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—Review—

Relationship between histology, development and tumorigenesis of mammary gland in female rat

Ján LÍŠKA[1)], Július BRTKO[2)], Michal DUBOVICKÝ[3)], Dana MACEJOVÁ[2)], Viktória KISSOVÁ[4)], Štefan POLÁK[1)], and Eduard UJHÁZY[3)]

[1)]*Institute of Histology and Embryology, Medical Faculty of Comenius University, Sasinkova 4, Bratislava 811 08, Slovak Republic*
[2)]*Institute of Experimental Endocrinology, Slovak Academy of Sciences, Vlárska 3, Bratislava 833 06, Slovak Republic*
[3)]*Institute of Experimental Pharmacology & Toxicology, Slovak Academy of Sciences, Dúbravská cesta 9, Bratislava 841 04, Slovak Republic*
[4)]*Department of Pathological Anatomy, University of Veterinary Medicine and Pharmacy, Komenského 73, Košice 041 81, Slovak Republic*

Abstract: The mammary gland is a dynamic organ that undergoes structural and functional changes associated with growth, reproduction, and post-menopausal regression. The postnatal transformations of the epithelium and stromal cells of the mammary gland may contribute to its susceptibility to carcinogenesis. The increased cancer incidence in mammary glands of humans and similarly of rodents in association with their development is believed to be partly explained by proliferative activity together with lesser degree of differentiation, but it is not completely understood how the virgin gland retains its higher susceptibility to carcinogenesis. During its developmental cycle, the mammary gland displays many of the properties associated with breast cancer. An early first full-term pregnancy may have a protective effect. Rodent models are useful for investigating potential breast carcinogens. The purpose of this review is to help recognizing histological appearance of the epithelium and the stroma of the normal mammary gland in rats, and throughout its development in relation to tumorigenic potential.
Key words: carcinogenesis, development, histology, mammary gland, rat

Introduction

Mammary glands present a modification of sweat glands. The rat mammary gland epithelium consists of branching ducts edging to the nipple. Morphogenesis and differentiation are mediated through the activities of systemic hormones and locally synthesized growth factors. Developmental changes impact women's life time breast cancer risk [31]. The breast undergoes structural and functional changes in association with epithelial expansion during puberty and cycles, secretory differentiation during pregnancy and lactation, and regression during involution. These stages occur in both rodents and humans [34] and there are similarities between rodent and human mammary gland development and carcinogenesis. Critical events include fetal mammary buds development, intensive epithelial outgrowth during puberty, and the rapid proliferation and differentiation in pregnancy and lactation [40]. Various studies point out that rodent mammary cancer imitate the human breast tumors and it is one of the main reasons for the use of rodent models for research of potential breast carcinogens [29, 30, 49]. In humans is the higher incidence of metastases compared with rats. This is related

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Address corresponding: J. Líška, Institute of Histology and Embryology, Medical Faculty of Comenius University, Sasinkova 4, Bratislava 811 08, Slovak Republic

to some extent to the limited length of the experiments and shorter life span. The laboratory mouse and also rat are widely accepted as an invaluable model for investigating of human disease generally because of its short life span, ease of experimental manipulation, limited genetic heterogeneity, and controlled handling environment [25].

Higher susceptibility of the mammary gland to carcinogenesis was observed during early adulthood in both humans and rodents [36], decreasing with aging. Factors that modify the developmental profile of the mammary gland may influence cancer risk. The mammary life cycle provides also useful model for the investigation of controlled angiogenesis as seen during pregnancy and lactation, and for controlled vascular regression during involution. Changes in tissue composition and activity require variable need for nutrients and oxygen and significant changes of microvascularization accommodate these needs [5].

The pubertal gland appears to be optimal model system which has been used to study mechanisms of epithelial morphogenesis and also of cancer susceptibility [15]. Breast cancer manifests itself in the mammary epithelium, but mammary stromal cells play an important role in tumorigenesis. Many of the properties associated with breast cancer appear in the mammary gland during its developmental cycle, and the stromal factors necessary for its development can promote or protect against breast cancer [53]. Some of these mechanisms may be disturbed and can not apply during the development and progression of cancer [30, 46].

Rat Mammary Gland Anatomy and Histology

Mammary glands appear in embryonic life in the form of cellular clumps proliferating from a longitudinal ridge of ectoderm. The number of these clumps may be different in various mammalian species. In humans milk lines regress, except for those in a small region of the chest. Ectodermal mammary crests in humans persist only in the pectoral area where they form the mammary placodes. Between weeks 7 and 8, the mammary parenchyma begins to invade the underlying stroma forming a primitive mammary disk. From the mammary proliferation originate epithelial buds. These buds branch out and extend to the epithelial–mesenchymal boundaries. The additional branching leads to the formation of solid epithelial cords and these undergo apoptosis of the internal epithelial cells. Skin surrounding the nipples proliferates forming the areola [32]. The rat has six pairs of mammary glands along the milk lines which extend along the ventral body wall. The thoracic, abdominal, and inguinal glands vary in their degree of development in the nulliparous rats, with the inguinal being the most differentiated, and the cervical glands being the least differentiated [35]. The postnatal proliferative activities of the epithelium and stromal cells of the mammary gland may contribute to its susceptibility to carcinogenesis [30].

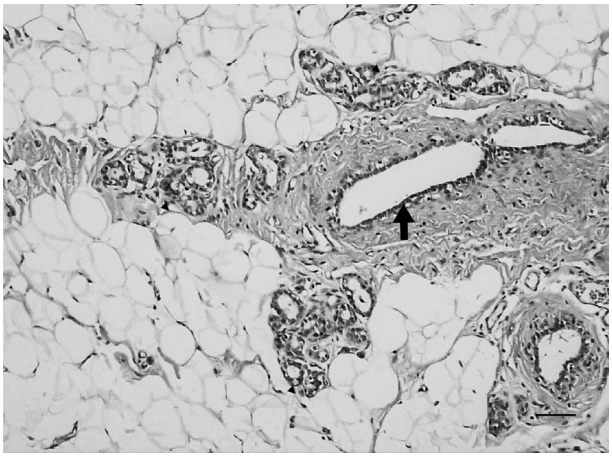

Fig. 1. Non lactating mammary gland of adult female rat. Duct (arrow) and terminal duct lobular units (arrowheads) surrounded by layer of eosinophilic connective tissue stroma embedded in adipous tissue. Hematoxylin and eosin staining (HE). Bar=20 μm.

The parenchymal compartment of the mammary gland is composed of different epithelial structures with distinct morphology and functional or proliferative activities, comprising the luminal epithelium of ducts, ductules, terminal end buds, alveolar buds, alveoli and the underlying myoepithelial layer [30]. The transition from stratified squamous epithelium of the nipple to two layers of cuboidal epithelium occurs in the lactiferous duct within the interior of the nipple. The lactiferous sinus is surrounded by a thick layer of connective tissue stroma. The alveoli, ductules, and ducts merge into one primary collecting duct (Fig. 1). One of the most evident difference between human and rodent mammary glands is the dense interlobular stroma and loose intralobular stroma of the human breast compared with the adipose-rich stroma in rodents [39]. The cell phenotype of breast cancers is mostly similar to the cell phenotype of the luminal epithelium of ducts, ductules, and alveoli. The

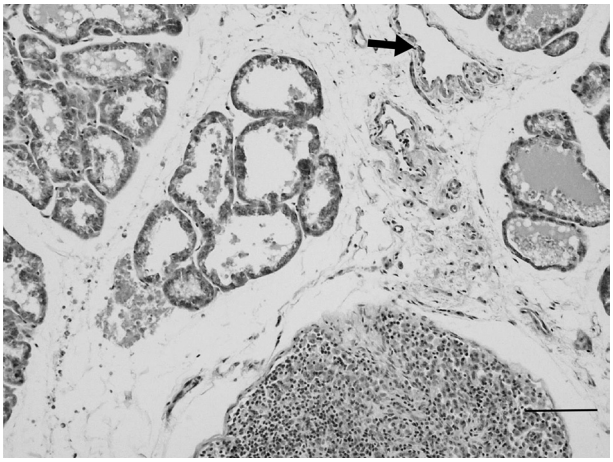

Fig. 2. Lymphatic follicle and lymphatics (arrow) in the lactating mammary gland (HE). Bar=20 μm.

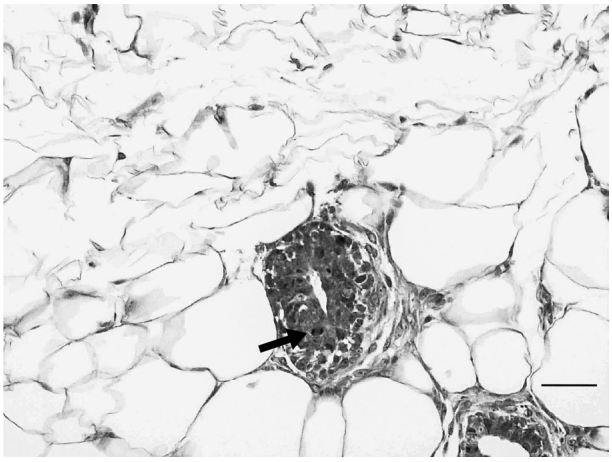

Fig. 3. Highly proliferative TEB with mitotic figures (arrow) in mammary gland of 14 days old female rat (HE). Bar=10 μm.

epithelial cells between the luminal epithelium and basement membrane form the myoepithelium that is contractible after the stimulation of the milk excreting oxytocin. Myoepithelium of the lactiferous ducts and lactiferous sinuses seems to be not fully differentiated as defined by lack of positivity to smooth muscle actin antibodies. The myoepithelial cells form a continuous layer and secrete the continuous basement membrane that separates the epithelium from the surrounding connective tissue. During pregnancy and lactation the layer of myoepithelial cells in alveoli is discontinuous [30]. In adhesion to the underlying basement membrane, the myoepithelial cells express higher levels of cell adhesion receptors and adhesion-associated molecules than the luminal epithelium [12, 30]. Absence of myoepithelial cells and basement membrane in breast cancer indicate invasive carcinoma. Degraded basement membrane fragments (laminin-5) play an important role in mammary epithelial cells migration [6].

Lymphatic nodules are present just in the mammary gland tissue of female rats. Lymphatic capillaries in the connective tissue of the lobule posses discontinuous basement membrane. Large lumen of the lymphatics is mostly irregular (Fig. 2).

Rat Mammary Gland Development and Tumorigenesis

The mammary buds develop in form of solid downgrowths from mammary crests of ectoderm extending from the axillary to the inguinal region. The postnatal period of mammary gland development is characterised by intensive cellular proliferation, extracellular matrix (ECM) remodeling, and epithelial invasion into the mammary fat pad, as ductal morphogenesis occurs [35]. Developmental stages in humans and in rodents are very similar [39]. This process involves growth, proliferation, migration, branching, invasion, apoptosis and these processes take place simultaneously during a few weeks to create a differentiated gland [16]. Processes present in normal gland development also occurs in neoplasia and the development of the malignant disease, as for the developmental pathways that influence branching, appear during the development or progression of cancer [53]. Mammary gland development is driven by terminal end buds (TEBs). TEBs contain undifferentiated epithelial cells, continuing in ductal elongation and branching. These highly proliferative structures are solid or semi-solid bulbous clusters of immature epithelial cells (Fig. 3) at the ends of ductules [30]. Mammary tissue during the development is embedded in a mass of preadipocytes, adipocytes and fibroblasts, referred to as the fat pad. The epithelial cells are separated from the fat pad by thin layer of colagenous stroma [16, 20, 44]. The growth of the mammary gland starts from the nipple area and progress into the fat pad (Fig. 4). Fatty stroma is also the appropriate environment for mammary tumor growth [7]. Bulb-shaped buds (the primary mammary rudiment) after penetration of the underlying mesenchyme contact the preadipocytes that become the mammary fat pad [53]. The branching of the ducts can begin in the fat pad environment and the embryonic mesen-

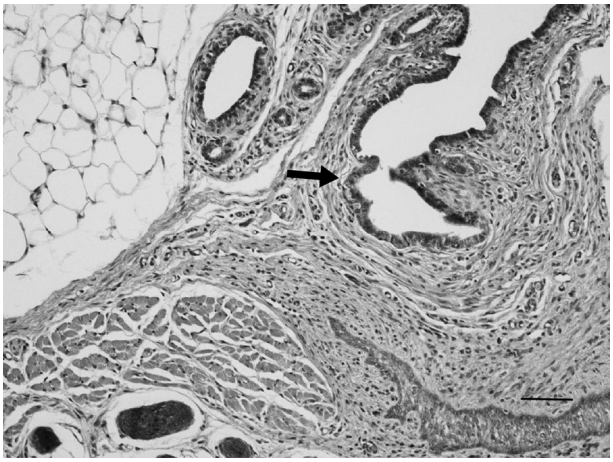

Fig. 4. The lactiferous duct (arrow) near the base of the nipple in mammary gland of 14 days old female rat (HE). Bar=20 μm.

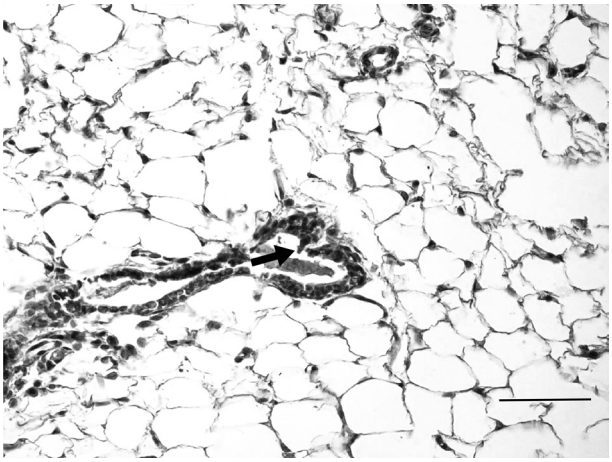

Fig. 5. Groving and bifurcation of TEB (arrow) in mammary gland of 14 days old female rat (HE). Bar=20 μm.

chym can transform into mammary-specific mesenchyme, which later develops into connective tissue with fibrous cords, forming blood vessels and the lymph system [15]. Fibrous tissue stroma serves as a communicating bridge between mammary epithelia and their environment during the development. Solid downgrowth of epithelial cells occurs in response to an inductive response from the mesenchyme and mesenchymal components control the branching pattern of the epithelium [53]. Stromal and epithelial cells communicate through the ECM. Stromal macrophages take part in ductal outgrowth and branching and in matrix remodelling [21]. Understanding of branching in the mammary gland is rapidly improving, but the complete mechanism and details are still object of study.

TEBs are apparent after 2 weeks of age, and reach their maximal number by 21–28 days of age in the rat [17]. These structures are of clinical importance because they are believed to be the most susceptible targets for chemical carcinogens [35, 41]. TEBs contain one or more loosely attached layers of cells at the leading edge, similar in appearance to the cap cells in mice [30]. Elevated levels of circulating growth hormone and estrogen attach to the epithelial cap cells on TEB tips through basement membrane. As the duct grows, multipotent cap cells can differentiate. These relatively undifferentiated epithelial stem cells can give rise to both luminal epithelium and myoepithelial cells. TEBs cavitate to form terminal ductules and this canalization involve antiadhesive mechanisms that promote the separation of apposed membranes and the apoptotic and consistent autophagic removal of cells from the forming luminal space [53]. Each terminal end bud bifurcates into two smaller ductules (Fig. 5) or alveolar buds [11, 30]. The term alveolar bud is used for those structures that appear morphologically more developed than the terminal end buds [37]. Alveolar buds are equivalent to terminal ductal lobular units (TDLU) in humans [30]. From day 21 of age, the alveolar buds increase in number [38], and generally consist of three to five blind ductules in a cluster [30]. The regulators of mammary gland morphogenesis include hormones, growth factors and receptors, cell cycle regulators and adhesion molecules. The assumption about role of the female steroid hormones in mammary gland development, was partly corrected by the ascertainment that proliferating mammary cells of the ducts and TEBs do not express steroid hormone receptors [2, 9]. The steroid receptor-expressing cells are distinct from, but often adjacent to the cells expressing markers of proliferation [16]. Adolescent branching requires estrogen receptor-α (ER-α), adult tertiary side-branching requires progesterone and its receptor, but embryonic branching is hormone independent, because it occurs also in mice lacking ER-α, ER-β, PR or the receptors for growth hormone and prolactin [3, 4]. The mammary gland develops during puberty to a branched epithelial network of ducts which can support alveolar development and subsequent milk production during pregnancy and lactation. The alveolar buds undergo differentiation to form lobules, which expand progressively over multiple estrous cycles (puberty is defined as the onset of estrous cycles, in the rat between days 35 and 42 of age).

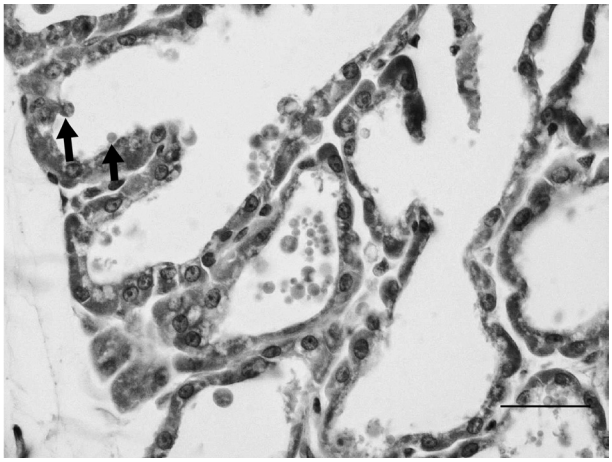

Fig. 6. Apocrine secretion (arrows) in lactating gland (HE). Bar=10 μm.

Intensive ovarian hormone-dependent increase in growth rate is observed shortly before the estrous cycling. After day 55 of age, the number of lobules remains stable. After day 50–55 of age development of mammary gland may be partially resemble early pregnancy [19, 30]. Fluctuations in mammary cell proliferation related to the oestrous cycle have been detected in pubertal rodents [26].With each estrous cycle, the TEB proliferate and branch, to give rise to terminal ductules and alveolar buds [14]. Hvid *et al.* [14] observed proliferation of the mammary epithelium mainly in the terminal end buds in the young animals whereas proliferating cells in mature animals were more equally distributed in the different compartments of the mammary gland. Epithelial proliferation was significantly increased during metestrus compared to the other phases. But increased proliferation during diestrus and proestrus may be also observed. The estrous cycle in female rats is of 4–5 days duration and in four phases: proestrus (12–18 h) estrus (25–38 h), metestrus (5–8 h) and diestrus (47–58 h). With increasing age, rats gradually develop an irregular estrous cycle and eventually persistent anestrus. In Sprague Dawley rats, gradually prolonged proestrus and estrus can progress to persistent estrus, which can be followed by pseudo-pregnancy after which persistent estrus can resume before persistent anestrus occurs. Estrous cycle irregularities and associated changes in endogenous concentrations of estradiol, progestrone and prolactin are likely to have an effect on mammary gland proliferation [14].

Throughout the estrous cycle variations in mammary gland development occur [30] and the gland undergoes a limited version of the life cycle observed during pregnancy, lactation and involution [1].

During pregnancy, intensive ductal side branching and lobuloalveolar formation are observed and the alveolar epithelium is near maximal in size. The extralobular and the intralobular stroma are decreased. Intensive increase in cell number occurs in mammary gland during lactation (Fig. 6), with a peak during the first days of lactation [11, 30]. Cell turnover during lactation is indicated by the presence of apoptosis although at a low frequency [28, 30, 33].

Early adulthood in rats is a time during which the breast is more susceptible to the initiation of carcinogenesis, decreasing with aging. Target cells will become the stem cells of the neoplastic event [37]. The higher incidence of breast cancer is observed in nulliparous women and in women having early menarche. The protection afforded by early pregnancy in women is justified by the higher degree of differentiation of the mammary gland. Cell proliferation is of importance for cancer initiation, while differentiation is a powerful inhibitor [37]. Practical experience suggests that parity-induced protection against breast cancer is principally dependent upon the timing of a first full-term pregnancy rather than on its occurrence itself [18]. Although sign of disease is often not evident until midlife, it has been hypothesized that breast cancer is initiated early in a woman's reproductive life. Differentiation of the mammary gland may inhibit carcinogenesis because cancer initiation requires the interaction of a carcinogen with an undifferentiated and highly proliferating mammary gland epithelium [28, 30]. Factors that modify the developmental profile of the gland may also modify breast cancer risk. One of important modulators of mammary growth and differentiation is inhibin. The synthesis of inhibin by the ovaries is stimulated by gonadotrophic hormones. Inhibin which regulates cellular proliferation and differentiation is expressed in a wide variety of tissues. Immunoreactive inhibin in breast tissue is synthetised by mammary epithelial cells [31]. The presence of inhibin is considered a marker of early differentiation and the decreased expression of inhibin in neoplastic cells is relevant for tumor growth [37].

Loss of the secretory alveolar epithelium during involution after weaning, is due to decreased circulating prolactin [10, 30], mild ischemia, factors related to milk producing cell death [51], and increased activity of base-

ment membrane-degrading enzymes [29, 30, 48]. In some other species, alveoli and secretory duct structures collapse by apoptosis and autophagy for lack of growth promoting factors either from the extracellular matrix or circulating hormones [53]. At the same time, apoptosis of blood capillary endothelial cells speeds up the regression of lactating ducts. Apoptosis of the luminal epithelium appears before changes in the basement membrane [8, 28, 30, 33]. During involution, an increase in the adipocyte areas and a decreased size of the alveolar lobules is apparent. Large ducts may contain cellular debris as well as basophilic amorphous exsudate, apoptotic nuclei, and lipid. Lobules contain increased interalveolar stroma [30].

The post-natal life cycle of the mammary gland involves morphological and functional changes reflecting its transient function of lactation. Puberty and pregnancy are characterised by epithelial cell proliferation and tissue organisation [22, 42]. More differentiated mamary gland during lactation exhibits high metabolic and synthetic activity, whereas during involution apoptotic cell death accounts for the return of the gland to the resting state [47]. Angiogenesis, the formation of new capillary networks from preexisting blood vessels is connected with higher requirement for nutrients and oxygenation, and is a process fundamental to both normal and malignant development. In the mammary gland the regulators of angiogenesis are produced by the mammary parenchyma thereby dictating its own vascularization. In contrast to most of organs, where the angiogenic potential is used in response to injury, the mammary parenchyma is constantly regulating its vasculature [5].

The finding that the most undifferentiated structures develop more aggressive neoplasia is clinically important because these structures are numerous in the breasts of nulliparous women who are at a higher risk of developing breast cancer [37]. Many aspects of mammary gland development are not completely clear. Mammary gland development requires hormones, growth factors, and cytokines that participate in cross-talk between its epithelium and mesenchyme. During morphogenesis of the gland, mesenchyme determines the way in which mammary epithelium will develop [43]. Sakakura *et al.* made experimental isografts of mouse mammary epithelium combined with salivary mesenchyme or mammary mesenchyme and transplanted them into syngeneic recipients [42]. Mammary gland morphogenesis was mesenchyme-dependent, and cytodifferentiation was epithelium specific. In the absence of mesenchyme, the epithelium degenerates. Several factors have been postulated to mediate the crosstalk between the stroma and epithelium during mammary gland development. One such factor is patched-1 (Ptc-1), the receptor for the secreted signaling protein hedgehog (Hh). Another factor is parathyroid hormone-related protein (PTHrP), which likely provides a direct epithelium-to-stroma signal. A third is insulin-like growth factor-I (IGF-I), a requisite factor in mammary gland development. IGF-I is induced by and mediates the function of growth hormone (GH) and the GH receptor [53]. The fibroblast growth factor (FGF) signaling pathway also contribute to mammary line specification [13]. Cell adhesion to ECM is necessary for development of the mammary gland, and to maintain the normal architecture and function of the gland. Cells adhere to the ECM via the integrin family of trans-membrane receptors, which signal to control mammary-specific gene expression and regulate cell proliferation and survival [24]. Integrins as main ECM receptors of mammary epithelium are checkpoints for the normal proliferation of mammary cells during development [27]. Integrins signal directly to cell cycle via the adhesion complex protein talin, which is necessary for focal adhesion kinase activation and p21 suppression, and thereby providing the conditions for growth factors to drive cell cycle [50]. The function of integrins as adhesion checkpoints for hormone and growth factor signalling is crucial for maintaining the normal architecture and integrity of mammary tissue [23]. Adult mammary fat pad retains the ability to support morphogenesis of fetal and adult mammary epithelia [43]. Histological evidence suggest that fatty stroma promotes elongation of ducts by synthesizing basement membrane components such as laminin. Each developmental state has a unique ECM protein content, which regulates mammary gland differentiation and gene expression. The changes in gene expression include not only laminins, but also fibrillar collagens type I, III, and V, bead-filament collagen VI, collagen IX, basal lamina collagen IV and collagen-associated proteins known to effect cross linking such as elastin, fibrillin 1, decorin, lumican, and biglycan [24]. The control of not only ECM deposition, but also degradation, is an important regulator of the formation of the ductal tree, as appropriate stromelysin-1, -3, gelatinase, and matrilysin are necessary for branching morphogenesis to occur [52]. Crosstalk be-

tween the mammary epithelium and extracellular environment is crucial for the proper patterning and function of the normal mammary gland. Disruption of the mentioned communication can induce cancer. On the other hand, many of the factors implicated in breast cancer are vital for mammary development [53]. It has been concluded already in the publication of Sinha *et al.* [45] that cell proliferation index at the time of carcinogen treatment is important for tumor induction but at the same time it does not determine whether or not the tumor is malignant; according to this authors the malignancy of induced tumor is determined by the presence or the absence of ovarian hormones. The process of mammary gland differentiation is the result of complex interactions of ovarian, pituitary, and placental hormones, which in turn induce inhibition of cell proliferation, downregulation of estrogen and progesterone receptors [46]. Among the hormonal influences, a major role has been attributed to the unopposed exposure to elevated levels of estrogens [15]. This knowledge has to be based on studies of the breast development, influence of hormones on the differentiation of individual structures, and their interactions in the pathogenesis of breast cancer. The development of breast cancer strongly depends on the ovary and on endocrine conditions modulated by ovarian function, such as early menarche, late menopause, and parity. Despite the numerous uncertainties surrounding the origin of cancer, there is evidence that breast cancer risk relates to endocrinologic and reproductive factors. However, the specific hormone combinations responsible for cancer initiation have not been identified, and their role as protective or risk factors is still incompletely understood [37]. Why the virgin mammary gland retains its higher susceptibility to carcinogenesis, and mechanisms through which an early pregnancy protects against cancer development remains not completely answered. Residual stem cell or progenitor population not removed by terminal differentiation is also under consideration by various researchers [5]. Whether and how perturbations in epithelial − mesenchymal interactions and extracellular matrix composition participate in neoplastic transformation in humans is yet also not resolved. These unresolved questions remain a challange to be continued in experimental research.

Conflict of interest

We declare that we have no conflict of interest.

Acknowledgments

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