synthesized and released into the mammary acini and ductal system as long as it is removed regularly from the mammary gland [3, 21]. It can be stored in the ductal system for up to 48 h, but thereafter the rates of synthesis and secretion begin to decrease. The accumulation of milk in the ducto-acinar lumina and within the cytoplasm of the lactogenic epithelial cells that occurs after weaning has an inhibitory effect on further milk synthesis. This effect is followed by a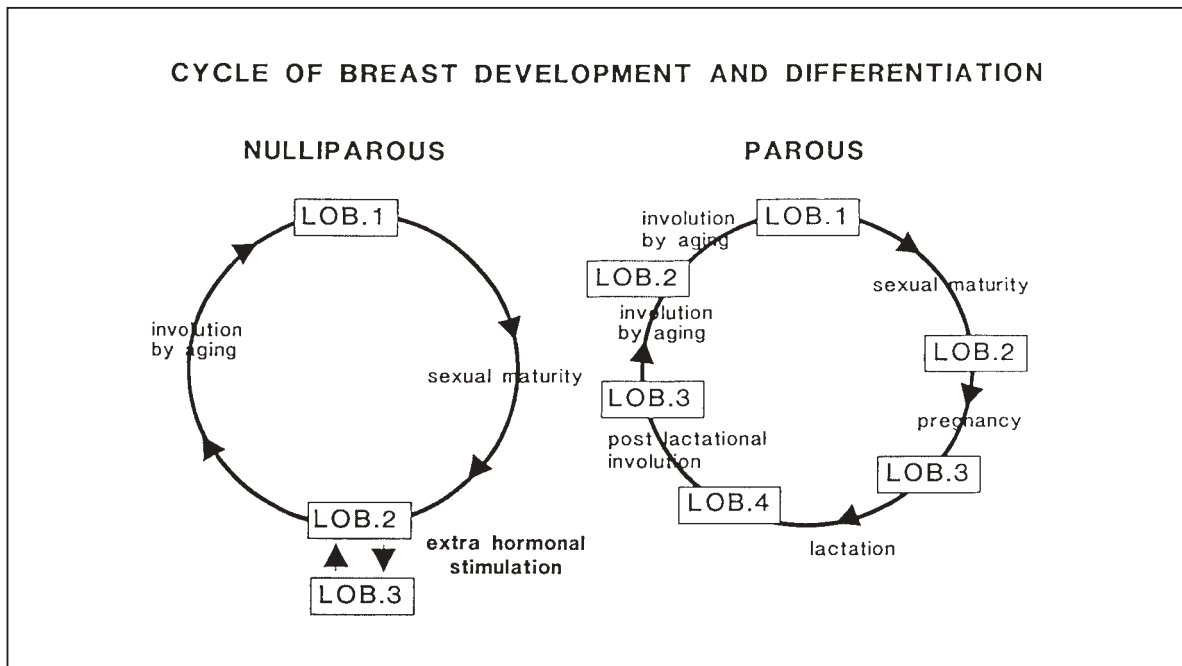 series of involutional changes in the mammary gland consisting of a multifocal asynchronous process of reduction in volume of the secretory epithelial cells and further inhibition of their secretory activity.

It is considered that post-lactational regression is due to two complementary mechanisms, cell autolysis, with collapse of acinar structures and narrowing of the tubules, and appearance of round cell infiltration and phagocytes in and about the disintegrating lobules, and finally, regeneration of the periductal and perilobular connective tissue with renewed budding and proliferation in the terminal tubules. Until menopausal involution sets in, the parous organ contains more glandular tissue than if pregnancy or pregnancy and lactation had never occurred [3, 21].

2.6 The Menopausal Breast

Menopause supervenes when more than 99% of the 400,000 follicles that were present in the ovaries when the fetus had a gestational age of 5 months become exhausted through ovulation or atresia. The lack of endometrial stimulation caused by the reduced or absent levels of ovarian estrogen and progesterone leads to the cessation of menses, or amenorrhea, the most characteristic sign of menopause. Gonadotropin-releasing hormone secretion is implicated in this phenomenon, indicating that the hypothalamus is also involved in the development of menopause. The years leading up to the final menstrual period, until menopause sets in, generally at around the age of 51 years, constitute the perimenopause. During this period many women ovulate irregularly, either because the rise in estrogen during the follicular phase is insufficient for triggering a surge of pituitary luteinizing hormone (LH), or because the existing follicles are resistant to the ovulatory stimulus. The increase in human longevity occurring in our society has caused a considerable increment in the number of women that will live one third or more of their lives in the menopausal period, namely without natural estrogen and progesterone. After menopause, the breast undergoes a regressive phenomenon both in nulliparous and parous women. This regression is manifested in the breast as an increase in the number

CYCLE OF BREAST DEVELOPMENT AND DIFFERENTIATION

Figure 2.13

Influence of parity on lifetime breast development. Diagrammatic representation based on the relative percentage of lobules present. In nulliparous women the breast contains primarily lobules type 1 (*Lob 1*) with some progression to type 2 (*Lob 2*), and only minimal formation of lobules type 3 (*Lob 3*). In parous women, pregnancy and lactation complete the cycle of lobular development through the formation of lobules type 4 (*Lob 4*), which regress to Lob 3 at post-weaning and to Lob 2 and Lob 1 after menopause. (Reprinted with permission from: Russo, J. et al. Breast Cancer Res. Treat. 23:211, 1992)

of Lob 1, and a concomitant decline in the number of Lob 2 and Lob 3. At the end of the fifth decade of life, the breast of both nulliparous and parous women contains predominantly Lob 1 [3, 21]. These observations led us to conclude that the understanding of breast development requires a horizontal study in which all the different phases of growth are taken into consideration. For example, the analysis of breast structures at a single given point, i.e., age 50 years, would lead us to conclude that the breast of both nulliparous and parous women appears identical. However, the phenomena occurring in prior years might have imprinted permanent changes in the breast biology that affect the potential of this organ to develop neoplasms, even though they are no longer morphologically identifiable. Thus, from a biological and quantitative point of view, the regression of the breast at menopause differs in nulliparous and parous women. In nulliparous women, the predominant breast structure is the Lob 1, which comprises 65 to 80% of the total lobular components and their relative percentage is independent of age (Fig. 2.7). Second, in frequency is the Lob 2 that represents 10 to

35% of the total. The least frequent is the Lob 3, which represent only 0 to 5% of the total lobular population. In premenopausal parous women, on the other hand, the predominant lobular structure is the Lob 3, which comprises 70 to 90% of the total lobular component (Fig. 2.8). Only after menopause Lob 3 decline in number, and the relative proportion of the three lobular types present approach that observed in nulliparous women (Figs. 2.7, 2.8, 2.13). These observa-
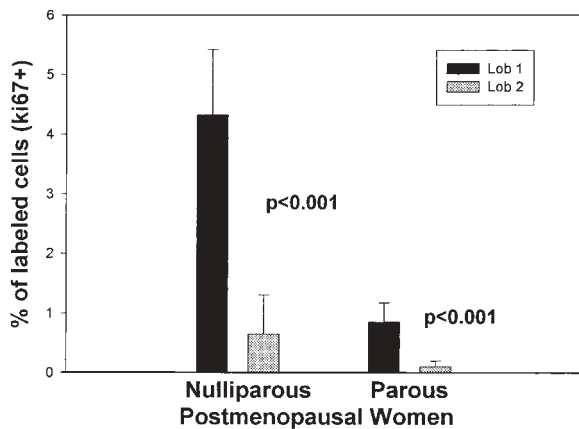
tions led us to conclude that early parous women truly underwent lobular differentiation, which was evident at a younger age, whereas nulliparous women seldom reached the Lob 3 stage, and never the Lob 4 stages (Figs. 2.13, 2.14) [21].

Even though during the post-menopausal years in the breast of both parous and nulliparous women the preponderant structure is the Lob 1, only the nulliparous women are at high risk of developing breast cancer, whereas parous women remain protected [21]. Since ductal breast cancer originates in Lob 1 (TDLU) [19], the epidemiological observation that nulliparous women exhibit a higher incidence of breast cancer than parous women [3, 4] indicates that Lob 1 in these two groups of women might be biologically different, or exhibit different susceptibility to carcinogenesis [17, 24–26]. The presence of Lob 1 in the breasts of parous women has also been interpreted as a failure of the mammary parenchyma to respond to the influences of pregnancy and lactation [21, 22]. It

is possible to postulate that unresponsive lobules that fail to undergo full differentiation under the stimuli of pregnancy and lactation are responsible of cancer development despite the parity history of a woman. If this were the case, then this unresponsive Lob 1 would be as sensitive to carcinogenesis as the lobules found in the breasts of nulliparous women. We have reported the presence of intralobular hyalinization and lower proliferative activity in the Lob 1 of the parous woman's breast, whereas hyalinization is absent and cell proliferation is higher in the Lob 1 of the nulliparous woman's breast. We have also shown that during the fourth and fifth decades of life there is a decrease in the number of Lob 2. We postulate that this type of lobule is the site of origin of both lobular hyperplasia and carcinoma in situ [19, 21, 27]. Since it has been reported that the incidence of atypical lobular hyperplasia decreases significantly with advancing age, it is possible to postulate that the observed diminution in Lob 2 is responsible for the decreased incidence of this type of preneoplastic lesions.

In addition to the differences in proliferative activity the three types of lobules exhibit variations in their in vitro growth characteristics. Lob l and Lob 2 grow faster; have a higher DNA labeling index, and a shorter doubling time than Lob 3 [18]. They also exhibit different susceptibility to carcinogenesis [15, 17]. Cells obtained from Lob l and Lob 2 express in vitro phenotypes indicative of neoplastic transformation when treated with chemical carcinogens, whereas cells obtained from Lob 3 do not manifest those changes [15, 17]. Collectively, our data establish a baseline for understanding the evolution of glandular development, and how it is influenced by age and parity. This knowledge is of utmost importance for understanding the role of differentiation in the protection of the mammary gland against carcinogenesis [27–30]. In addition, these data establish well-defined endpoints for studying the response of the mammary gland to hormonal or chemopreventive agents, which could be utilized in modulating the susceptibility of the breast to carcinogenesis.
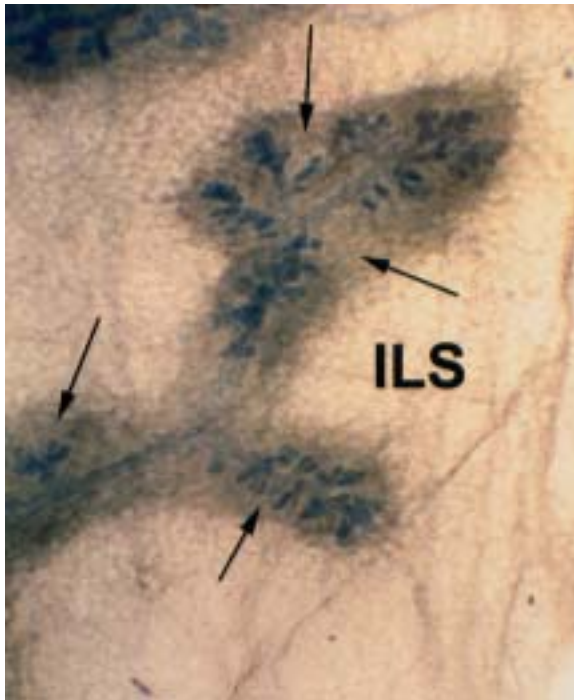
2.7 Parenchyma-Stroma Relationship

Breast development occurs through a process of ductal elongation, branching and sprouting of ductules or acini, a process that requires extensive cell proliferation and penetration of the ductal epithelium into the stroma [3]. Both the intralobular and the interlobular stroma are affected simultaneously during development, pregnancy, lactation, and involution (Figs. 2.15, 2.16). These processes occur, in turn, in a synchronous manner in response to specific hormonal and growth factor stimuli [27]. Two major mechanisms are considered to be involved in the in-

teraction of the stroma with epithelial cells, the production of soluble growth factors and a modification of the composition of the extracellular matrix. This interaction seems to be bi-directional, such that epithelial cells are also capable of influencing stromal cell behavior and governing gene expression [31, 32]. The study of the stroma-parenchyma ratio in 14 breasts of pubertal, post-pubertal, parous, and pregnant women, showed that the relationship between parenchyma and stroma is a dynamic process. At puberty, almost 90% of the mammary gland is made up of stroma: the intralobular stroma, that represents 17% of the total, consists of the loose connective tissue that surrounds each individual ductule, and the interlobular stroma, composed of fat and connective tissue, that separates one lobule from another (Fig. 2.15). The parenchyma of the pubertal breast comprises 10% of the mammary area; it is made up almost exclusively of Lob 1 and ductal structures. In post-pubertal and young nulliparous women's breast, the parenchyma increases from 10 to 30% of the total area of the gland (0–10% is composed of Lob 1, 10–18% of Lob 2, and 1–3% of Lob 3) (Fig. 2.16). The intralobular stroma represents about 28% of the total bre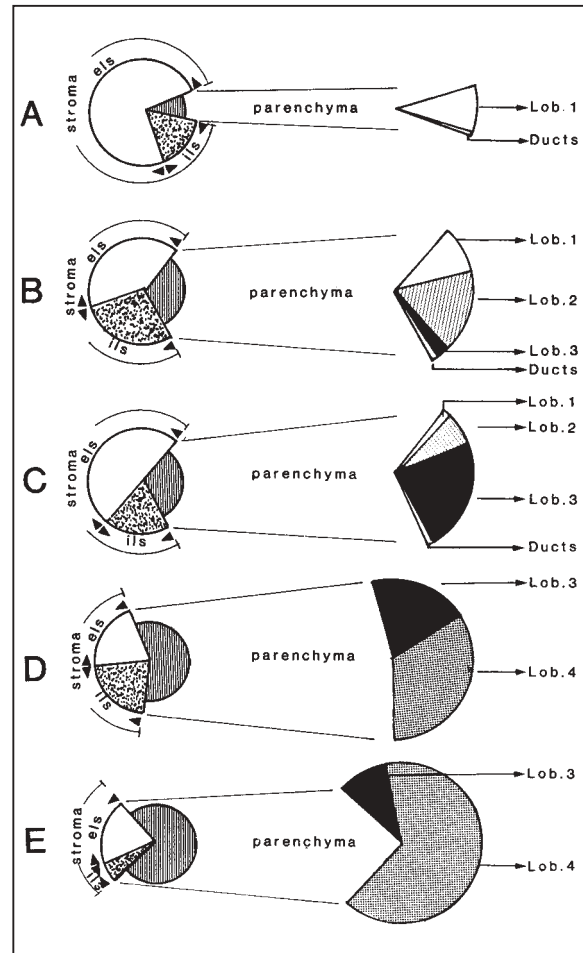ast area [21]. Parity induces significant differences in mammary gland development. The breast of non-pregnant parous women is mostly composed of Lob 3 with a markedly reduced proportion of Lob 1 (Fig. 2.16).

2.8 Genetic Influences in Breast Development

Genetic influences are responsible of at least 5% of the breast cancer cases; they also seem to influence the pattern of breast development and differentiation, as evidenced by the study of prophylactic mastectomy specimens obtained from women with familial breast and breast/ovarian cancer, or proven to be carriers of the BRCA1 gene, as determined by linkage analysis [33]. Morphological and architectural analysis of prophylactic mastectomy specimens revealed that these characteristics were similar in breasts obtained from either nulliparous or parous women. In both groups of women the breast tissues were predominantly composed of Lob 1, and only a few specimens contained Lob 2 and Lob 3, in frank contrast with the predominance of Lob 3 found in parous women without familial history of breast cancer (Fig. 2.17) [33]. We concluded that the developmental pattern of the breast of parous women of the familial breast cancer group was similar to that of nulliparous women, and less developed than the breast of parous women without history of familial breast cancer. The breast of women belonging to the familial breast cancer group also presented differences in the branching pattern of the ductal tree, observations that suggested that the genes that control lobular development might have been affected in women carrying breast cancer predisposing genes [33].

Table 2.3. Influence of parity on the lobular composition and proliferative activity of the breast. *Lob 4* lobule type 4

Group	Number of cases	Donor's age	Lob 1		Lob 2	
			Lobular structures[a]	Ki 67 index[b]	Lobular structures[a]	Ki 67 index[b]
Nulliparous	7	40.85 ± 10.93	81.13 ± 6.90	4.97 ± 2.51*	18.87 ± 6.89	0.62 ± 0.30
Parous	25	48.76 ± 11.00	66.84 ± 7.55	1.56 ± 0.29*	23.10 ± 6.37	0.85 ± 0.31

[a] Percentage of each type of lobular structures over total number of structures present
[b] Ki67 index, proliferative activity determined as the percentage of Ki67 positive cells
* Proliferative activity of Lob 1 nulliparous vs. Lob 1 parous, t = 2.44, p = <0.02
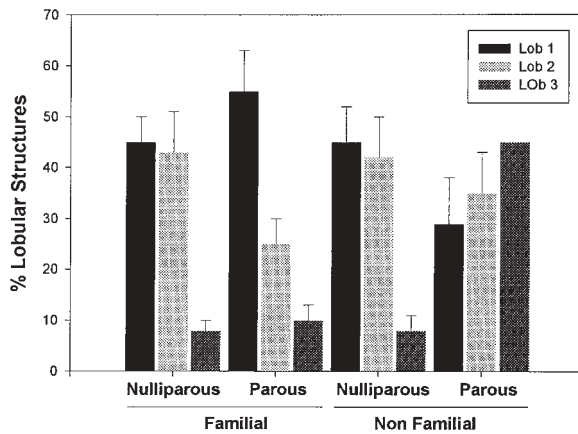
Figure 2.17

Relative percentage of lobules type 1, 2, and 3 (Lob 1, Lob 2 and Lob 3) (ordinate) in the breast of nulliparous and parous women with history of familial breast cancer in comparison with that of women with no family history. (Adapted from: Russo, J. and Russo, I.H. Prog. Clin. Biol. Res. 396:1–16, 1997)

2.9 Cell Proliferation and Hormone Receptors in Relation to Breast Structure

The determination of the breast proliferative activity, expressed as the percentage of cells that react positively with the antibody Ki67 (Ki67 or Proliferative Index) revealed that it is maximal in those Lob 1 present in the breast of nulliparous women (Table 2.3). It is two-fold lower in Lob 1 of parous women ($p < 0.02$) (Table 2.3), but in both groups cell proliferation decreases progressively and proportionally as the lobules mature to Lob 2 and Lob 3 (Table 2.3). These differences are not abrogated when the phases of the menstrual cycle are taken into consideration [25]. Parity, in addition to exerting an important influence in the lobular composition of the breast, as described above, profoundly influences the proliferative activity of the mammary epithelium. Lob 1 and Lob 2 present in the breast of premenopausal nulliparous women exhibit a significantly higher proliferative activity than the same type of lobules found in the breast of parous women. Even though in the breast of menopausal women the proliferative activity of the mammary epithelium decreases, the differences in Proliferation Index between nulliparous and parous women still persist.

Estrogens and progesterone are known to promote proliferation and differentiation in the normal breast epithelium. Both steroids act intracellularly through a receptor which, when activated by its binding with the hormone, regulates the expression of specific genes [34]. However, the mechanism by

Lob 3		Lob 4		Ducts
Lobular structures[a]	Ki 67 index[b]	Lobular structures[a]	Ki 67 index[b]	Ki 67 index[b]
00.00	00.00	0.0	0.0	2.00 ± 0.75
8.03 ± 4.67	0.22 ± 0.18	0.0	0.0	1.47 ± 0.19

which these molecules exert their mitogenic and differentiation effects has not been clearly established [35–45]. One of the accepted mechanisms of action of steroid hormones postulates that the proliferation of cells is the response to direct stimulation, as the result of the interaction of the estradiol bound to the classical estrogen receptor alpha (ER α) with the DNA [35]. Measurements of the levels of ERα and progesterone receptor (PgR) in normal breast in the cytosol fraction, using standard biochemical techniques, is inaccurate because of the low cellularity of the tissue. The use of monoclonal antibodies that specifically recognize ERα and PgR makes it possible to identify and to quantify the cells expressing these receptors [45]. Both ERα and PgR are present in the nucleus of epithelial cells. The percentage of cells expressing these receptors, however, varies as a function of the degree of lobular development of the breast, and therefore of the type of lobular structure analyzed. Lob 1 are the structures more consistently containing a higher percentage of ERα and PgR positive cells than Lob 2, 3 and 4, an observation that indicates that a progressive decrease in the percentage of cells exhibiting an immunocytochemically positive reaction for these markers occurs as the structures become more differentiated. These data allowed us to conclude that degree of differentiation of the breast is an important determinant in the expression of both ERα and PgR, in addition to modulate the proliferative activity of the breast epithelium. Age and parity history do not affect the percentage of cells reacting with these receptors except through their influence on the lobular composition of the breast [45].

2.10 Extracellular Matrix Protein Expression in the Normal Breast

The molecular organization of the extracellular matrix consists of multifunctional proteins, which play an important role in cell growth, adhesion, migration and differentiation. It cannot be seen as a simple amorphous gel anymore. Indeed we know today that, besides its mechanical properties, the extracellular matrix can show a variety of biochemical changes in response to minimal variations in the parenchyma's metabolism [46, 47]. We have identified by immunohistochemistry tenascin and elastin, two proteins of the extracellular matrix that might have been produced or repressed during the differentiation process of the breast. These proteins play an important role in the cell-cell, cell-substratum interactions and malignant transformation. Studies of tenascin's structure, tissue distribution and in vitro models have indicated that this is a multifunctional glycoprotein participating in cell adhesion, motility and migration pathways, shedding of epithelial cells from surfaces, promotion of cell growth, demarcation of tissue boundaries, angiogenesis, tissue remodeling and immune modulation. Tenascin is also largely confined to areas of epithelial-mesenchymal interactions during the process of development of the embryo [48] suggesting an important role during the differentiation process.

2.10.1 Angiogenic Index in the Lobular Structures

Using factor VIII as a marker of endothelial cells for identifying blood vessels, we determined the angiogenic index (AI), based on a count of the number of identifiable vascular spaces are counted in the intralobular stroma of Lob 1, Lob 2 and Lob 3 (Fig. 2.18, Table 2.4). AI was 1.64 in Lob 1, which was significantly higher ($p < 0.00000$) than in Lob 2 and Lob 3, in which AI was 0.84 and 0.55, respectively. There were no differences in AI between nulliparous and parous women when comparisons were done between similar lobular types (Table 2.4), and the same phenomenon was observed with aging (Table 2.5) [22].

Table 2.4. Angiogenic index in the normal breast

Group	Number of cases	Age[a]	Lob 1	Lob 2	Lob 3
All women	48	43.36 ± 7.59	1.64 ± 0.40	0.84 ± 0.28	0.55 ± 0.11
Nulliparous	13	40.16 ± 8.32	1.66 ± 0.35	0.82 ± 0.19	0.57 ± 0.09
Parous	35	44.36 ± 7.29	1.63 ± 0.42	0.84 ± 0.31	0.55 ± 0.13

[a] Age in years, mean ± standard deviation

Table 2.5. Effect of age on parous women's breast angiogenic index

Number of cases	Donors' age	Angiogenic index		
		Lob 1	Lob 2	Lob 3
4	30–39	1.54 ± 0.30	0.75 ± 0.03	0.59 ± 0.11
10	40–50	1.42 ± 0.41	$0.76 \pm 0.$	0.44 ± 0.14
4	>50	1.88 ± 0.58	0.97 ± 0.02	0.47 ± 0.02

Table 2.6. Influence of parity on elastin reactivity. *NA* no Lob 4 available for examination

Number of cases	Group	Percentage of elastin-positive lobules			
		Lob 1	Lob 2	Lob 3	Lob 4
44	All Women	$10.35 \pm 1.55^{a,b,c}$	19.82 ± 2.37^{a}	$36.64 \pm 4.63^{b,d}$	$33.80 \pm 20.75^{c,d}$
11	Nulliparous	$7 37 \pm 174^{e,f}$	$19.36 \pm 5.92^{f,g}$	$28.71 \pm 7 74^{f,g}$	NA
33	Parous	$11.50 \pm 2.03^{h,i,j}$	$19.92 \pm 2.64^{h,k,l}$	$40.60 \pm 5.61^{h,k,m}$	$84.52 \pm 452^{j,l,m}$

[a] Lob 1 vs. Lob 2; $t = 3.47$, $p < 0.0009$
[b] Lob 1 vs. Lob 3; $t = 6.58$, $p < 0.00000001$
[c] Lob 1 vs. Lob 4; $t = 2.89$, $p < 0.005$
[d] Lob 3 vs. Lob 4; $t = 3.47$, $p < 0.001$
[e] Lob 1 vs. Lob 2; $t = 2.58$, $p < 0.02$
[f] Lob 1 vs. Lob 3; $t = 3.30$, $p < 0.004$
[g] Lob 2 vs. Lob 3; not significant

[h] Lob 1 vs. Lob 2; $t = 2.87$, $p < 0.006$
[i] Lob 1 vs. Lob 3; $t = 7.91$, $p < 0.000000$
[j] Lob 1 vs. Lob 4; $t = 9.75$, $p < 0.0000000$
[k] Lob 2 vs. Lob 3; $t = 5.66$, $p < 0.000004$
[l] Lob 2 vs. Lob 4; $t = 8.68$, $p < 0.000000$
[m] Lob 3 vs. Lob 4; $t = 3.74$, $p < 0.002$

Table 2.7. Influence of age on elastin reactivity

Donor's age	Number of cases	Percentage of elastin-positive lobules			
		Lob 1	Lob 2	Lob 3	Lob 4
20–40	17	14.53 ± 3.57	20.02 ± 3.73	40.91 ± 5.76	84.52 ± 4.52
41–50	18	8.21 ± 1.83	20.47 ± 3.46	34.61 ± 7.26	NA
51–80	9	7.70 ± 2.65	12.25 ± 5.90	54.15 ± 20.85	NA
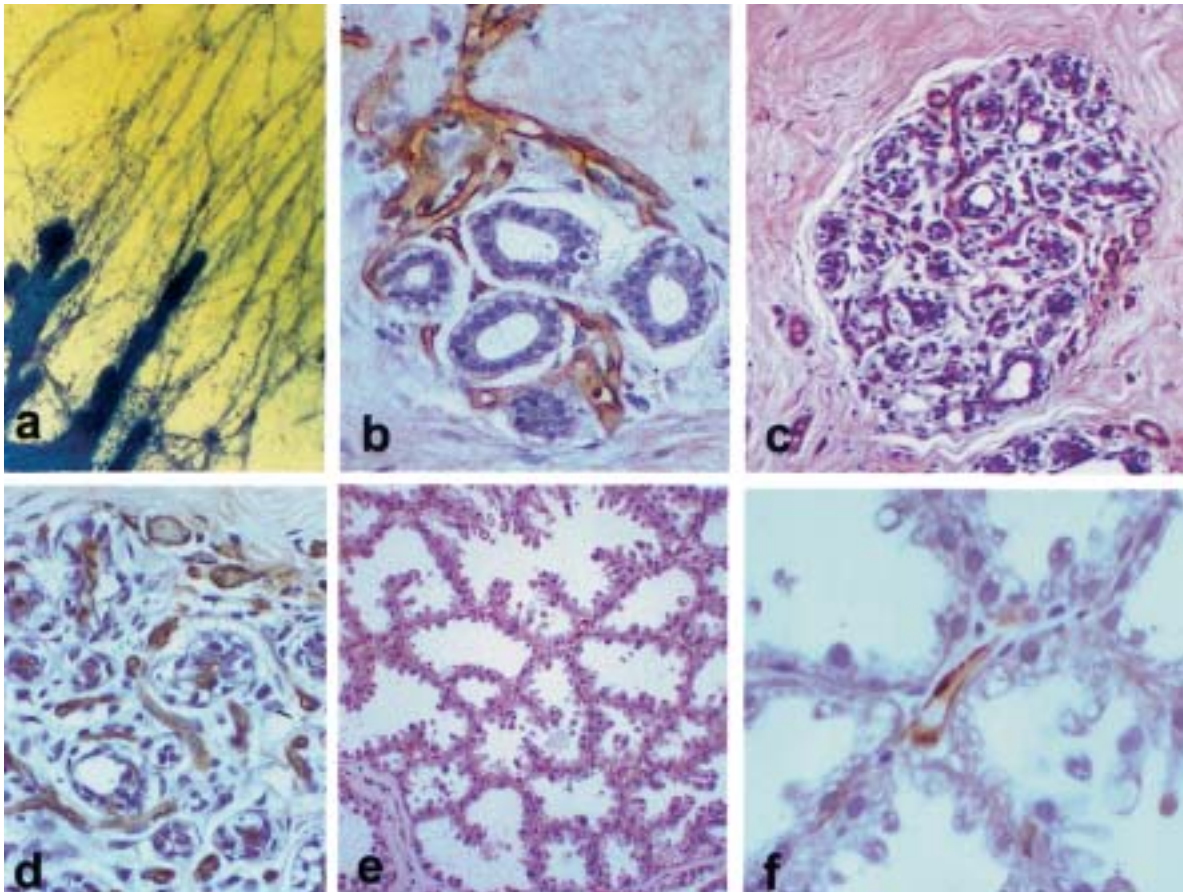
Figure 2.18 a–f

a Breast tissue of a 19-year-old nulliparous woman showing a network of capillaries surrounding terminal ductal structures and extending into the adjacent stroma. (Whole mount preparation, toluidine blue, ×2.0). b Cross section the terminal ductal structures shown in a, showing the endothelial cells of blood vessels reacting positively with anti-factor VIII antibody used for determining the angiogenic index (AI), based on a count of the number of identifiable vascular spaces in the intralobular stroma (DAB counterstained with hematoxylin [H] (×20); c–f endothelial cells of blood vessels reacting positively with anti-factor VIII antibody (DAB counterstained with H). Lob 1 c (×4), and d (×10); Lob 4 e (×20), f (×40), showing progressive reduction in the ratio of ductule/acinus to intralobular blood vessels with lobular differentiation

2.10.2 Elastin in the Lobular Structures

Elastin interacts with microfibrillar proteins; it has also been demonstrated that peptide segments of elastin elicit a chemotactic response by fibroblast and monocytes, suggesting that this protein has domains with biologically important activities other than those related to its mechanical properties [49].

Immunocytochemical reactivity reveals elastin to surround each individual ductule as a continuous layer (Fig. 2.19a). Although the intensity of the reaction was similar in all the ductules and acini that were positive, the number of structures positive varied depending upon the type of lobules. Lob 1 had the lowest percentage of positive ductules (10.35%), which increased to 19.82% in Lob 2, and to 36.64% and
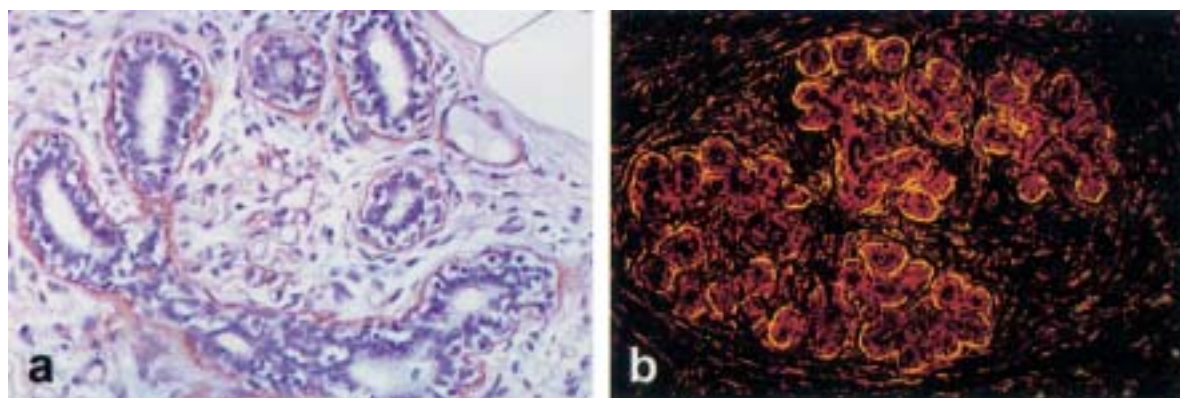
Figure 2.19 a, b

a Lobule type 1 stained with anti elastin antibody, (DAB-H & E, ×10). b Lobule type 2 stained with anti-tenascin antibody (DAB-H & E, ×4)

33.80% in Lob 3 and Lob 4, respectively (Table 2.6). Whereas parity slightly increases the percentage of elastin positive Lob 1 and Lob 3 the differences were not statistically significant (Table 2.6). Aging did not affect the percentage of lobules presenting positive elastin reactivity (Table 2.7) [22].

2.10.3 Tenascin in the Lobular Structures

Tenascin is identified in the intralobular stroma of all the lobular structures of the human breast (Fig. 2.19b). The percentage of lobules reacting with tenascin is varies from 44% and 41% in Lob 1 and Lob 2, respectively, to 30% in Lob 3, with Lob 4 exhibiting the highest percentage of reactivity, 89.25% (Table 2.8). Parity and aging did not affect the percentage of structures reacting for tenascin [20].

In conclusion, there is a direct relationship between proliferative activity and angiogenic index, which is, in turn, inversely proportional to the degree of differentiation of the lobular structures. Thus, Lob 1, that are the less differentiated ones, have the highest proliferative index and AI. Elastin, on the other hand, follows an inverse pattern, being expressed more highly in the more differentiated Lob 3 and Lob 4. Tenascin is moderately expressed in the less differentiated Lob 1 and Lob 2, and highly expressed in the stroma of Lob 4, with the lowest level of expression in Lob 3, and indication that its expression is not linear with the degree of differentiation of the lobules. Overall, age and parity do not affect the expression of elastin and tenascin independently of the degree of lobular development. The only significant difference induced by pregnancy is the proliferative activity of Lob 1, which is two fold lower in the breast of parous women than in those of nulliparous women.

Table 2.8. Tenascin expression in lobular structures

Number of cases	Lob 1	Lob 2	Lob 3	Lob 4
43	$44.43 \pm 16.28^{a,b,c}$	$41.53 \pm 18.22^{b,d}$	$30.67 \pm 22.87^{b,d}$	$89.25 \pm 0.91^{c,d}$

[a] Lob 1 vs. Lob 2; not significant
[b] Lob 1 vs. Lob 3; $t = 2.35$, $p < 0.02$
[c] Lob 1 vs. Lob 4; $t = 3.84$, $p < 0.00003$
[d] Lob 3 vs. Lob 4; $t = 3.50$, $p < 0.0004$

2.11 Genomic Profile of Lobular Structures in Nulliparous and Parous Women's Breasts

For assessing whether the differentiation of the breast induced by pregnancy imprints permanent genomic changes in the mammary epithelium, we compared the genomic profile of the well differentiated Lob 3 from he breast of parous women with that of the undifferentiated Lob 1 from the breast of nulliparous women. Reduction mammoplasty specimens free from pathological lesions were obtained from three parous and in three nulliparous premenopausal women. Epithelial cells from Lob 3 and Lob 1 of the parous and nulliparous women's breasts respectively were microdissected by laser capture microdissection (LCM). Total RNA was extracted and 20–25 μg were hybridized to cDNA array membranes that contained 1,176 human genes (Clontech Human Cancer 1,2 array). Microarrays were hybridized to fluorescent cDNA probes that were synthesized with control total or poly(A)+RNA through indirect incorporation of Cy3/Cy5 fluorescent label using aminoallyl-dNTP incorporation and chemical coupling of the dyes. cDNA expression microarrays and gene clustering analysis performed utilizing BioDiscovery's Ima-Gene and GeneSight software applications reveal that the level of reproducibility between replicate microarray hybridizations was >90%; the average coefficient of covariance per gene between replicate array data sets was ~14%; and the level of falsely detected genes as differentially expressed when the cut-off range was a 2-fold change in expression) was less than 1%.

The genomic signature of Lob 3 of parous women differed by 82 genes from that of Lob 1 of nulliparous women (Fig. 2.20). We have clustered these genes according to functional properties (Table 2.9). With this array, it is clear that Lob 3 have a gene expression profile significantly different from that of Lob 1 of the nulliparous breast. Of interest is the Rho-E gene, which is amplified 14 folds in the Lob 3 of the parous breast (Table 2.9). This gene belongs to a small G protein superfamily that includes the Ras, Rho, Rab, Arf, Sarl, and Ran families. Members of the Rho family of small guanosine triphosphatases (GTP) have emerged as key regulators of the actin cytoskeleton, and furthermore, through their interaction with multiple target proteins, they ensure coordinated control of other cellular activities such as gene transcription and adhesion [50]. Rho-E is a Rho protein that binds GTP but lacks intrinsic GTPase activity and is resistant to Rho-specific GTPase-activating proteins. Within a region that is highly conserved among small GTPases, RhoE contains amino acid differences specifically at three positions that confer oncogenicity to Ras. Replacing all three positions in Rho-E with conventional amino acids completely restores GTPase activity [51, 52]. Rho-E may act to inhibit signaling downstream of Rho-A, altering some Rho-A-regulated responses, such as stress fiber formation, but not affecting others, such as peripheral actin bundle formation. In vivo, Rho-E is found exclusively in the GTP-bound form, suggesting that unlike previously characterized small GTPases, RhoE may be normally maintained in an activated state [51, 52]. This might be of importance in maintaining this gene activated in Lob 3, even after its involution Lob 1 in the postmenopausal state. Another gene that is significantly overexpressed (five-fold) in Lob 3 is the protein tyrosine phosphatase (HPTPCAAX1 or PRL-1) (Table 2.9). This gene encodes a unique nuclear protein-tyrosine phosphatase that is regulated by a mechanism different from those of other immediate early genes such as *c-fos* and *c-jun* [53]. Importantly, this gene has been shown to be upregulated in villus enterocytes and in confluent differentiated colon carcinoma cells, but not in crypt enterocytes or undifferentiated proliferating Caco-2 colon carcinoma cells, respectively. In other systems this gene has been found to be related to differentiation, development and regeneration [54–57]. Therefore in the breast epithelial cells of Lob 3 its function may be related to differentiation rather than to proliferation, since Lob 3 have a lower proliferative activity than Lob 1 (Table 2.9).

Insulin-like growth factor binding protein-3 (IGFBP-3) is significantly overexpressed in the Lob 3 over the Lob 1 of the breast of premenopausal women. This gene codes a specific binding protein for the insulin-like growth factors (IGFs). Insulin-like growth factor binding protein 3 (IGFBP-3) modulates the mitogenic and metabolic effects of IGFs [58]. IG-
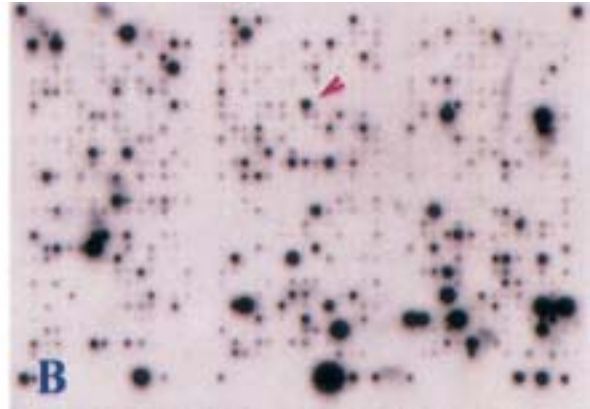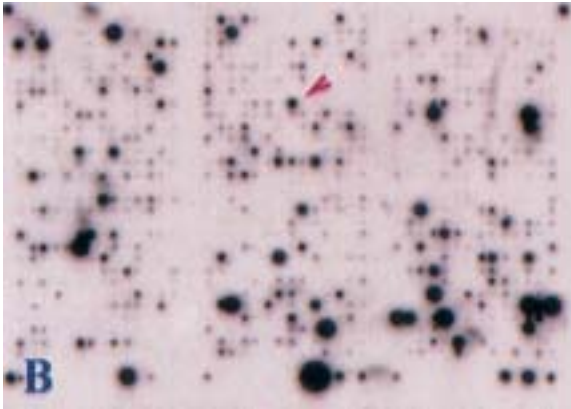
Figure 2.20 a, b

Gene expression analysis of the human breast. For Panel **a** RNA was extracted from Lob 1 of a 28-year-old nulliparous woman, and for Panel **b** from Lob 3 of a 26-year-old parous woman. RNAs were hybridized to Atlas Human cDNA Expression Array membranes. The *arrow* points to the amplification spot for the IGFBP-3 gene

Figure 2.21 ▲

Verification of cDNA microarray results with semi-quantitative RT-PCR. Genes PRL-1, Rho-E and IGFBP-3, which were overexpressed in Lob 3 show a more intense band, whereas beta actin, that was not modified, gives two equally intense bands in Lob 1 and Lob 3. *Nu-Pa* nulliparous

FBP-3 forms a ternary complex with IGF-I or IGF-II and an 85 kDa glycoprotein acid-labile subunit ALS. IGFBP-3 may also play more active, IGF-independent, roles in growth regulation of cancer cells [59]. IGFBP-3 protein levels are developmentally regulated and influenced by a number of hormonal stimuli both in vitro and in vivo. p53 may regulate apoptosis in tumor cells via transactivation of the IGFBP3 gene [59–61]. It has been shown that IGFBP-3 can be modulated by hCG, and this could represent an important pathway in the differentiating effect of this hormone on the mammary gland. IGFBP-3 expression in the Lob 3 is a new finding that requires further investigation.

The final biological significance of changes in gene expression in relation to the process of breast differentiation, post-menopausal involution, and how they are influenced by a reproductive event in women, is not known. Neither there is a clear understanding of the role of these known or still unidentified genes play for conferring protection to the breast through pregnancy. It was of interest the observation that the expression of cytoskeletal proteins, such as cytokeratins 18, 19, and 8, did not differ between Lob 1 and Lob 3, even though they are overexpressed in tumor cells [62]. Known genes that are overexpressed in breast cancer, such as HER-2/neu [63] and mucin [64, 65], were not expressed in any of the lobular structures. The levels of expression of other genes, such as fibroblast-like growth factor 1 (FGF-1), insulin-like growth factor-1 (IGF-1) binding protein-2, and Zinc-a-2-glycoprotein [66], which are generally up or down regulated in neoplastic lesions, did not differ between Lob 3 and Lob 1. The fact that cells derived from the undifferentiated Lob 1 express transforma-

Table 2.9. Gene expression in Lob 3 of parous women versus Lob 1 of nulliparous women

Gene category/name[a]	Fold increase (- decrease) Mean ± SD[b]	Gene category/name[a]	Fold increase (- decrease) Mean ± SD[b]
Intracellular kinase network		**Oncogenes, tumor suppressor genes, transcription activators and repressors, intracellular transducers, effectors and modulators**	
– CDC7-related kinase	2.4 ± 0.8	– c-jun proto-oncogene: transcription factor AP-1	2.8 ± 0.5
– c-jun N-terminal kinase 2 (JNK2)	4.5 ± 1.2	– Interferon-inducible protein 9–27	2.0 ± 0.3
– Ribosomal protein S6 kinase II	1.6 ± 0.4	– Neurogenic locus notch protein (N)	3.0 ± 0.7
Caspases		– c-myc oncogene	–1.5 ± 0.4
– Caspase-4 (CASP4)	2.3 ± 0.5	– c-myc binding protein MM-1	9.5 ± 1.2
Protein turnover		– Cyclin-dependent kinase 4 inhibitor, p16	–2.0 ± 0.4
– Ubiquitin-conjugating enzyme E2	–2.0 ± 0.5	– Epidermal growth factor receptor (EGFR)	1.6 ± 0.3
– Basigin precursor (BSG)	–2.0 ± 0.4	– Fos-related antigen (FRA1)	5.0 ± 0.9
– Heterogen nuclear ribonucleoprot K	3.2 ± 0.8	– Active breakpoint cluster region-related protein	2.5 ± 1.0
Calcium binding proteins		– ETS-related protein	1.6 ± 0.4
– Calmodulin 1	7.0 ± 1.6	– ETS domain protein elk-3, NET	3.0 ± 0.8
– Guanine nucleotide-binding protein	10.5 ± 2.5	– Purine-rich single-stranded DNA-binding protein	–1.6 ± 0.4
Metabolism		– Early growth response protein 1 (hEGR1)	2.0 ± 0.2
– Cytosolic superoxide dismutase 1 (SOD1)	1.6 ± 0.3	– Neutrophil gelatinase-associated lipocalin precursor	2.0 ± 0.5
– Glutathione-S-transferase (GST) homolog	2.0 ± 0.5	**Extracellular matrix, cell adhesion, cellular matrix**	
– L-lactate dehydrogenase M subunit (LDHA)	6.5 ± 1.5	– Cadherin 3 (CDH3); placental cadherin precursor	–2.0 ± 0.7
– L-lactate dehydrogenase H subunit (HDHB)	3.2 ± 0.8	– Integrin beta 6 precursor (ITGB6)	4.5 ± 0.9
Replication factors, DNA damage and repair		– Integrin beta 4 (ITGB4): CD104 antigen	–1.8 ± 0.5
– G/T mismatch-specific thymine DNA glycosylase	3.5 ± 0.9	– Integrin beta 8 precursor (ITGB8)	4.0 ± 1.2
– MutL protein homolog, DNA mismatch repair	–3 ± –0.8	– Paxillin	1.6 ± 0.3
– Proliferating cyclic nuclear antigen (PCNA); cyclin	3 ± 0.8	– Alpha catenin (CTNNA1)	2.2 ± 0.6
Cell cycle and proliferation related		– Desmoplakin I & II (DSP, DPI & DPII)	–2.0 ± 0.2
– G1/S-specific cyclin D1 (CCND1)	3.5 ± 1.6	– Polycystin precursor	–3.3 ± 1.8
– Cell cycle protein P38–2G4 homolog, HG4–1	3.0 ± 1.1	– Wnt-8B	–2.3 ± 0.9
– Cyclin-dependent kinase inhibitor 1 (CDKN1A)	–1.5 ± 0.9	– Vimentin (VIM)	1.6 ± 0.8
– Btg protein precursor	–3.6 ± 1.1	– Type I cytoskeletal 13 keratin	–2.4 ± 1.2
– Cdc2-related protein kinase PISSLRE	–2 ± 0.6	– Nm23-H4; nucleoside-diphosphate kinase	–2.5 ± 0.5
– PTPCAAX1 nuclear tyrosine phosphatase (PRL-1)	5.0 ± 0.0	– Type II cytoskeletal 2 epidermal keratin (KRT2E)	–2.0 ± 0.5
DNA binding nuclear proteins		– BIGH3	3.0 ± 0.8
– DNA-binding protein CPBP	2.2 ± 0.4	– Fibronectin precursor (FN)	5.2 ± 1.6
Undefined		**Receptors**	
– Interferon-induced 56-kDa protein (IFI-56 K)	1.6 ± 0.5	– Arylhydrocarbon receptor (AH receptor)	5.0 ± 1.1
– TRAM protein	13.0 ± 1.9	– Signaling lymphocytic activation molecule	–3.0 ± 0.6
– BENE	4.0 ± 0.8	– Bone morphogenetic protein 4 type II receptor precursor	1.8 ± 0.4
Metalloproteinases and protease inhibitors		– Oncostatin M-specific receptor beta subunit	1.6 ± 0.5
– PRSM1 metallopeptidase	–2.0 ± 0.7	– IgG receptor FC large subunit P51 precursor	–3.0 ± 1.0
– Matrix metalloproteinase 3 (MMP3)	16.0 ± 1.9	– CD59 glycoprotein precursor	2.0 ± 0.4
– Matrix metalloproteinase 7 (MMP7); matrilysin	4.0 ± 1.3		

– Metalloproteinase inhibitor 3 precursor	3.2 ± 1.0
– Leukocyte elastase inhibitor (LEI)	3.0 ± 0.5
– Bikunin; hepatocyte growth factor activator inhibitor 2	–1.5 ± 0.7
– Metalloproteinase inhibitor 1 precursor (TIMP1)	2.9 ± 1.0
Growth factors and hormones	
– Vascular endothelial growth factor precursor (VEGF)	2.6 ± 0.6
– Macrophage inhibitory cytokine 1 (MIVI)	2.1 ± 0.4
– Insulin-like growth factor binding protein 3 precursor (IGF-bind)	9.5 ± 1.8
– Interleukin-1 beta precursor (I)	1.5 ± 0.3
Intracellular transducers, effector modulators, symporters and antiporters	
– 14–3-3-protein sigma, stratifin; epithelial cell marker protein	–2.0 ± 0.4 –1.5 ± 0.7
– T3 receptor-associating cofactor 1	–2.0 ± 0.4
– Growth factor receptor-bound protein 2 (GRB2) isoform	2.5 ± 0.7
– Elongation factor 1 alpha (EF1 alpha)	1.6 ± 1.3
– ADP/ATP carrier protein	3.0 ± 0.2
– ATP synthase coupling factor 6 mitochondrial precursor	1.6 ± 1.7

[a] Genes classified according to function

[b] Mean fold increase (- decrease) of gene expression in Lob 3 in three parous women compared with Lob 1 of three nulliparous women ± SD, standard deviation of the mean

tion phenotypes upon in vitro carcinogen treatment indicates that genomic changes detected in tumors are a late event in the process of cancer progression [13, 15, 67].

The differential expression of some those genes that were detected to be overexposed in the Lob 3 by microarray analysis were quite consistently confirmed by semiquantitative RT-PCR (Fig. 2.21).

2.12 Novel Differentiation-Associated Serpin is Upregulated During Lobular Development

For understanding the molecular basis of biological differences between Lob 1 and Lob 3 we used differential display technique for comparing gene expression in breast epithelial cells of the more differentiated Lob 3 with that of cells from the undifferentiated Lob 1; two cDNA fragments, identified as L342C and L346C, which were preferentially expressed in the breast epithelial cells of Lob 3 (Fig. 2.22). The differential expression of these genes was then confirmed by reverse Northern blot analyses, which showed that plasmid DNA containing either L342C or L346C cDNA hybridized to the labeled cDNA synthesized from Lob 3 RNA, but not to that from Lob 1 RNA.

Sequence analysis indicated that L342C and L346C consisted of 665 and 416 nucleotides (nt), respectively. Remarkably, the sequence of L346C was identical to L342C, except that a stretch of 249 nucleotides in L342C was missing in L346C. Search for homology in the gene bank databases revealed that L342C and L346C each were nearly identical to two stretches of two sequences, accession Nos. K01500 [68] and J05176 [69] of a human α1-antichymotrypsin (α1-ACT), a serine protease inhibitor or serpin [68, 69].). When the three sequences were aligned, it became apparent that L342C and L346C contained 327-nt and 576-nt deletions, missing nucleotides 1041–1367 and 783–1367 of the α1-ACT, respectively. These results suggested that these two differentially expressed genes represented potentially alternatively spliced transcripts of a novel serpin. For differentiating this novel serpin from human α1-ACT and for detecting its isoforms, an antisense riboprobe was synthesized using the RSA-linearized L342C plasmid DNA. The
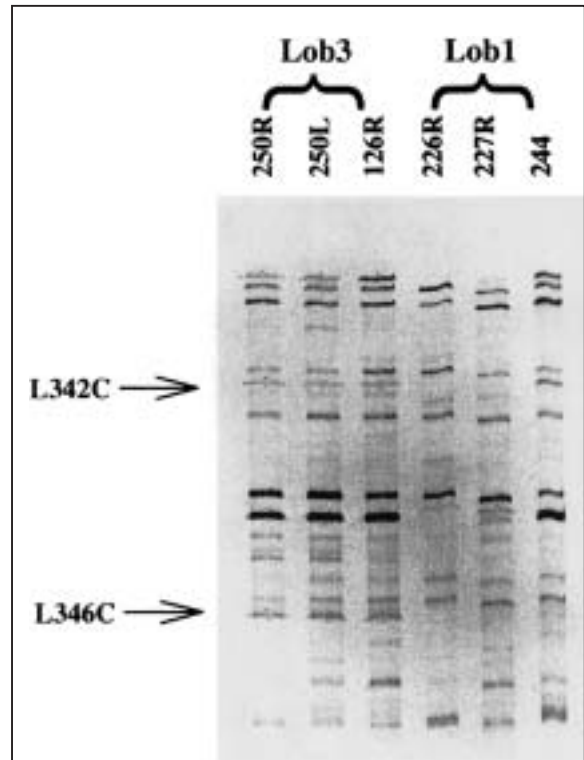

Figure 2.22

Differential display between mRNA from human breast epithelial cells (HBEC) obtained from Lob 3 of breast samples 250R, 250L, and 126R and from Lob 1 of samples 226R, 227A, and 244). cDNA bands that were preferentially expressed in HBEC from Lob 3 were identified as L342C and L346C. (Reprinted with permission from: Russo J. et al., Microsc. Res. Tech. 52:204, 2001)

antisense riboprobe was approximately 464 bp in full length and contained 68 bp of vector sequences and covered the entire region that was missing in L346C (Fig. 2.23). The riboprobe was predicted to protect two fragments in human α1-ACT transcripts, a 228-bp fragment protected by its 3'-end and a 168-bp fragment protected by its 5'-end. These two fragments were expected to be protected equally well. In addition, if the novel serpin was present as two isoforms in human breast cells, the riboprobe was predicted also to protect two fragments, a full-length
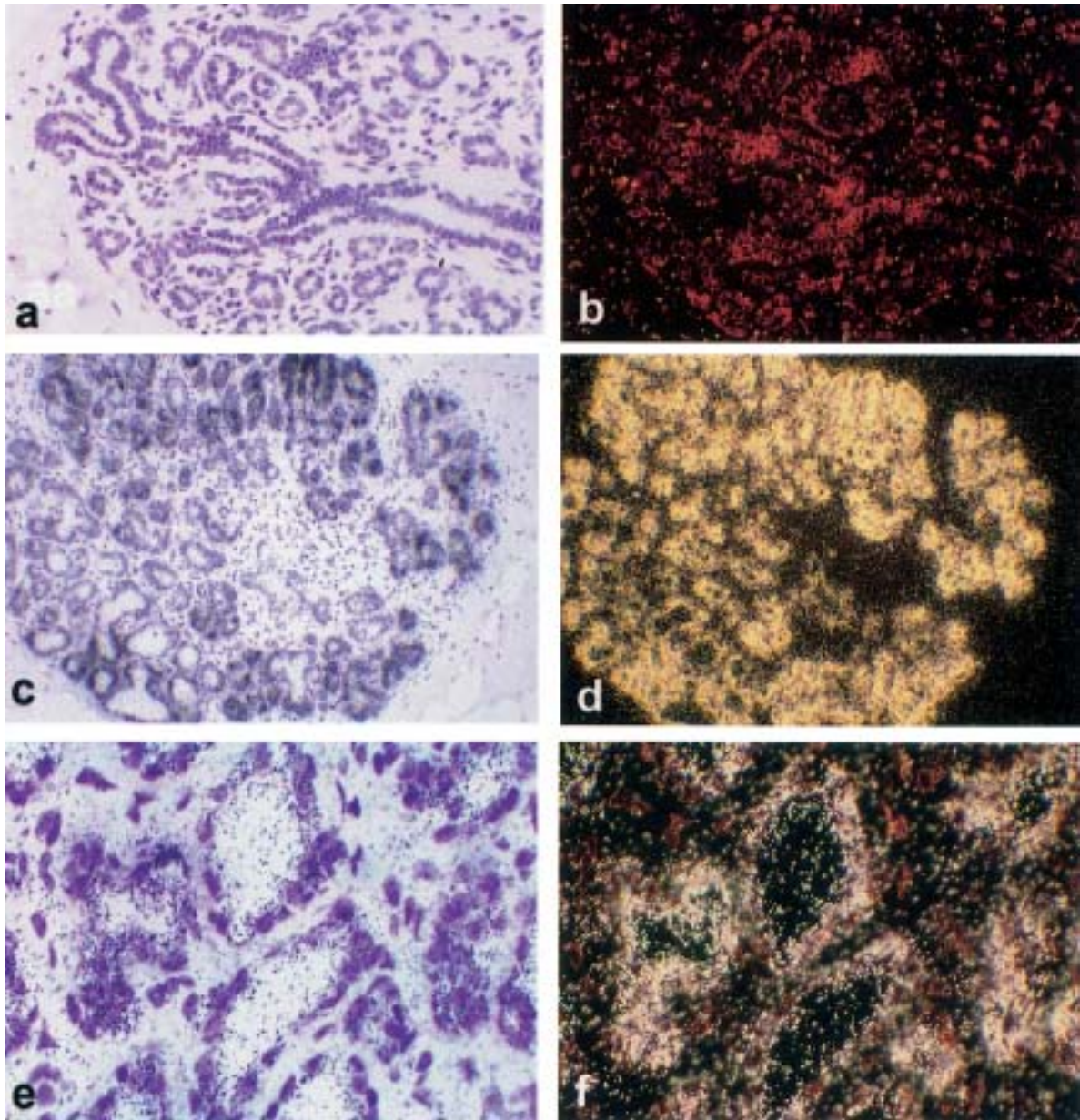
Figure 2.23

RNase protection assays of the novel serpin L342C (*top*) and, β-actin (*bottom*) in cultured human breast epithelial cells (HBECs), placenta and liver tissues. The sizes of the protected fragments are indicated by *arrows* on the left side of yeast tRNA band; samples used for analysis are listed on top of the lanes. The samples include four primary cultures of HBECs obtained from normal human breast tissues, samples number 227, 244, 248, and 250; a mortal HBEC line cultured in low calcium medium, 130, and the same cell line cultured in high (*H*) calcium medium, sample 130H; the normal immortalized HBEC line MCF-10F and transformed cells derived from BP treated MCF-10F cellsBP1 and BP1E. HBL-100, another normal HBEC line and T47D, BT20, and MCF-7, three breast carcinoma cell lines, were also tested. Yeast tRNA with or without RNase digestion was used as control. RNase protection analysis of β-actin expression served as controls of equal sampling. (Reprinted with permission from: Russo J. et al, Microsc. Res. Tech. 52:204, 2001)
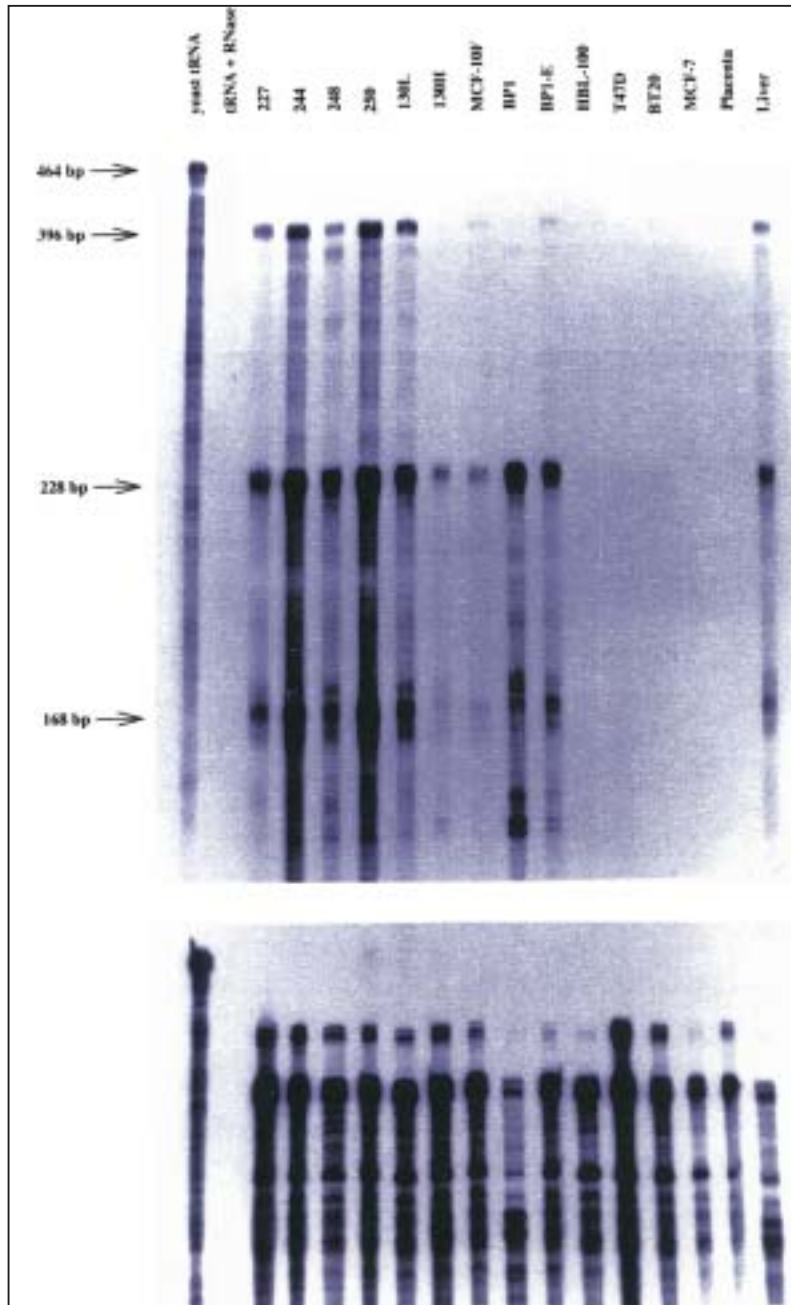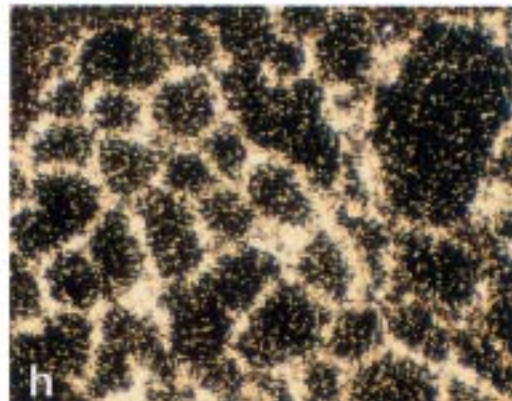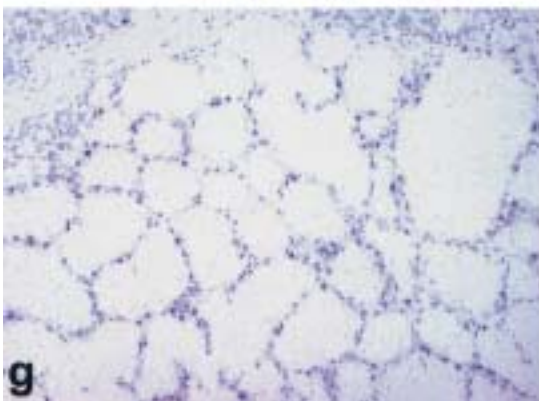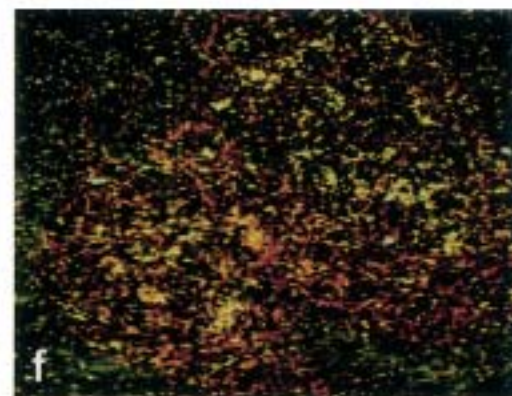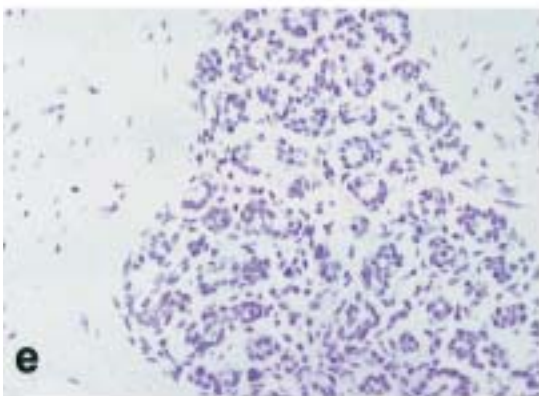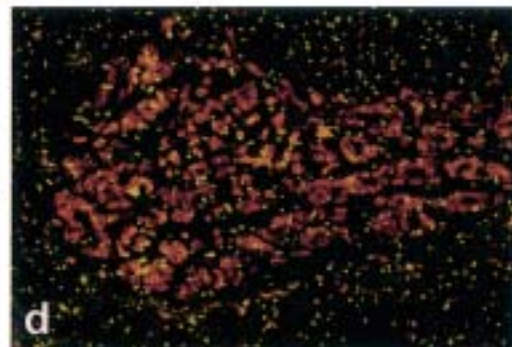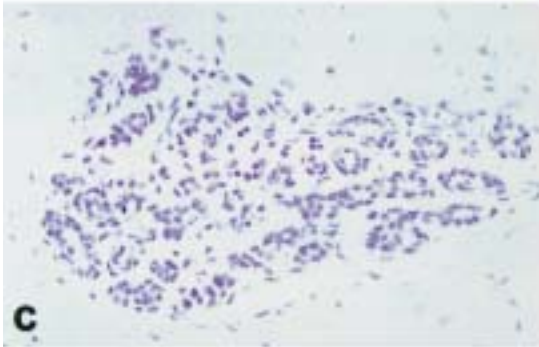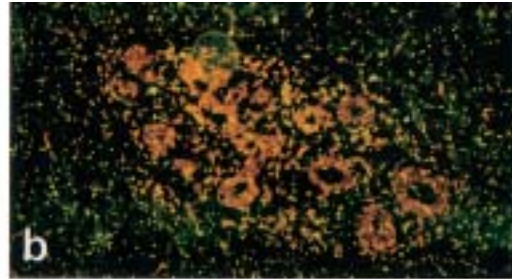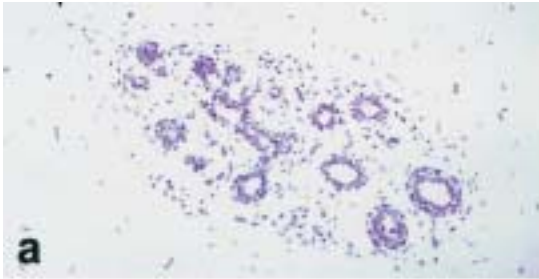
396-bp fragment found in L342C and a 168-bp fragment found in L346C. Results from the RNase protection assay showed that the antisense riboprobe protected three fragments in human breast cells, namely, the full-length 396-bp fragment found in L342C and the 168-bp fragment expected for its isoform as well as the 228-bp fragments apparently from α1-ACT. Similarly, the same antisense riboprobe protected three differently sized transcripts in liver, but not in full-term human placenta (Fig. 2.23). Yeast tRNA, used as control, did not protect any fragments from RNase digestion (compare lanes yeast tRNA versus yeast tRNA + RNase in Fig. 2.23), and a sense riboprobe did not protect any of the RNA fragments from the same panel of cells (data not shown). The amount of various transcripts protected by the antisense riboprobe varied with the status of immortalization and neoplastic transformation in the panel of cells we studied. Protection of the full-length 396-bp fragments indicative of expression of the long transcripts of the novel serpin was highest in primary cultures of human breast epithelial cells (HBECs) obtained from normal human breast tissues containing well differentiated Lob 3, samples number 244 and 250, and less intense in samples 227, 248, and in the mortal HBEC line 130L, cultured in 0.04 M calcium concentration, or low (L). Protection was low in line 130H, cultured in high (H) calcium medium, in MCF-10F cell line, and in BP1 and BP1-E, two transformed cell lines derived from benzo(a)pyrene (BP)-treated MCF-10F cells,. It was completely absent in HBL-100, a normal HBEC line, and in T47D, BT20, and MCF-7, three breast carcinoma cell lines. In contrast, expression of the human α1-ACT, determined as the 228-bp fragments, was evident in the four primary cell lines tested, the mortal HBECs 130L and 130H, in MCF-10F cells and in BP-transformed cell lines. A similar pattern of expression was also observed for the 168-bp fragment (Fig. 2.23). β-actin gene expression, used as control of equal sampling, appeared to be similar in all the samples tested by RNase protection analysis (Fig. 2.23). These results, confirmed by Northern blot analysis, indicated that we have cloned a novel serpin different from human α1-ACT that was present as two isoforms in differentiated human breast epithelial cells and in liver tissue [22].

The physiological function of this novel serpin in human breast is completely unknown. It is clearly a biomarker of lobular differentiation because of its preferential expression in the more differentiated HBEC obtained from Lob 3 as compared to those obtained from Lob 1, as determined by differential display and reverse Northern analyses. In fact, it is of interest to note that expression of the novel serpin, as determined by RNase protection analyses, was higher in primary cultures established from breast tissues containing more of the well-differentiated structures than those containing a lower amount of the more differentiated structures. Since degree of mammary lobular differentiation is a critical determinant of mammary susceptibility to carcinogenesis [30], the preferential expression of the novel serpin in more differentiated epithelium may confer protection against breast cancer development, as has been proposed for other differentiation-associated genes [15, 24]. Thus, it is reasonable to speculate that silencing of the serpin in the normal undifferentiated Lob 1, which is known to be the site of origin of ductal carcinomas [19, 70], may render the mammary cells susceptible to events leading to carcinogenesis [15, 24]. This notion is supported by our evidence that the full-length transcription of the novel serpin is indeed down regulated during the process of immortalization and neoplastic transformation of human breast epithelial cells in vitro. We have evaluated the expression of the differently sized transcripts of the novel serpin in relation to lobular differentiation and found that the Lob 1 has no expression, whereas the Lob 3 has sufficient expression of this serpin (Fig. 2.24) [22].

Figure 2.24 a–f

Histological characteristics and localization of a serpin-like mRNA by in situ hybridization in normal breast tissues with varying degree of lobular differentiation. **a** Low level of expression in lobule type 1 (Lob 1) (bright field, $\times 2.5$). **b** Lob 1 shown in **a** in dark field ($\times 2.5$). **c** intense reactivity in Lob 4 (bright field, $\times 2.5$). **d** Lob 4 shown in **c** in dark field ($\times 2.5$). **e** Acini of Lob 4 showing intense reactivity in luminal epithelial cells (bright field, $\times 40$), and **f** same as acini shown in **e** in dark field ($\times 40$)

2.13 Mammary-Derived Growth Inhibitor

Mammary-derived growth inhibitor (MDGI) has been shown to induce rodent mammary gland differentiation, to suppress human breast cancer cell growth in vitro, and carcinogenesis in transfected human breast cancer cells [71]. It was first detected in ascitic fluid; where it inhibited the growth of Ehrlich ascites carcinoma cells [72]. MDGI belongs to a multigene family of fatty acid-binding proteins (FABP) known to bind long-chain fatty acids, retinoids, and eicosanoids [73]. The amino-acid sequence of MDGI and/or the gene encoding it have been sequenced in cattle [74], rat [75] mouse [76], and human heart [71]. The human heart FABP3, which is considered to be an identical homologue to the bovine MDGI, covers an 8-kb region of genomic DNA [77]. This gene has been localized to chromosome 1p32–35 [71], a locus that has been associated with frequent loss of heterozygosity in sporadic breast cancer [78–80], suggesting that MDGI serves as a tumor suppressor gene.

We have cloned MDGI from cultured HBECs as a 640-bp fragment that contained a single open reading frame encoding a protein of 133 amino acids identical to MDGI/FABP3. In situ hybridization analysis of paraffin-embedded normal human breast tissues revealed that HBEC-MDGI was highly expressed in the most differentiated Lob 4, poorly expressed in moderately differentiated Lob 3, and absent in the least differentiated Lob 1 and Lob 2 (Fig. 2.25). In addition, HBEC-MDGI was not expressed in breast tissues that contained ductal hyperplasia, carcinoma in situ or invasive carcinomas. Our results suggest that HBEC-

MDGI is a biomarker of lobular differentiation in the human breast, and its expression is silenced in poorly differentiated lobules as well as in the early and late stages of breast cancer progression [15, 24]. In light of a strong correlation between MDGI expression and lobular differentiation, and the fact that the degree of mammary differentiation is a critical determinant of mammary susceptibility to carcinogenesis [17], it is tempting to speculate that the protective effects of pregnancy against breast cancer may result from expression of MDGI, which might be in turn responsible for pregnancy-associated mammary differentiation, however, more studies in this direction are necessary.

2.14 Conclusions

The human breast undergoes a complete series of changes from intrauterine life to senescence. These changes can be divided into two distinct phases; the developmental phase and the differentiation phase. The developmental phase includes the early stages of gland morphogenesis, from nipple epithelium to lobule formation. In lobule formation, both processes, development and differentiation, take place almost simultaneously. For example, the progressive transition of Lob 1 to Lob 2, Lob 3, and Lob 4 requires active cell proliferation, to acquire the cell mass necessary for the function of milk secretion. This later process implies differentiation of the mammary epithelium. Therefore, the presence of Lob 4 is the maximal expression of development and differentiation in the adult gland. It is important to point out that even though the presence of proteins that are indicative of milk product synthesis, such as α-lactalbumin, casein, or milk fat globule type membrane protein, are indicators of breast epithelial cellular differentiation, only when all the milk components are coordinately synthesized within the appropriate structure can full differentiation of the mammary gland be acknowledged.

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