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REVIEW

Anatomy of the Human Mammary Gland: Current Status of Knowledge

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Mammary glands are unique to mammals, with the specific function of synthesizing, secreting, and delivering milk to the newborn. Given this function, it is only during a pregnancy/lactation cycle that the gland reaches a mature developmental state via hormonal influences at the cellular level that effect drastic modifications in the micro- and macro-anatomy of the gland, resulting in remodeling of the gland into a milk-secretory organ. Pubertal and post-pubertal development of the breast in females aids in preparing it to assume a functional state during pregnancy and lactation. Remarkably, this organ has the capacity to regress to a resting state upon cessation of lactation, and then undergo the same cycle of expansion and regression again in subsequent pregnancies during reproductive life. This plasticity suggests tight hormonal regulation, which is paramount for the normal function of the gland. This review presents the current status of knowledge of the normal macro- and micro-anatomy of the human mammary gland and the distinct changes it undergoes during the key developmental stages that characterize it, from embryonic life through to post-menopausal age. In addition, it discusses recent advances in our understanding of the normal function of the breast during lactation, with special reference to breastmilk, its composition, and how it can be utilized as a tool to advance knowledge on normal and aberrant breast development and function. Finally, anatomical and molecular traits associated with aberrant expansion of the breast are discussed to set the basis for future comparisons that may illuminate the origin of breast cancer. Clin. Anat. Clin. Anat. 00:000–000, 2012.

Key words: breast; mammary gland; anatomy; histology; stem cells; lactocytes; lactation; breast cancer

INTRODUCTION

The mammary gland is an organ unique to the class Mammalia, with the specific function to synthesize, secrete, and deliver milk to the newborn upon demand for its optimal nourishment, protection, and development (Medina, 1996). Milks from different mammalian species vary in composition and are uniquely appropriate for the species for which the milk was synthesized. In humans, the life cycle of the female mammary gland is epitomized by drastic

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changes in composition, architecture, and functionality, mediated by marked changes in gene expression, that characterize its physiological stages of development, all of which are aimed at allowing it to perform its function as a milk-producing organ with the birth of the infant. The key mammary developmental stages include fetal growth, infant (prepubertal) growth, pubertal expansion, pregnancy- and lactation-associated remodeling, and post-lactational and post-menopausal involution (Russo, 2004; Geddes, 2007). A sound knowledge of the development, anatomy, physiology, and regulation of the breast is integral in the understanding of both the normal biology and function of this organ and its benign or malignant pathologies and their successful treatment.

Unlike most other organs of the body, which develop to a relatively mature state during embryonic life, the mammary gland reaches a mature functional state only during the pregnancy-lactation cycle (PLC) in the adult female. Therefore, this is the most important developmental stage of the breast when it is characterized by a very high metabolic demand, requiring about 25% of daily maternal energy intake during lactation to produce milk (Hartmann, 2007). Human breastmilk has a unique biochemical and cellular composition, providing the infant with optimal nutritional, protective, and developmental factors. Due to this, the American Academy of Pediatrics (AAP, 2005) and the World Health Organisation (WHO) recommend breastfeeding (or the provision of mother's own milk) for all infants, including high risk and premature infants. Despite this, few women, especially in developed countries, reach current recommendations for breastfeeding duration, which state exclusive breastfeeding to six months postpartum, with breastfeeding to continue up to two years or beyond. This situation suggests inadequate support of the mother-infant dyad (term and preterm) to achieve and maintain a successful lactation, demonstrating a clear need to bridge scientific investigation of breast function and development of evidence-based medical treatment.

In addition to pathologies associated with successful lactation, breast cancer is another devastating pathology of the mammary gland, with current statistics reporting it as the most frequent cancer and cancer-related cause of death in women worldwide (AIHW, 2010). Despite considerable scientific effort into elucidating the primary cause of breast cancer, the differing responses to therapy and characteristics of its various subtypes (Perou et al., 2000) together with the lack of basic understanding of the physiology of the breast have hindered the development of appropriate preventative guidelines and treatment options for these patients. This review summarizes the current knowledge of the normal anatomy of the human breast and the distinct changes it undergoes during the key developmental stages that characterize it. In addition, anatomical and molecular traits associated with aberrant expansion of the breast are discussed to set the basis for future comparisons that may illuminate the origin of breast cancer.

DEVELOPMENT OF THE BREAST

Breast development during life follows a time course of distinct phases. Beginning with the formation of the mammary crest and subsequent primitive mammary buds during embryonic life, it continues with minimal growth during infancy followed by a rapid growth phase at puberty in the female. Breast development culminates during the pregnancy and lactation cycle (PLC) when the mammary gland undergoes complete remodeling, maturating into a functional milk-secretory organ. Regression of PLC-induced growth is initiated as weaning is commenced and is completed after involution when the breast regresses to a resting state. Remarkably, the PLC-induced mammary growth and subsequent involution can be repeated at multiple pregnancies during the reproductive life of a female. The cycle is completed with a further phase of involution post-menopause. Full development of the breast during lactation is critical to provide the appropriate volume and composition of breastmilk for the growth, protection, and development of the infant. Further, it has been shown that the PLC is protective against the development of breast cancer in the long term (Key et al., 2001), particularly when it occurs before the age of 30 and with an interval of less than 14 years between menarche and the first pregnancy. Multiparity confers slightly more protection; however, the effect is not as great as that of the first pregnancy (Russo, 2011). The above strongly suggest that pregnancy and lactation induce permanent breast changes through which they exert a protective, yet still not well understood, effect against breast malignancy.

Fetal Development

Embryonic development of the breast is initiated by six weeks of gestation from a thickened ectodermal ridge extending from the groin to the axilla on the anterior surface of the embryo (milk line). The entire ridge apart from the pectoral region (second to sixth rib) regresses to form the mammary gland. Supernumerary glands may develop at any location along the ectodermal ridge presenting as either mammary glands or accessory nipples in 2–6% of women (Vorherr, 1974; Schmidt, 1998; Russo, 2004).

Between week 7 and 8 of gestation (embryo 10–11 mm), the mammary parenchyma invades the stroma forming an elevated portion termed the mammary crest. A basement membrane separates the invading ectodermal cells from the underlying mesoderm. Between 10 and 12 weeks gestation (embryo 30–68 mm), mammary epithelial buds form, a phase that marks the commencement of distinct differentiation patterns. At this stage, subtle differences can be seen between males and females, such as the female ovoid versus male spherical budding shape, and the female smaller bud volume. The smooth musculature of the areola and nipple are formed between 12 and 16 weeks. The appearance of the mammary buds does not change significantly until weeks 13–20, when a depression is formed at the surface of the buds and

proliferation of distinct epithelial progenitor populations results in secondary bud formation and branching. At around 20 weeks gestation (fetus = 10 cm), secondary buds that have gradually elongated appear as 15–25 solid cords, which grow into the stromal tissue reaching the subcutaneous tissue below the mesenchyme (Hovey, 2002; Russo, 2004). Branching and canalization of the cords results in the formation of monolayered primary milk (lactiferous) ducts by 32 weeks gestation (Hovey, 2002). The degree of mammary development at birth varies from simple tubules to branching ducts; however, no relationship has been reported between the developmental state of the breast at birth and its potential to expand and functionally mature during life (Anbazhagan and Gusterson, 1994; Osin et al., 1998; Howard and Gusterson, 2000).

Formation of the mammary vascular system begins as mesenchymal cells differentiate at 7 weeks into erythroblasts and primitive blood vessels. This is followed by the appearance of small capillaries between 9 and 10 weeks, and subsequently the formation of a concentric vascular network 12–13 weeks gestation. Development of the vascular system is complete by week 16 as blood is circulated to the skin and the secretory, adipose, and connective tissues of the gland.

In the final eight weeks of gestation, the periductal stroma increases in density along with a limited amount of lobulo-alveolar development (Naccarato et al., 2000). The ducts open onto the nipple area at 32 weeks gestation (Tobon and Salazar, 1974). Pigmentation of the skin around the nipple and development of Montgomery glands also occurs. It is believed that the adipose tissue of the mammary gland, which is considered essential for mediating further growth of the mammary parenchyma via signaling cascades, is formed by specialized connective tissue from the deeper subcutaneous mesenchyme that has lost its capacity to form fibers (Vorherr, 1974). Growth hormone is thought to play an important role in mammary epithelial expansion and development both during fetal and adult life, as evidenced by mouse studies (Nandi, 1958). Interestingly, maternally derived lactogenic hormones present in the fetal circulation at birth can cause production of small amounts of colostrum that can be expressed from the infant's mammary glands shortly after birth. Regression of the infant's mammary gland normally occurs spontaneously within four weeks postpartum and coincides with a decrease in infant prolactin levels (Vorherr, 1974; Russo et al., 1982).

Neonatal and Pre-pubertal Development

The breast of a newborn consists of rudimentary ducts that have small club-like termini that regress soon after birth. Until early childhood, the mammary gland remains at an immature resting state with minimal further development and virtually no differences in structure between males and females (Russo, 2004). Growth of the gland is isometric prior to puberty with allometric growth of both the mammary epithelium and stroma initiated at puberty (8–12 years) (Russo, 2004).

Puberty

Puberty induces rapid breast growth driven by ovulation and the establishment of regular menstrual cycles. Increase in breast size is mainly due to increased deposition of adipose tissue within the gland (Russo et al., 1987). However, distinct changes of further epithelial and stromal development are also observed and are fueled by an ovarian hormonal circuit that acts on mammary stem cell (MaSC) populations thought to exist in the basal ductal layer (Neville et al., 2002; Anderson and Clarke, 2004; Russo, 2004; Visvader, 2009; Asselin-Labat et al., 2010). These changes include elongation of the existing ducts and branching into secondary ducts at the termini of which bi-layered epithelial buds appear and form clusters called lobules (Russo and Russo, 1992; Russo, 2004). Typically, the mammary mini-remodeling at each menstrual cycle does not fully regress at the end of the cycle. Thus, compounding epithelial development continues gradually during adolescence through to adult life until approximately the age of 35 (Russo and Russo, 1992; Russo, 2004). In the mature virgin, three distinct types of lobules have been observed, which have been classified according to their degree of development. Lob 1 consists of approximately 11 ductules, Lob 2 of about 47 ductules, and Lob 3 of approximately 80 ductules (Russo, 2004). The proportions of each lobule type vary widely among individuals. Despite this, a shift from Lobs 1–3 to a fourth lobule type (Lob 4) containing mature alveoli is typically observed in women during pregnancy.

Pregnancy

Although a mini-remodeling of the breast occurs at each menstrual cycle, it is not until a PLC that a complete remodeling of the breast occurs as it gradually transforms into a fully mature functional organ. This remodeling takes effect via changes in circulating hormonal complexes that activate MaSCs.

The maturation stages are directly regulated by increased levels of the circulating lactogenic hormone complex (estrogen, progesterone, and prolactin) that induces ductal branching, alveolar morphogenesis, and secretory differentiation (Pang and Hartmann, 2007). Other hormones and growth factors that are direct regulators of mammary expansion during pregnancy include placental lactogen, epidermal growth factor, TGFα, and stromal paracrine factors, whereas insulin, growth hormone, glucocorticoids, and fibroblast growth factors are involved indirectly (Medina, 1996; Czank, 2007). Blood placental lactogen has been shown to be strongly correlated with breast growth (Hartmann, 2007), probably via stimulation of stem/progenitor cell proliferation. During pregnancy, an initial phase of cellular proliferation of MaSCs and progenitor cells results in *de novo* synthesis of new ducts, elongation of the existing ducts via mitotic activity in the terminal end bud, extensive epithelial branching, and formation and expansion of spherical structures called alveoli at the terminal buds (mammogenesis) (Sternlicht et al., 2006a; Sternlicht et al., 2006b). Each alveolus is embedded within the stroma

and separated from it via a basement membrane. It consists of a basal mesh-like layer of myoepithelial cells surrounding an epithelial cell layer that encapsulates the alveolar lumen (Russo et al., 2001; Russo, 2004; Sternlicht et al., 2006c; Watson and Khaled, 2008). The myoepithelial cells display phenotypic and functional properties of smooth muscle cells. Triggered by bound oxytocin stimulated by infant suckling, they contract resulting in the expulsion of milk from the alveolus through the ductal lumen toward the nipple (milk ejection). Oxytocin is released in a pulsatile fashion resulting in milk flow from the alveoli, expansion of the ducts and increased intraductal pressure. Milk ejection is the period of time when milk is available to be removed by either the breastfeeding infant or the breast pump, and in its absence little milk can be removed. It has been recently demonstrated that each mother has a distinct and relatively consistent pulse profile, at least during breast expression, with wide differences observed among women (Prime et al., 2011).

In the second trimester of pregnancy and following the expansion phase (alveolar development/mammogenesis), gradual increases in prolactin levels stimulate cellular differentiation at the alveolar sites, where mammary epithelial cells of the luminal layer further differentiate into lactocytes (Czank, 2007). This secretory differentiation (Lactogenesis I) occurs around 24 weeks gestation and is often accompanied by accumulation of first secretion (colostrum) within the alveoli and ducts.

Pregnancy-induced breast changes are clinically reflected as an increase in breast volume and in most women are complete by week 22. However, there is an enormous variation in breast growth between women ranging from either little or no increase to a considerable increase in size that can occur either rapidly during the first trimester or more gradually over the entire pregnancy (Cox et al., 1999). Therefore, breast size during pregnancy is not a reliable indicator of lactation potential, particularly since it does not reflect the amount of secretory tissue contained in the breast. It is of note, however, that mothers who deliver preterm ($<$28 weeks) may interrupt the development of the breast. This may impact on lactation efficiency, resulting in delayed secretory activation (Lactogenesis II) and a reduction in milk production in the first week(s) postpartum (Henderson et al., 2003). Further studies are required to elucidate the degree of mammary development in term versus preterm women and investigate potential avenues for improvement of lactation efficiency in preterm women. Importantly, the cellular component of mammary secretions may be used as a potential indicator of mammary development, thus providing a useful tool of assessment of lactation potential.

Lactation

Secretory activation allows a rapid up-regulation of milk synthesis and typically occurs 48–72 hrs after parturition triggered by a decrease in circulating progesterone (after delivery of the placenta) and further increase in prolactin levels (Suzuki et al., 2000; Czank, 2007; Pang and Hartmann, 2007). Blood prolactin levels are high during early lactation and gradually decrease as lactation progresses (Jacobs, 1977; Cox et al., 1996). The effects of prolactin in the mammary gland during the PLC are complex as it has been shown to stimulate not only milk synthesis, but also cell proliferation (Neville et al., 2002). This may be a potential mechanism allowing concurrent regeneration and differentiation of the lactating epithelium and dynamic maintenance and turnover of the secretory tissue during the course of lactation. It may also suggest changing/various function(s) of prolactin during the course of lactation (Czank, 2007). After parturition, a characteristic change in the integrity of the basement membrane separating the mammary stroma from the epithelium is also observed, with tightening and reduced permeability. This serves to control systemic and stromal signaling to the mammary epithelium as well as movement of milk components or their precursors from the systemic circulation into either the alveolar lumen or the lactocyte.

Colostrum is usually present for the first 3–5 days after parturition followed by transitional milk until about weeks 2–3 postpartum, after which breastmilk is considered mature. Colostrum has a distinct biochemical and cellular composition aimed at providing enhanced immunological protection and nutritional and developmental support to the newborn. In addition to high concentrations of factors providing immunological support/protection, such as immunoglobulins, lactoferrin, oligosaccharides, and active viable immune cells, it contains cell proliferation-inducing factors that are thought to promote development of the newborn's gastrointestinal tract and stimulate hematopoiesis and immune maturation (Bessler et al., 1996; Playford et al., 2000; Bode and Jantscher-Krenn, 2012). It has been suggested that the one–two-day delay in the onset of secretory activation after parturition in women may function to maximize exposure of the infant to the immune-modulatory protective factors of colostrum (Hartmann, 2007) at a period when its own immune system is still immature. At the same time, colostrum contains a higher protein content (30–70 g/L or 3–7%) than mature breastmilk (7–25 g/L or 0.7–2.5%), which may provide an additional benefit to the newborn in the first few days after birth (Saint et al., 1984; Playford et al., 2000; Mitoulas et al., 2002; Saarela et al., 2005). It remains to be established how the very low protein content of mature breastmilk meets the growth requirements of the human term infant at a period when human growth rate is at its maximum across the lifespan. Indeed, when compared to bovine milk, human milk contains at least three times less protein (Frank, 1988). This suggests that animal milk-based infant formulas containing higher amounts of protein and different ratios of protein types (e.g. caseins versus whey proteins) may induce changes in infant development that may be associated with diseases in both the short- and long-term. For example, it is well established that the higher rate of curding of cow milk-based infant formulas in the baby's stomach is associated with slower gastric emptying and different col-

onization of the gut compared to infants fed breastmilk (Meier et al., 2010). Additionally, formula-feeding has been associated with higher risk of overweight and obesity later in life (Stettler, 2011; Pirila et al., 2012). Future research addressing the superiority of human milk protein content and quality for human infant growth may provide a basis for improvement of current recommendations for not only infant nutrition, but also adult nutrition.

The current understanding is that both the composition of the mature breastmilk and the structure and composition of the breast do not change significantly during lactation until the reduction and/or cessation of milk removal from the breast. Nevertheless, changes in the fat and cell milk composition in response to feeding have been previously reported (Kent et al., 2006; Hassiotou et al., 2012b). It is not yet clear whether these short-term cellular changes observed in breastmilk reflect regional short-term changes in the alveolar micro-development and structure. At the same time, a cross-sectional study examining cellular populations of breastmilk collected at different stages during lactation demonstrated changes in biomarker expression at the protein and the mRNA levels with lactation, suggesting corresponding changes in the breast epithelium (Hassiotou et al., 2012e). This, together with the epidemiologic evidence documenting a protective effect of long breastfeeding duration against breast cancer support the notion that the PLC induces permanent changes in the breast, which are at least partially effected during the course of lactation.

Although pregnancy and lactation in women are generally studied as separate phenomena, it is not uncommon for women to breastfeed one child while being pregnant with the next. This is especially common in more traditional societies where women breastfeed their children for long periods (Merchant et al., 1990). Pregnancy concurrent with lactation has not been investigated in detail in women, but it usually results in reduction of milk supply and/or cessation of milk production, particularly in the second half of pregnancy, as it is also evident in dairy cows (Merchant et al., 1990). Merchant et al. (1990) reported that among the women examined who were pregnant and concurrently breastfeeding, 41.4% continued to breastfeed into the second trimester of pregnancy, with this value decreasing to 3.2% in the third trimester. Milk composition and appearance also change during this period, with milk having a more yellowish colostrum-like color. The mechanisms involved in pregnancy-induced reduction/cessation of milk production are poorly understood. Some studies in cattle suggest an association with increasing levels of plasma estrogen (Robertson and King, 1979; Bachman, 1982). It can be postulated that the increase in proliferation-induction factors during the course of pregnancy may counteract milk synthesis induction factors, since these two exert antagonistic effects. Furthermore, a feedback signaling loop between the lactocytes and the stem/progenitor cells in the mammary epithelium may exist, resulting in down-regulation of lactocyte milk synthesis and/or differentiation during a period critical for MaSC expansion. The above merit further investigation.

The effect of nutrition on the development of the mammary gland during the PLC has not been extensively studied in women. However, recent studies have highlighted that overweight and obese women are less likely to initiate lactation (Hilson, 1997; Donath, 2000; Li, 2003; Kugyelka et al., 2004; Mok, 2008; Liu, 2010), more likely to experience feeding difficulties due to problematic infant attachment (Mok, 2008), have shorter durations of lactation (Hilson, 1997; Donath, 2000; Li, 2003; Kugyelka et al., 2004; Baker, 2007; Mok, 2008; Liu, 2010), and are twice as likely to fail at breastmilk expression as women of normal weight. Interestingly, overweight and obese women who do express breastmilk tend to have longer breastfeeding durations than those who do not express (Leonard, 2011). Animal studies have shown similar results, drawing an association between obesity and lactation failure (Lovelady, 2005; Rasmussen, 2007). It has been shown that dietary intake in excess of energy requirements impairs mammary development and subsequent lactation performance (Sejrsen et al., 1982; Sejrsen and Purup, 1997; Kamikawa et al., 2009), suggesting a strong link between nutrition and normal mammary development and functionality. A 40% increase in energy intake in mice resulted in markedly abnormal alveolar development and subsequent delay in initiation of lactation (Flint et al., 2005). Conversely, a 40% restriction of energy intake in the first part of pregnancy in a rat model resulted in 46% increase in mammary cell proliferation and a 14% increase in milk yield (Kim and Park, 2004). It is therefore evident that diet and nutritional intake can influence lactation initiation, efficiency, and performance. The effects of diet on lactation performance or efficiency are likely to be mediated via modulation of hormonal action on the mammary gland (Ceriani, 1974). Given the numerous beneficial effects of breastfeeding and breastmilk feeding for both infants and mothers, it is important to further elucidate the impact of maternal nutrition on breast development and lactation.

The Pregnancy/Lactation Cycle and Breast Cancer

Breast cancer is a devastating disease affecting an increasing population of women. It develops during multiple alterations in the molecular signatures, function, and structure of the affected cells and is therefore characterized by various stages (Medina, 1996). These include cellular immortality, hyperplasia, tumorigenicity, and invasiveness and are structurally evident as an initial epithelial hyperplasia, which develops into cellular atypia and occlusion of the duct, intra-ductal carcinoma, and progression to a locally invasive carcinoma, which can metastasize to various organs, such as the lung, bone, and liver (Medina, 1996; Lu et al., 2009; Oskarsson et al., 2011). Breast cancer is a heterogeneous disease with different subtypes that are characterized by a distinct molecular signature, have different responses to therapy, and show differences in patient survival (Perou et al., 2000). It has been

suggested that distinct cell types or transformation of MaSCs with arrest at different developmental stages are behind each breast cancer subtype (Prat and Perou, 2009; Visvader, 2009). It is therefore important to elucidate what transforms a cell and under which conditions, with emphasis on protective factors that may reduce risk.

The protective effect of extended breastfeeding duration against development of breast cancer in the long term is well documented (Key et al., 2001). Indeed, a recent study demonstrated that breastfeeding for at least one year significantly reduced the risk of breast cancer diagnosed under the age of 50 (Kotsopoulos et al., 2012). Currently, the mechanisms through which these protective effects are mediated are unknown. However, a number of theories have been proposed. Initiation of breast cancer is considered to be associated with the degree of maturity of the mammary gland (Pike et al., 1983; Russo and Russo, 1992). Early and complete differentiation of the mammary gland conferred via breastfeeding has been suggested to confer protection against the development of breast cancer (Key et al., 2001; Kotsopoulos et al., 2012). Typically, the breast of nulliparous women (with or without breast cancer) comprises predominantly Lob 1 structures, whereas parous women free of cancer display a larger proportion of Lob 3 structures. By contrast, parous women with breast cancer have been shown to have a greater percentage of Lob 1 and a lower proportion of Lob 3 structures compared to parous women without breast cancer (Russo, 1994). Increasing evidence is showing that different types of breast cancer originate from different mammary epithelial subtypes, with the more aggressive breast cancers originating from mammary stem/progenitor cell populations and the less aggressive from more differentiated mammary cells (Stingl and Caldas, 2007; Visvader, 2009). Indeed, more cancer stem cells are present in poorly differentiated tumors than in well-differentiated tumors, and the former are associated with poor survival and high invasiveness (Pece et al., 2010; Zhou et al., 2010). It is suggested that longer breastfeeding duration allows for better depletion of the mammary gland in the stem/progenitor populations with proliferative capacity, therefore reducing the risk of development of aggressive breast cancer.

A recent meta-analysis examined critical pathways in breast development in the mouse using gene set enrichment analysis (Zhao et al., 2012). It was found that during the PLC, pathways associated with a number of different cancers are down-regulated in the mammary gland. This was partly in agreement with an earlier study, which had shown that stromal factors playing fundamental roles during mammary development are also associated with breast carcinogenesis, some protecting from and some promoting breast cancer (Wiseman and Werb, 2002). Indeed, the mammary gland during the PLC displays transient breast cancer-related characteristics, with a study in mice presenting evidence of Epithelial to Mesenchymal Transition (EMT), a breast cancer key feature, in the terminal end buds during the PLC (May et al., 2011). This was recently confirmed in humans (Hassiotou et al., 2011), strongly suggesting that at least some of the signaling pathways that support normal mammary morphogenesis during the PLC are also involved in breast cancer initiation and progression when they are aberrantly activated or suppressed. There is a need to further examine these pathways in the lactating breast and the cancer-infected breast with comparative studies to identify deregulation that might lead to cancer. Since it is difficult to obtain human lactating breast tissue specimens, breastmilk may offer a non-invasive and plentiful alternative to access the cellular hierarchy of the mature mammary gland.

Further research to elucidate how breastfeeding-induced mammary differentiation confers protective effects against breast cancer and to provide mechanistic insights into the pathways associated with normal and aberrant mammary development is necessary. In this respect, breastmilk is used as a useful tool for accessing the normal cellular hierarchy of the fully differentiated gland and comparing it with the cellular hierarchy of breast cancer subtypes (Hassiotou et al., 2012a). Indeed, self-renewal transcription factors have been shown to be shared between normal breastmilk stem cells and certain types of aggressive breast tumors, suggesting that it is the imbalance of certain gene regulatory networks that is at the origin of this disease (Hassiotou et al., 2012a).

Exposure to carcinogens through diet and other media as well as imbalanced levels of hormones and growth factors during the PLC, despite its brevity, are thought to influence breast cancer development via effects on gene expression, cell proliferation, and invasiveness. The relationship between cause and effect is still unclear; however, the migration-related increases in cancer incidence and the significantly reduced incidence of breast cancer in wild mice versus experimental mice suggest that high breast cancer incidence is anomalous reflecting a dietary origin of this disease, which may be preventable (Medina, 1996; Grover and Martin, 2002). Indeed, the American Cancer Society guidelines for cancer prevention highlight diet as a key factor in cancer development, stating that ''most of the variation in cancer risk across populations and among individuals is due to factors that are not inherited'' (Byers et al., 2002). An increasing body of evidence suggests that animal-based foods promote cancer whereas plant-based foods prevent it (Michaud et al., 2001; Byers et al., 2002; Kris-Etherton et al., 2002). This may be associated with certain growth factors present in animal-based foods which promote cell proliferation as well as with the presence of carcinogens generated during cooking or digestion of these foods (Grover and Martin, 2002). Certainly, long breastfeeding durations can reduce breast cancer risk potentially via depletion of the mammary cell population that is sensitive to factors inducing mutations and/or aberrant proliferation. A logical question that follows is whether elimination of these factors from our diet and lifestyle may reduce breast cancer risk further.

Post-Lactational and Post-Menopausal Involution

Cessation or significant reduction (weaning) of milk removal from the breast results in post-lactational involution, during which the mammary gland transitions to a resting non-lactating state (Hurley, 1989). Although in animals such as mice involution occurs within a few days after cessation of milk removal, in women it is a more gradual prolonged process, even at abrupt weaning (Hartmann and Kulski, 1978). During involution, clearing of the mammary alveolar cells occurs, to allow the regression of the breast into a non-functional organ until the next PLC. How cessation of milking or suckling and milk stasis trigger involution is still unclear, although some evidence suggests an inflammatory response associated with alveolar cell apoptosis (Hughes, 2012). Milk components such as α-lactalbumin have been suggested as potential apoptotic triggers (Hakansson et al., 1995; Hakansson et al., 1999). Undoubtedly, this is an orchestrated process that involves regulation of both systemic and local mammary-derived factors that control lactation and is associated with milk stasis. In addition to post-lactational involution, the breast undergoes a second phase of involution during menopause. Post-menopausal mammary involution is associated with ovarian functional decay and is characterized by reduction of the glandular breast tissue and increase in the adipose surrounding tissue (Hutson et al., 1985). It is not well established whether post-menopausal breast remodeling eliminates the potential for the breast to become functional again, for example, upon hormonal stimulation.

ANATOMY OF THE BREAST

An understanding of the gross anatomy of the breast and its variations has many clinical applications ranging from breastfeeding/lactation support to the detection, diagnosis, and removal of benign and malignant lesions. In this context, the epithelial cells lining the duct walls are at the origin of the majority of breast malignancies (Li et al., 2003), underscoring the importance of a thorough understanding of the anatomy of the breast.

The incidence of pathologies of the lactating breast is rising due to the increased age at which women are having their first child (Ventura, 1989; Stensheim et al., 2009). While lesions of the breast are less common during lactation, it is possible that a mass, either benign or malignant, may obstruct milk flow. Depending on the location of the mass, this can lead to a cascade of events where milk stasis causes blockage of ducts/lobes and often, mastitis. If not successfully managed, the above may result in reduction of milk synthesis and eventual involution of the glandular tissue proximal to the obstruction. Therefore, a fundamental knowledge of the anatomy of the breast enables better diagnosis and treatment of women whether or not lactating.

Mature Non-Lactating Breast

Current descriptions of breast anatomy are based on Cooper's dissections of lactating breasts (Cooper, 1840). However, there is renewed interest in investigating breast anatomy, particularly that of the ductal system, with the motive of better understanding the origins of breast cancer and the potential of localized intra-ductal therapies (Going and Mohun, 2006). The breast is composed of glandular (secretory) and adipose (fatty) tissue supported by a loose framework of fibrous connective tissue called Cooper's ligaments. The secretory tissue is drained by a ductal system that stores and transports milk to the nipple during lactation.

Nipple. The nipple is composed of longitudinal and horizontal smooth muscle fibers relating to the nipple base. These muscles either remain separate or are intermixed with longitudinal fibers often associated with the nipple ducts (Tezer et al., 2011). The nipple ducts are crenulated and approximately 0.5 mm in diameter (Taneri et al., 2006; Rusby et al., 2007), with horizontally orientated muscles located distally and which provide a sphincter-like function (Tezer et al., 2011). A median of 23–27 ducts at the base of the nipple has been consistently documented in histological sections of mastectomy specimens (Going and Moffat, 2004; Taneri et al., 2006; Rusby et al., 2007). These results are in conflict with other methods of investigation such that some ducts can be cannulated, but do not appear to enter the breast, and much fewer ducts (five to nine) appear to yield milk (Love and Barsky, 2004). Ductal branching within the nipple does not account for this discrepancy (Going and Mohun, 2006), making patency of the ducts a more likely explanation. Indeed, some ducts within the nipple cannot be traced to the nipple tip (Going and Moffat, 2004; Going and Mohun, 2006; Rusby et al., 2007). Rusby (Rusby et al., 2007) has further described the morphology of the nipple ducts in that they narrow substantially before exiting the nipple through pores on the surface of the nipple.

Breast ductal system. Standard textbook descriptions depict the ductal system as numerous small ductules that drain the alveoli merging to culminate in one main duct that dilates slightly to form a lactiferous sinus (2–4.5 mm) (Venta et al., 1994). The main duct then narrows at a 'waist' before it passes through the nipple and opens onto the nipple surface (Rusby et al., 2007). Generally, dilated ducts in the non-lactating breast identified by ultrasound imaging are associated with pathologies such as ductal ectasia, fibrocystic disease, intra-ductal adenoma, or malignancy (Stavros, 2004).

Lobes. In women, the glandular tissue is composed of lobes that comprise lobules containing 10–100 alveoli that are approximately 0.12 mm in diameter (Hartmann, 1991). Each breast lobe is generally considered to exist as a single entity (Cooper, 1840; Going and Moffat, 2004; Love and Barsky, 2004). However, serial sections (100-μm thick) of a mastectomized breast of a 69-year old woman in one study identified two connections between different lobes (16 lobes identified in total) (Ohtake et al.,

2001). Cooper (Cooper, 1840) had only ever-encountered one anastomosis during all of his dissections. Textbooks have also long described the lobes to be of equal size and arranged in a radial fashion, despite Cooper describing the intertwined nature of the lobes consistent with the inability to surgically excise a solitary lobe from the breast. Since then, the arrangement and volume of tissue associated with each lobe within the breast has been confirmed to be highly variable, showing up to 20–30-fold differences in lobe volume (Moffat and Going, 1996).

Histology. The resting breast consists of ductal epithelial tissue embedded within a fibrous stroma. Each duct wall is lined by two layers of epithelial cells: an inner layer that encapsulates the ductal lumen, and which contains cuboidal epithelial cells, some of which (typically those of the terminal duct) have the potential to further differentiate into milk-secretory cells (lactocytes) during lactation; and a basal/outer layer of contractile myoepithelial cells that tightly surround the luminal layer and have properties of smooth muscle cells (Figs. 1A–1D). The basal layer lies on the basement membrane and is thought to contain bi-potent MaSC populations (Visvader, 2009).

The presence of stem cells in this organ was first postulated based on its ability to expand and regress in a repeated fashion throughout adult life (Taylor-Papadimitriou et al., 1977). Passaging and maintenance of mammary luminal and myoepithelial cells in 2D cultures supported this argument. Elegant 3D mammosphere assays proved the presence of self-renewing bi-potent MaSCs and uni-potent progenitors in the resting epithelium (Dontu et al., 2003). The reconstitution of a cleared mammary fat pad by a single sorted MaSC, which formed a fully functional mammary gland in a mouse model (Shackleton et al., 2006) revolutionized the field of mammary stem cells. Nevertheless, most studies have been conducted in mice, and the human gland has mostly been studied in its resting state, which is not representative of the mature functional organ. Thus, the scarcity and quiescent state of MaSCs in the resting breast may partially explain the slow progression in the identification of markers specific to MaSCs. Increasing evidence is suggesting that the profile $CD49f^{high}/CD29^{+}/CD24^{low}$ characterizes a bi-potent stem cell population in the resting breast, able to differentiate into both the luminal and the myoepithelial cells of the mammary epithelium (Visvader, 2009; Asselin-Labat et al., 2010; Joshi et al., 2010). MaSCs are also thought to be marked by expression of Cytokeratin 5 (CK5), which seems to be highly specific to the basal layer both in the resting and the lactating breast (Fig. 1E).

Differentiation between cell types further along the mammary hierarchy is generally done using Cytokeratin 19 (CK19) for ductal luminal cells, Cytokeratin 18 (CK18) for alveolar luminal cells, and Cytokeratin 14 (CK14) for the myoepithelial cells, which are also positive for smooth muscle actin (SMA). Although cytokeratin expression is widely used to differentiate between these mammary epithelial subtypes, some investigations have shown that small numbers of CK19/CK14 or CK18/CK14 double positive cells are also present in the mammary epithelium, potentially reflecting transitioning cells, as CK14 is also thought to be a marker for MaSCs or myoepithelial progenitors (Gusterson et al., 2005; Villadsen et al., 2007). This further reinforces the presence of a hierarchical continuum within the mammary epithelium and signifies the need for better markers specific to the different cellular developmental stages along this continuum.

A group of ductal structures can be associated in a single lobule, with the central lobule ducts often being somewhat ''squeezed'' compared with the outer lobule ducts. The mammary stroma is highly fibrous compared with other species such as mice, consisting of dense fibrous connective tissue, which embeds adipose tissue (inter-lobular stroma). Enclosing the lobules, the intra-lobular stroma consists of mesenchymal cells that are highly responsive to hormonal micro-environmental cues and have been associated with initiation and progression of various stages of mammary development via cross-talk with the mammary epithelium (Bissell et al., 1999; Wiseman and Werb, 2002). Little is known about the signaling cascades between the stroma and the epithelium that fuel mammary development as well as of the histological changes within the stroma and the epithelium during the PLC and markers that identify specific cell types in the resting and the fully mature gland. We have recently shown that the cellular hierarchy of the lactating breast is represented in breastmilk, including early-stage stem cells, progenitor cells, and more differentiated myoepithelial and milk-secretory cells as key cellular types in a mammary developmental continuum (Hassiotou et al., 2012d).

Lactating Breast

Few studies have focused on the anatomy of the lactating breast since Cooper's (1840) extensive dissections of the breast of women that had died during lactation. Interestingly, Cooper used lactating cadavers because the structures of the breast of non-lactating women were too technically challenging to provide adequate information.

Nipple. Nipples differ widely in size and appearance between women. Nipple size typically increases during pregnancy and is related to plasma prolactin levels (Cox et al., 1996). Documented nipple diameters range from 9 to more than 23 mm (Cox et al., 1996; Ramsay et al., 2005; Wilson-Clay and Hoover, 2005) and large nipples have been implicated in breastfeeding difficulties, potentially due to problematic infant attachment.

Breast ductal system. While texts describe 15-20 ducts and lobes in the breast, Cooper identified up to 22 ducts but found that only 7–12 were generally patent (Cooper, 1840). Studies have since confirmed this number. Love et al. (2004) and Love and Barsky (2004) observed five patent nipple openings (range 1–17) in lactating women and five to nine nipple orifices in 10 non-lactating mastectomy

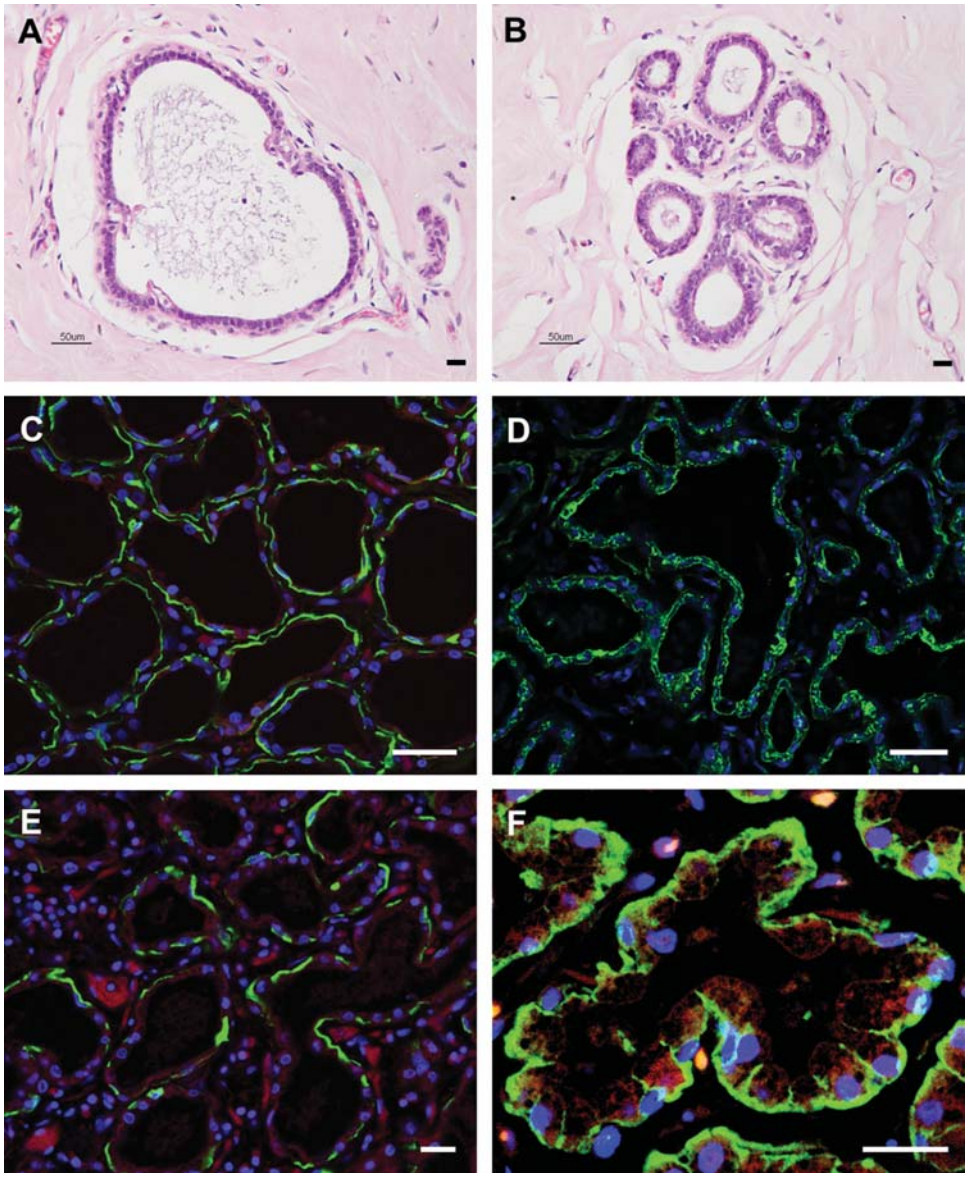

Fig. 1. Anatomy and histology of the human lactating breast. **A, B:** IHC of the human lactating breast showing **A:** a duct and **B:** a group of alveoli, embedded within the fibrous stroma. **C–F:** IF staining of human lactating breast tissue. **C:** Alveolar myoepithelial cells shown in green via staining of smooth muscle actin. **D:** Alveolar lactocytes shown in green via staining of alpha-lactalbumin. **E:** Mammary stem cells identified by Cytokeratin 5 staining (green) in the basal layer of a group of alveoli. **F:** Polarized luminal alveolar cells stained for EpCAM (green). Nuclei are shown in blue (DAPI staining). Actin is shown in red (phalloidin staining). Scale bars: 20 μm.

nipples. Ramsay et al. (2005) resolved an average of nine main milk ducts (range 4–18) at the base of the nipple of the lactating breast with 2D ultrasound, while Going and Moffat (2004) found that only four nipple ducts were connected to functional lobes in a lactating mastectomy. This supports the notion of localized activity and/or differential maturation/differentiation of different lobules within the same breast, suggesting that not all ductal systems need to be simultaneously functional to meet the demands of the infant (Gooding et al., 2010).

The sizes of the ducts in the lactating breast are often assumed to be larger than that of the non-lactating breast despite lack of supporting evidence. Ramsay et al. (2005) and Gooding et al. (2010) found the main ducts to be relatively small (2 mm, range: 1.0–4.4 mm), which is not dissimilar to that of non-lactating women (2–4.5 mm) (Venta et al., 1994), and which was further supported by ductography. This suggests that enlargement of the ducts does not necessarily occur. Variation in duct diameter within women is likely to be due to the amount of

milk contained or synthesized at any time within the breast (Ramsay et al., 2006), with increased milk associated with increased duct diameter. In addition, PLC-associated changes in duct diameter may be either linked to or controlled by ductal cell proliferation and expansion, which may differ among women, being regulated by gene expression and factors influencing it. Transient increases in duct diameter are also associated with milk ejection (Ramsay et al., 2004).

Cooper (1840) originally described the proximal ducts to be large 'sac-like' structures that contained significant amounts of milk. However, since then recent ultrasound studies have shown that these areas did not appear as typically described. Ramsay et al. (2005) found that the main milk ducts were relatively small with expanded areas coinciding with merging of ducts. This has since been confirmed by a small pilot study using 3D ultrasound imaging demonstrating enlarged ducts deep in the breast, with narrower ducts often visualized between the larger ducts (Gooding et al., 2010). The ducts beneath the areola are superficial (Ramsay et al., 2005) and are easily compressed. In this context, compression of ducts has been suggested as one factor that may to contribute to milk stasis and consequently blocked ducts (Geddes, 2009).

Lobes. Despite vast improvements in imaging techniques, the volume of glandular tissue in the breast has not been quantified. This is due to the intermingling of glandular and adipose tissue throughout the breast. Semi-quantitative measurement of both glandular and adipose tissue in ultrasound images obtained from 21 Caucasian mothers reported that the ratio of glandular to adipose tissue is approximately 2:1 (Ramsay et al., 2005). Enormous variability in proportions of tissue are observed among women, similarly with the non-lactating breast, with up to half the breast comprising glandular tissue to almost the whole breast consisting of glandular tissue. This provides further evidence that breast size is not indicative of lactation potential. Furthermore, the amount of fat situated amongst the glandular tissues is also highly variable and can vary not only between women, but also in a woman throughout adult life.

Histology. The breast reaches its full development only during pregnancy and lactation, when a complete remodeling of the different breast tissue types occurs. Under the effect of the lactogenic hormone complex and via cross-talk between the stroma and the epithelium, a massive expansion of the epithelium is observed, which results in reversal of the resting stromal/epithelial ratio. By the end of pregnancy, the breast is mainly composed of lobular highly branched epithelial tissue separated by some fibrous stroma. Toward the third trimester of pregnancy, secretory differentiation occurs in some luminal cells of the alveoli, resulting in formation of fat globules that are visible within the cells. Often, some colostrum can be expressed before birth, but it is the withdrawal of progesterone after birth that stimulates a cascade of signals associated with secretory activation and copious milk synthesis.

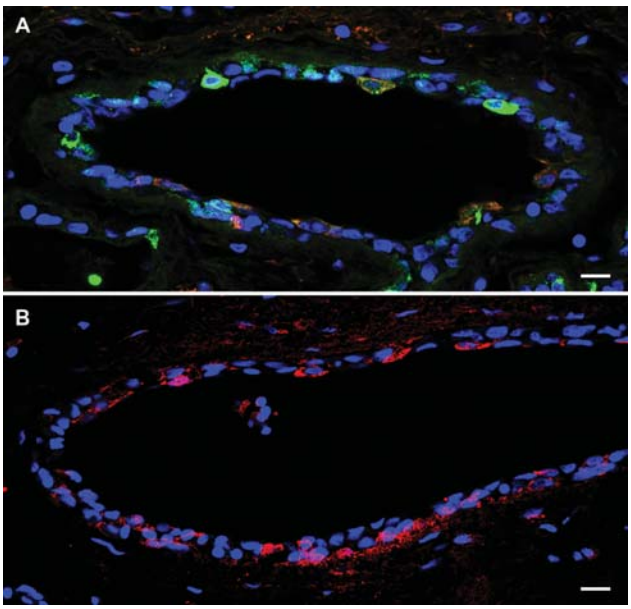

Fig. 2. Transitioning luminal cells with milk-secretory features in the ductal area close to the alveoli. **A:** Cytokeratin 18 (green) and lactoferrin (red) in a duct. Single and double positive transitioning cells can be seen. **B:** Alpha-lactalbumin (red) in a duct. Nuclei are shown in blue (DAPI staining). Scale bars: 5 μm.

At the duct termini, alveoli are formed which contain the lactocytes in the luminal layer, surrounded by the myoepithelial cell layer (Fig. 1A–1D) (Sternlicht, 2006; Watson and Khaled, 2008). The lactocyte is a cuboidal highly polarized cell (Fig. 1F), and this polarization ensures the movement of milk components toward the lumen (Lavialle et al., 2000). Although lactocytes are typically restricted to the alveolar compartment, some transitioning luminal cells with milk-secretory features can also be seen in the ductal area close to the alveoli (Fig. 2). Under the effect of oxytocin, the SMA^+ myoepithelial cells contract resulting in milk secretion from the $CK18^+$ lactocytes into the alveolar lumen. Milk is then pushed through the duct lumen toward the nipple during breastfeeding, containing not only the biochemical factors secreted by lactocytes, but also a number of cells from the epithelium.

Recent advances in our laboratory have identified various cell types present in breastmilk *ex vivo*, from early-stage stem cells (termed human breastmilk stem cells, hBSCs) with embryonic-like features and multi-lineage differentiation potential, to the previously known $CD49f^+$ MaSC population described in the resting breast, to cells with progenitor characteristics, to the mature myoepithelial and milk-secretory cells (Fig. 3) (Hassiotou et al., 2012d; Hassiotou et al., 2012e), revealing a cellular hierarchy along a complex developmental continuum characteristic to the fully mature organ. We have examined the localization of these different breastmilk cell populations in the lactating breast using rare human normal lactating breast tissue specimens (Fig. 1) and compared

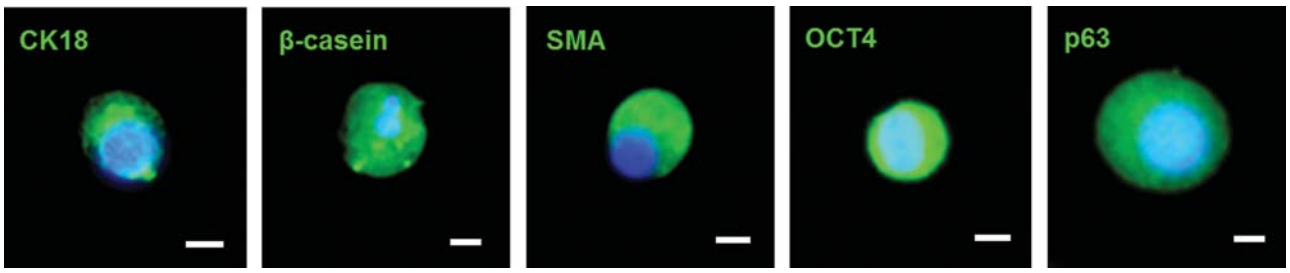

Fig. 3. Breastmilk contains a cellular hierarchy. A variety of cell types can be found in breastmilk, including cells positive for the luminal epithelial marker Cytokeratin 18 (CK18), the lactocyte marker β-casein, the myoepithelial cell marker smooth muscle actin (SMA), the stem cell marker OCT4, and the epithelial progenitor marker p63. Nuclei are shown in blue. Scale bars: 5 μm.

marker expression with the resting breast. In contrast to the lactating breast, minimal or no representation of hBSCs was found in the resting breast, suggesting the absence or quiescent state of these cells outside the PLC (Hassiotou et al., 2012d). It is not known whether and to what extent in different women the menstrual cycle influences gene expression in the resting breast outside the PLC and whether it transiently activates the embryonic gene network characteristic of hBSCs to induce transient cell proliferation. Indeed, this is probable given activation of the previously described $CD49f^{+}$ MaSC population upon the effect of progesterone increase during the luteal dioestrus phase of the menstrual cycle in mice (Joshi et al., 2010) and merits further investigation. Importantly, we have now shown that cells with stem cell phenotypes are found not only in the basal myoepithelial layer but also in the luminal layer in the lactating breast (Hassiotou et al., 2012d; Hassiotou et al., 2012e). These cells can be accessed via breastmilk and are capable of forming spherical alveolar- and ductal-like structures in 3D culture (Thomas et al., 2011; Hassiotou et al., 2012d) (Fig. 4), offering a non-invasive alternative to biopsies of human lactating breast tissue, which are extremely rare. Therefore, breastmilk rises as an extremely useful source of cells to study the interplay between different cell types in the breast during normal lactation and factors associated with lactation difficulties, as well as the potential role(s) of these cells for the breastfed infant. It also offers a potential ethical, non-invasive, and plentiful source of stem cells for regenerative medicine.

The cellular component of breastmilk is thought to represent the lactating breast as a whole, but a more correct interpretation would include only those ducts that ejected milk at the time of collection. It is difficult therefore to draw conclusions as to the histological characteristics and maturation stage of different alveolar batches within the same breast. Indeed, the breast of a lactating woman is not histologically homogeneous. Differences have been reported in the maturation of different groups of alveoli (lobules) within the same breast (Molenaar et al., 1992). More recent studies in our laboratory have suggested that not all alveoli are at the same developmental stage in a breast, based on expression of stem cell, progenitor, and functional differentiation markers (milk proteins) (Fig. 5). This is in agreement with the presence of some ''non-functioning'' ducts at any given time point in the lactating breast (Going and Moffat, 2004). Further work is needed to illuminate the signaling cascades that influence alveolar development and differentiation and factors that allow/effect differing developmental patterns between different lobules. Vascularization of individual lobules may be implicated in the functional heterogeneity of the lactating breast.

Post-lactational Involution

Breastmilk production continues until weaning and cessation of lactation, when a rapid reduction in

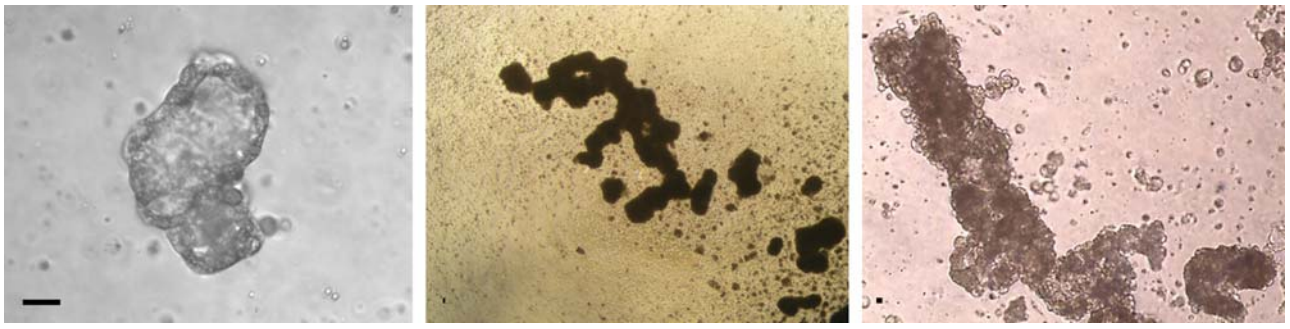

Fig. 4. Stem cells isolated from human breastmilk form functional alveolar- and ductal-like structures in 3D culture. Scale bars: 20 μm.

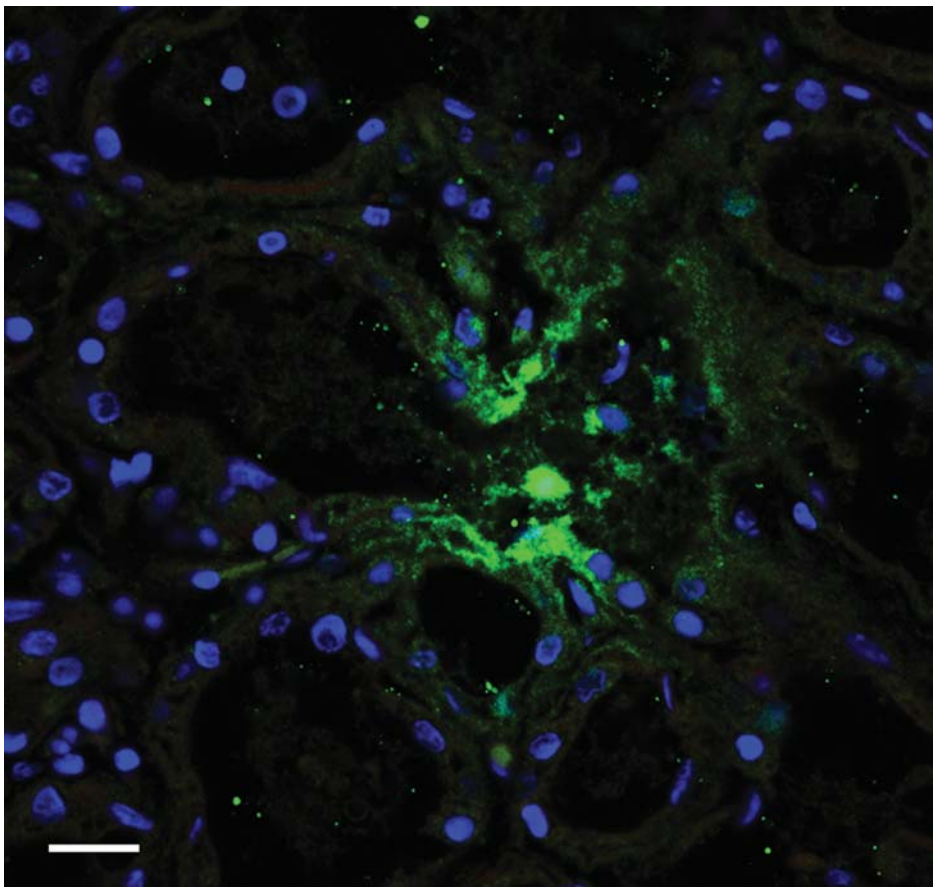

Fig. 5. Developmental heterogeneity in the human lactating breast at the alveolar and cellular levels. A group of alveoli stained for the mature lactocyte marker β-casein (green). Nuclei are shown in blue. Scale bar: 10 μm.

breastmilk production is followed by cessation of breastmilk synthesis and mammary involution. Breastmilk accumulation and stasis in the ducts initiates involution, though the mechanisms and factors involved are not well understood. Whether it be a physical phenomenon of milk accumulation and stasis in the ducts and/or be effected by one or more biochemical factors in the milk and/or blood (Pang and Hartmann, 2007; Sutherland et al., 2007), involution comprises two distinct phases.

The first phase is characterized by activation of programmed cell death and regression of both the epithelial and stromal tissues in the breast (Monks and Henson, 2009). Clearing of residual milk components is thought to be aided by an increase in hydrolytic and other enzymes (Hurley, 1989). Phase I may last up to two weeks and is reversible, i.e., frequent removal of milk from the breast can re-establish milk supply. Phase II of involution is irreversible and is characterized by luminal cell loss and extensive stromal remodeling. The latter is thought to be affected by epithelial-stromal signaling that stimulates expression of matrix remodeling factors in stromal fibroblasts (Wiseman and Werb, 2002; Monks and Henson, 2009). Luminal epithelial cells that have initiated an apoptotic process are shed into the alveolar lumen and gradually cleared. It is not known whether all alveolar batches enter Phase I and II of involution simultaneously or whether differences exist between different epithelial compartments within a breast.

At the conclusion of involution, the breast returns to a resting (non-lactating) state. However, the structure and morphology of the gland is not identical to the pre-pregnancy state (nulliparous stage) (Russo et al., 2001; McDaniel et al., 2006; Watson, 2006). Considerably more Lob 2 and Lob 3 structures are present in the parous gland, and less often, even Lob 4 structures compared to the nulliparous gland, accompanied by changes at the cellular level. Wagner et al. (2002) proposed that some partially differentiated epithelial cells escape clearing during involution and act as "memory precursor cells" in subsequent pregnancies. This was recently supported by Van Keymeulen et al. (2011) in a murine model of mammary development, which demonstrated the presence of unipotent epithelial progenitor cells that persist after involution and fuel mammary remodeling in subsequent pregnancies. Further work is needed to establish the properties of these cells and position in the mammary developmental hierarchy, how they are regulated, and whether they

may act as targets of malignant transformation influencing breast cancer risk. The milk secretion from the involuting gland may provide significant insights into the developmental changes occurring in the gland during involution in women.

Most of our knowledge on the mechanisms and process of involution as well as the composition of the secretion from the involuting gland is based on animal models, particularly the dairy cow and the mouse, due to the commercial interest in the first and the availability of study animals in the second. The limited research in women has demonstrated marked changes in milk biochemical composition during involution, including increased concentrations of sodium, potassium, and protein, and decreased concentrations of lactose and potassium, with the secretion of the involuting gland being more similar to that of colostrum rather than mature breastmilk (Hartmann and Kulski, 1978). Levels of lactoferrin, hydrolytic enzymes, and immunoglobulins increase consistent with a pro-inflammatory response that is thought to take place in the breast, reflecting a changing biochemical and cellular environment mediated by marked changes in gene expression (Hurley, 1989; Humphreys et al., 2002; Kreuzaler et al., 2011; Watson and Kreuzaler, 2011; Hughes, 2012).

A number of studies in the dairy cow have suggested that macrophages populate the mammary gland during involution, aiding the phagocytosis of apoptotic epithelial cells (Hurley, 1989). However, studies in the mouse supported the notion that a subpopulation of mammary epithelial cells may be capable of phagocytosis (Monks et al., 2002). More recently, a murine model of involution showed that a population of viable epithelial cells engulfs intact apoptotic epithelial cells effecting clearing of the glandular tissue within four days (Monks et al., 2008). Immune inflammatory cells such as macrophages seemed to contribute neither to the epithelial clearance nor to residual milk clearance, and both processes were found to be associated with the epithelium itself (Monks et al., 2008). This is in agreement with other recently described events of ''phagocytosis'' of apoptotic cells by neighboring nonhematopoietic cells (Henson, 2005; Gardai et al., 2006). Further research is warranted to elucidate the cell types involved in/activated during the involution process in the human breast, their properties and gene regulatory networks involved, and the mechanisms through which involution is initiated and successfully completed to allow subsequent activation of the mammary gland in the next PLC.

Post-menopausal Involution

Post-menopausal involution is triggered by a declining ovarian function and thus circulatory levels of sex-steroid hormones (estrogen, progesterone). This results in further regression and atrophy of the glandular tissue of the breast and in a concurrent increase in the adipose tissue (Vorherr, 1974; Williams, 1995). The reduction of the glandular tissue can be up to approximately a third of its original volume (Tavassoli, 1992), although the ratio of adipose to glandular tissue varies among women during this period. Nevertheless, a decrease in this ratio together with reduced elasticity of the supporting connective tissue is generally seen (Hutson et al., 1985). The parallel decrease in the volume of adipose tissue starts at the periphery of the breast and progressing inward toward the nipple (Vorherr, 1974; Williams, 1995). The menopausal effect on breast anatomy can be altered by hormone replacement therapy (HRT). The increased mammographic density (glandular tissue) observed in women prescribed HRT is due to hormonally induced epithelial and/or stromal proliferation (Greendale et al., 2005). However, HRT significantly increases breast cancer risk and is not considered safe (NH&MRC, 2005). The molecular switches that stimulate epithelial and/or stromal expansion during HRT may be similar to those involved in the normal PLC-induced breast remodeling and the aberrant mammary cell transformation in breast cancer. What differentiates between the two is of substantial scientific interest for the understanding of both normal mammary biology and aberrant conditions of the breast.

BLOOD SUPPLY

Few extensive descriptions of the blood supply to the breast exist and are based mainly on the classic dissections of lactating cadavers made by Cooper (Cooper, 1840). Investigative methods of mammary vasculature include injection of either colored wax or mercury into the blood vessels (Cooper, 1840), surgical dissection (Anson, 1939) of specimens, injection of a suspension of fine lead, and radiography of the blood supply in a non-lactating woman (Salmon, 1939). More recently injections of latex into the mammary vessels of cadavers prior to dissection has been carried out (van Deventer, 2004).

The majority of the blood supply is derived from the anterior and posterior medial branches of the internal mammary artery (60%) and the lateral mammary branch of the lateral thoracic artery (30%) (Vorherr, 1974; Cunningham, 1977; Doughty et al., 1996). Blood vessels and capillaries are housed within the mammary stromal matrix delivering biochemical and cellular components essential for the function of the gland and milk synthesis (Hennighausen and Robinson, 2005). Blood supply is variable between women and studies are often conflicting. Cooper (Cooper, 1840) showed that the four anterior perforating branches of the IMA were of similar size, yet Maliniak (1934) showed the branch at the level of the second intercostal space to be much larger and supply the majority of blood. Anson et al. (Anson, 1939) showed two main branches. Recently, Aljazaf (2005) demonstrated that most frequently one dominant artery is present with multiple arteries occurring less often. The lateral thoracic artery is considered to supply up to a third of the blood to the breast, yet the LTA is absent in up to a third of women (Doughty et al., 1996). There is wide variation in the proportion of blood supplied by each artery (Doughty et al., 1996; Geddes et al., 2012).

Minor sources of arterial blood also include the posterior intercostal arteries and the pectoral branch of the thoracoacromial artery (Freeman et al., 1981; Williams, 1995). Interestingly, the course of the arteries does not follow the ductal breast system (Cooper, 1840), and there is little evidence of arterial symmetry between breasts (Anson, 1939; Aljazaf, 2005).

The rapid growth phase in pregnancy is reflected by a doubling in mammary blood flow (MBF) 24 weeks gestation after which it remains constant during lactation (Vorherr, 1974; Thoresen and Wesche, 1988; Geddes et al., 2012). The increase in blood flow is also accompanied by an increase in the size of the superficial veins of the breast, making them more visible during pregnancy and lactation. It has long been thought that mammary blood flow drives (in part) milk synthesis in that an increase in blood flow produces a reciprocal increase in milk production. An alternative theory based on recent evidence proposes the reverse, in that the metabolic activity of the mammary gland regulates MBF (Prosser et al., 1996) and milk yield varies independently of MBF. This process is illustrated in goats where increasing MBF to only one mammary gland did not result in increased milk production compared with the control gland (Lacasse and Prosser, 2003) and that hourly milking increased milk secretion without an accompanied increase in MBF (Maltz et al., 1984). It is likely however that a minimum threshold of MBF necessary for adequate milk production exists in both animals (Prosser et al., 1996) and women (Geddes et al., 2012).

The ratio of blood flow to milk yield is approximately 500:1 (Linzell, 1960; Christensen et al., 1989) in lactating animals and women (Geddes et al., 2012), with high variability demonstrated in women and other species such as the sow (Renaudeau et al., 2002) and goat (Lacasse and Prosser, 2003). Transient changes in MBF have been documented in women at milk ejection with a reduction in flow of 40–50% prior to milk ejection followed by an increase 1–2 minutes later (duration of one milk ejection). These changes in MBF are replicated with intravenous injections of oxytocin (Janbu et al., 1985). Significant decreases in MBF at milk ejection have also been observed in other species (Pearl et al., 1973; Davis et al., 1995; Eriksson et al., 1996).

More recently, color Doppler ultrasound has been used to study the lactating breast with the aim to produce reference values in women that will allow comparison of lactation pathologies (Geddes et al., 2012). This noninvasive method of investigating MBF provides an opportunity to determine the role of MBF in milk synthesis such that factors thought to influence milk production could be monitored. Reference parameters will also be useful when investigating conditions in which a disruption in MBF may be suspected such as delayed secretory activation (McClellan, 2008), lactation failure, and maternal medications that are believed to decrease milk supply (Aljazaf et al., 2003) or conversely an increase in MBF that would be expected with mastitis.

Blood supply to the breast as well as lymphatic vessels are thought to be the source of viable hematopoietic cells present in the milk. These cells primarily include immune cell (leukocyte) populations, which are thought to diapedese through the basement membrane via the paracellular pathway and enter the milk (Seelig and Beer, 1981; Lin et al., 1995). Maternal milk immune cells have been hypothesized to confer active immunity to the infant, but also to be involved in the protection of the mammary gland from/during infection (Zhou et al., 2000; Vidal et al., 2001; Lonnerdal, 2003). They can comprise granulocytes, B and T lymphocytes, monocytes and macrophages, and have been thought to constitute an important part of the cellular portion of colostrum, decreasing in mature breastmilk. Literature on the content of milk in immune cells has focused on colostrum, with few publications on mature breastmilk. And although a highly variable milk immune cell content is reported among women during the course of lactation, few of these studies have appropriately considered the health status of the mother-infant dyad. Moreover, the response to and effect of breast or systemic infections of the mother or the breastfed infant on milk immune cell populations and content have not been examined. We have recently demonstrated a close association between the mature breastmilk immune cell content and mother or infant infections across the course of lactation (Hassiotou et al., 2012c). The specific responses and role(s) of recruited milk immune cells for the breastfed infant and in the breast merit further investigation, particularly in the diagnosis of mastitis and other medical conditions of the lactating breast. An important study by Zhou et al. (2000) in a murine model demonstrated that maternal milk immune cells pass unharmed through the digestive tract of the infant into the systemic circulation and engraft in various tissues. Maternal milk immune cell engraftment in tissues of the pup has also been shown for other species, such as lambs and baboons (Michie, 1998), but it remains to be investigated in the human.

LYMPHATIC DRAINAGE

Until 1840, Gasparo's (1662, 1962) theory persisted that chyle was transported to the breast via the lymphatic vessels for the synthesis of milk. Cooper (1840) dissected and injected the lymphatic vessels of the lactating breast and concluded that fluid in the vessels flowed away from the breast rather than toward it. The lymphatic drainage of the breast has been extensively investigated due to its implication in the spread of breast carcinoma.

Lymph is drained by two main pathways; to the axillary nodes (Turner-Warwick, 1955) and to the internal mammary nodes (Hultborn et al., 1955; Turner-Warwick, 1955; Vendrell-Torne et al., 1972). The axillary nodes receive more than 75% of the lymph from both the medial and lateral portions of the breast (Turner-Warwick, 1959; Borgstein et al., 2000). The internal mammary nodes receive lymph mainly from the deep portion of the breast (Aukland and Reed, 1993). The pattern of drainage is highly variable however and less common pathways have

been demonstrated. Lymph may pass through either the interpectoral nodes (Williams, 1995) or the intraparenchymal lymph (Tanis et al., 2001), and drain into the posterior (Turner-Warwick, 1959) and anterior intercostal nodes (Tanis et al., 2001). Direct drainage to the supraclavicular nodes (Tanis et al., 2001) and retrosternal passage of the lymph into the contralateral internal mammary nodes may also occur. Since Coopers (1840) work there has been no investigation of the lymphatic drainage of the lactating breast despite its importance in clinical conditions such as breast engorgement and mastitis.

INNERVATION

The second to sixth intercostal nerves innervate the breast and they are located superficially in the gland (Cooper, 1840) dividing into superficial and deep branches. The nipple and glandular tissue are supplied by the deep branches, and the nipple and areola by superficial branches with a complex and variable distribution. However, the anterior and lateral cutaneous branches of the second to fifth intercostal nerves follow the ducts and always supply the nipple and areola (Craig and Sykes, 1970; Sarhadi et al., 1996; Schlenz et al., 2000). The lateral supply of the nipple and areola is less variable than the medial supply. The lateral supply is provided by the fourth lateral cutaneous nerve (Farina et al., 1980; Schlenz et al., 2000), and it most often takes a subglandular course within the pectoral fascia to the posterior aspect of the nipple (Craig and Sykes, 1970; Schlenz et al., 2000). Less commonly it takes a superficial course (Cooper, 1840; Farina et al., 1980; Sarhadi et al., 1996). Detailed descriptions of the course of the anterior cutaneous branches are scant and conflicting. A deep course is described by Craig and Sykes (Craig and Sykes, 1970), whereas Sarhadi et al. (1996) and Schlenz et al. (2000) describe a superficial course.

Nerves have been demonstrated along the major duct system with none identified near the smaller ducts (Linzell and Peaker, 1971). Distribution of the nerves of the areola and nipple is sparse with all concentrated at the base of the nipple, few at the side of the nipple, and virtually none in the areola (Montagna and Macpherson, 1974). These nerves are sensory in nature and together with the lack of motor innervation of both the lactocytes or myoepithelial cells suggest that both the synthesis and secretion of milk is independent of neural stimulation. However, motor innervation of the smooth muscle of the areola and nipple (Courtiss and Goldwyn, 1976) and the mammary arteries (Cowie, 1974) is apparent.

Investigation of the innervation and sensitivity of the breast has focused on the effect of breast surgery such as reduction mammoplasty. Only one study investigated the sensitivity of the breast during lactation. Areola and nipple sensitivity is markedly heightened 24 hrs postpartum (Robinson and Short, 1977) decreasing in the following days. In addition Kent et al. (personal communication) found limited sensory discrimination of the skin of the breast, areola, and nipple using the two-point discrimination method in women with established lactation (one to six months), which is consistent with reports of reduced sensitivity of the nipple epidermis (Vorherr, 1974). Clinical evidence supports the limited distribution of mammary nerve fibers, based on observations of women experiencing pain associated with a distended breast, who are often unable to accurately localize their sensation (Cowie et al., 1980). Furthermore, often the first signs of mastitis in women are influenza-like symptoms despite tenderness and/or localized changes in their breasts.

CONCLUSIONS

There still remain many questions about the physiology and pathology of the breast, and ongoing investigation will improve knowledge of its normal anatomy and histology, assisting in addressing these questions. Among those, the orchestration of local and systemic interactions regulating the functional maturation of the breast and milk synthesis should be a research priority. The complexity of the breast, its normal function involving the breastfed infant, and its pathologies call for collaborations between different disciplines, including the anatomist, the cell biologist, the biochemist, the epidemiologist, the cancer biologist, the nutritionist, the lactation consultant, and the physician. Such collaborations will assist in addressing key questions involving the mother and the infant, such as, for example, the very low proportion of the infant's protein-derived energy intake during exclusive breastfeeding and how it allows the infant to optimally grow in a period when the human growth rate is the maximum across life. Unraveling the mechanisms behind the changes in milk biochemical and cellular composition in the short- and long-term (diurnally and across lactation) will also be instrumental in better understanding the function of the breast, its pathologies and the role of the different milk components for the optimal development of the infant. Further studies on breastmilk cellular composition and its regulators are much needed if we are to better understand how these cells contribute to successful lactation as well as the role of milk in providing optimal nourishment, protection, and development to the infant. To this end, the cellular hierarchy of breastmilk together with lactating tissue specimen analysis may aid understanding of the cellular inter- and intra-alveolar and lobular heterogeneity and factors regulating milk synthesis and mammary cell cycle. The above may prove instrumental in managing low milk supply and other conditions of the breast. Furthermore, the potential of breastmilk stem cells to form mammary structures *in vitro* offers a new promising opportunity for *in vitro* studies of mammary gland biology and its regulators without the need for biopsy. Importantly, the presence of viable stem cells with multi-lineage potential in breastmilk poses the question of the potential integration within the infant's tissues and differentiation, contributing to optimal tissue development and regeneration early in life.

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