Comparative Aspects of Mammary Gland Development and Homeostasis

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Abstract
Mammary glands are crucial to the reproductive strategy of mammals, and the milk of domesticated ruminants serves as an important source of nutrients for the human population. The majority of mammary gland development occurs postnatally, and the mammary gland undergoes cyclical periods of growth, differentiation, lactation, and regression that are coordinated to provide nutrients for offspring or are driven by strategies to manage reproduction and milk production of domesticated species. Growth and maintenance of the mammary epithelium depends on the function of mammary stem cells and progenitor cells. In this review, we provide an overview of postnatal mammary gland development, cyclical phases of mammary gland regression (regression during lactation and between successive lactations), and mammary stem cells and progenitor cells. Where possible, these processes are related to animal production and compared across species, particularly bovine, porcine, murine, and human.
INTRODUCTION

Before birth, essentially all the important cell lineages in the mammary gland are established, and a mammary rudiment forms. In contrast to the functional development of requisite tissues (e.g., cardiac, gastric, muscular), the mammary rudiment essentially is nonfunctional at birth. However, very shortly after birth, pronounced developmental changes occur that continue through the prepubertal period, after puberty, during gestation, and even into lactation and involution. Because the requirement for mammary functionality is transient, the cyclical phases of development and involution are decoupled in some respects from normal developmental processes. Despite the difference in developmental timing, the mammary parenchyma undergoes extensive branching morphogenesis, alveolar development, functional differentiation, regression, and even redevelopment. The cyclic changes in mammary development result from endocrine, nutritional, and management factors (e.g., milking) associated with reproduction. The purpose of this review is to describe the developmental changes that precede and follow lactating periods in a range of domesticated species, to highlight the role of mammary stem cells (MaSC) in maintaining mammary functionality and homeostasis, and to present an updated perspective on the status of MaSC research in domesticated livestock species.

POSTNATAL MAMMARY GROWTH AND DEVELOPMENT

The mammary gland undergoes considerable growth before puberty, although it is described by many authors as a period during which little development occurs. Classic reports of mammary development in rodents (1–3), cattle (4), and sheep (5) demonstrate prepubertal allometric mammary development throughout most of the prepubertal phase. More recent reports on cattle (6, 7), goats (8), and pigs (9, 10) also provide data supporting the notion of significant development during early postnatal life. During the prepubertal period of development, the mammary parenchyma in all species is characterized by a lack of alveolar structures, abundant cell proliferation, and branching morphogenesis, the extent of which varies with species. Species differences in the overall parenchymal architecture exist (Figure 1), and recent reviews have highlighted many of the salient histologic features of mammary development in agricultural species (10, 11). The most obvious distinction is that in rodents the mammary parenchyma is embedded in a nearly homogeneous depot of unilocular adipose tissue with very limited and sparse collagenous stromal tissues. Actively growing ducts are characterized by the presence of bulbous expansions, called terminal end buds (TEBs), at the distal end (relative to the teat) and ductal elongation in the absence of significant side branching. The TEBs eventually regress as the ducts reach the glandular perimeter (1), and after the onset of estrous activity, some ductal side branching occurs. In contrast to rodents, the prepubertal mammary ducts of cattle, other species with pendulous mammae, and humans are more arborescent and embedded in a collagenous stroma with distinct inter- and intralobular heterogeneity. Arborescent structures called terminal ductal units (TDUs) (referred to as terminal ductular lobuloalveolar units in human breast) are present instead of TEB. Each TDU is characterized by a radiating array of roughly three to eight ductules arranged around a subtending duct (12). Although each ductule initially has minimal luminal space, the ductal lumens expand as the TDU grows, and new TDUs are subsequently formed as outgrowths that penetrate deeper into the mammary fat pad. With the onset of puberty, regular reproductive cycles, and pregnancy, the ductal framework of the mammary gland begins to undergo alveolar development. During the estrous cycle and predominantly during gestation, alveolar development occurs in response to elevated progesterone, which leads to the establishment and expansion of milk secretory tissue.
As documented in numerous studies, both steroid and protein hormones play prominent roles in the development of the mammary gland (13, 14). Estrogens, the primary drivers of ductal development, appear to act through paracrine mechanisms to stimulate cell proliferation because proliferative cells [Ki-67-positive or bromodeoxyuridine (BrdU)-labeled] are estrogen receptor-α (ESR1)-negative in ruminant (12), rodent (15), and human (16) mammary epithelia. Amphiregulin appears to serve as the paracrine mediator of estrogenic signals in the murine mammary gland (17). Joshi et al. (18) used a cell-transplantation approach to demonstrate that during periods of elevated progesterone, the MaSC populations expanded 14-fold, likely through modulation of the Wnt4 and receptor activator of NF-κB-ligand (RANKL) pathways. Others (19, 20) have shown that interactions between the progesterone receptor and RANKL mediate a significant fraction of cell proliferation in postpubertal mice and that the mammary phenotype in PR-knockout mice can be rescued by directed RANKL expression. Similar data are not available for bovine mammary glands or other agricultural species owing to the lack of practical mammary transplantation and gene-knockout models.

In addition to their importance for mediating estrogen signaling, a variety of paracrine mediators and cell-cell interactions play important roles in modulating mammary growth and development. For example, recent developments in our understanding of the regulation of mammary development pertain to the involvement of immune cells and the utilization of immune-related signaling molecules as important players in mammary development processes. Watson and

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Figure 1
Comparative structure of terminal end bud (TEB) and terminal ductal unit (TDU). Murine TEBs consist of a bulbous outgrowth at the distal tip of the developing duct. TDUs, as found in cattle and other species, lack the well-defined central lumen found in TEBs during initial growth phases. In both structures, undifferentiated luminal cells (blue) rest on top of myoepithelial cells at various stages of functional differentiation (pink, red). Frequently, the epithelium of the TDU is more stratified than that of TEB and its subtending ducts in the mouse mammary gland, such that an embedded layer(s) of cells is readily visible in the epithelium of the TDU and the immature subtending ducts of intact heifers. Stratification in the TEB is generally restricted to the area immediately behind the cap cells, the region of so-called body cells (yellow).
colleagues (21, 22) have demonstrated that the same STAT6 and IL4 axis involved in helper T cell development is active in mammary development. Other indications of a role for immune-system components in mammary development come from murine model data in which deletion or removal of macrophages, eosinophils (23, 24), or mast cells (25) can impair mammary development. Interactions between the immune system and the mammary gland are consistent with the current theory of the evolution of lactation (26).

**Lactation**

After parturition, milking or suckling by the neonate brings the lactogenic events of pregnancy to fruition. The amount of milk produced by a mammary gland is a function of the number of secretory epithelial cells and the activity per cell. Furthermore, rates of cell proliferation and death, along with changes in secretory activity of mammary epithelial cells, account for the shape of the lactation curve. There are notable differences in these parameters between ruminants and litter-bearing species.

In litter-bearing species, the amount of mammary growth that occurs during early lactation is key to meeting the nutrient demands of the suckling litter. Mammary growth during early lactation in sows (27) and rodents (28, 29) may double the number of mammary cells present at parturition. This growth is promoted by milk removal and the suckling stimulus, and generally it parallels the increase in milk production. In contrast, mammary growth during early lactation in dairy ruminant species is less pronounced than that in litter-bearing species. In dairy goats and sheep, mammary growth during early lactation accounts for approximately 20% of the total number of mammary cells (30). In dairy cows, there is little evidence for continued accretion of mammary cells during early lactation (31), although increased milking frequency during the first weeks of lactation may elicit a proliferative response and an increase in milk yield that persists after reverting to less-frequent milking (32).

With the onset of lactation, there is an increase in the metabolic and secretory activity per cell as the lactation becomes established (27, 29, 31, 33). Changes in the secretory epithelium involve closure of the tight junctions between adjacent cells (34–36), and increased differentiation of the secretory cells, which includes increased prominence of the endoplasmic reticulum and mitochondria (37–39).

**Figure 2**

Population dynamics of the mammary epithelium during lactation and after cessation of milk removal. (a) Factors contributing to changes in milk yield during a bovine lactation are depicted. Prior to peak lactation, milk yield increases owing to increased secretory activity per cell. After peak lactation, milk yield declines primarily because of a decline in epithelial cell number owing to apoptotic cell death. Factors that decrease loss of cells decrease the decline in milk yield (i.e., increase persistency) (blue curve). Factors above the lactation curves increase milk yield and persistency, and those below decrease milk yield and persistency. The subsequent lactation is depicted as a truncated curve. Adapted from Capuco et al. (41). (b) Two cell populations that differ by rates of cell proliferation and cell death. Circles show the initial population of cells (blue), new cells formed by cell proliferation (green), and cells that die during this period (gray). In both cases, net regression is the same, but the populations differ fourfold with regard to cell turnover or replacement. (c) Mammary alveoli during the transition from the cessation of milk removal to the following lactation. Changes during regenerative involution of the mammary gland of a pregnant animal and changes during mammary involution in a nonpregnant animal are shown. Alveolar structure is maintained during regenerative involution, and senescent (darkened) epithelial cells (blue) and progenitor cells (yellow) are replaced. Mammary involution in a nonpregnant animal results in the destruction of the alveolus, which is not restored until the next pregnancy. Myoepithelial cells are depicted as pink cells. Abbreviations: bST, bovine somatotropin.
Peak lactation

- Increased activity
- Increased differentiation
- Increased growth?

Initial cells

Dead cells

New cells

Increased activity
Decreased cell number
Regenerative involution

Day of lactation

- bST/IGF
- Long daylight
- Increased milk frequency
- Antioxidants
- Mastitis
- Neutrophil activity
- Stress
- Incomplete milking
- Decreased blood flow
- Stressors
- bST/IGF
- Long daylight
- Increased milk frequency
- Antioxidants

Milk yield

Increased activity
Decreased cell number
Regenerative involution

Dry period

Pregnant

Epithelial cell

Progenitor cell

Senescent cells

Myoepithelial cell

Regenerative involution

Nonpregnant

Late lactation

Involution

Lactation

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Knight & Peaker (33) estimated the relative contributions of mammary cell number and secretory activity during lactation in goats. This was accomplished by using multiple biopsies to evaluate changes in nucleic acid concentrations and enzymatic activity. When coupled with measures of udder volume, the data were extrapolated to whole-udder measures. This study demonstrated that increases in milk production during early lactation are the result of an increase in mammary cell number followed by an increase in secretory activity per cell. After peak lactation, decreased milk yield with advancing lactation is primarily the result of declining cell number. But during late lactation, when goats were concomitantly pregnant, the secretory activity per cell also declined. Declining milk yield during extended lactation in rats is primarily a result of reduced secretory activity per cell (29), whereas a decline in cell number and secretory activity per cell contributed to the decline in milk yield during an extended lactation in mice (28). These factors have not been evaluated during lactation in sows.

Analysis of changes in mammary cell number and secretory activity during bovine lactation was performed utilizing an approach that also permitted evaluation of mammary cell–population dynamics (31). Based upon measures of total udder DNA, increased milk yield during early lactation was attributed to increased secretory activity per cell and the decline in milk yield with advancing lactation to declining cell number (Figure 2a). The secretory activity per cell increased prior to peak lactation but did not change significantly with advancing lactation in non-pregnant multiparous cows. (When cows are concomitantly lactating and pregnant, a decline in secretory capacity per mammary cell likely accompanies advanced pregnancy, consistent with the coordinated adaptations to meet the metabolic demands of gestation and lactation. This is readily apparent during extended lactation, when the number of mammary epithelial cells increases simultaneously with a rapid decline in milk production.) Rate of proliferation was assessed by incorporation of BrdU by mammary epithelial cells over a 24-h, in vivo labeling period and rate of apoptosis through extrapolation from an apoptotic index. Measures of proliferation rate and apoptotic rate were consistent with the observed decline in mammary cell number. The decline in cell number after peak lactation was predicted by the estimates of proliferation (0.3% per day) and apoptotic rate (0.56% per day). If none of the cells that died by apoptosis were those that proliferated during lactation, then 100% of the mammary cells remaining at the conclusion of a typical lactation were predicted to have been formed during that lactation. Although absolute rates of cell proliferation and cell death during lactation appear to be very low, the balance of these processes promotes gradual cell loss but considerable cell turnover during a typically lengthy (~300-day) bovine lactation. These measures provide the first quantitative demonstration that apoptotic death could account for the decline in mammary cell number during lactation (31). In contrast to cell turnover during a bovine lactation, approximately 75% of mammary cells are maintained throughout a typical lactation in rats (40). This lack of extensive cell turnover is probably a result of the shorter length of lactation in rodents than in cows.

Many factors that can influence the persistency of lactation are outlined in Figure 2a and are described more fully in a recent review (41). Some factors with the potential to enhance persistency of lactation by increasing cell proliferation or decreasing apoptosis are: (a) bovine somatotropin (bST) and components of the GH/IGF axis; (b) prolactin, through potential interactions with IGF survival activity; (c) photoperiod, likely mediated by prolactin and the IGF axis; (d) increased milking frequency; and (e) antioxidants. Some factors that can increase apoptotic cell death and decrease persistency are: (a) mastitis, in which many stimuli may be involved, including bacterial toxins, reactive oxygen species generated by phagocytic activity, and damage owing to neutrophil influx into the gland; (b) neutrophil surveillance activity in the absence of infection; (c) decreased milking frequency; (d) decreased blood flow; (e) stressors; and (f) pregnancy. Later stages of
gestation may impact milk production negatively owing to a coordinated shift in nutrient partitioning to support fetal development and hormonally by apoptotic effects of estrogens.

**Mammary Involution Versus Regenerative Involution**

Cessation of milking or weaning in the young promotes involution of the mammary glands. This process is characterized by the apoptotic death of mammary epithelial cells and their removal by phagocytes, both macrophages and epithelial cells (42, 43). Apoptosis is regulated by local and endocrine factors, although local factors often appear to dominate (42, 44). Many features of mammary involution are common across species, but the rapidity and degree of involution vary. However, an overriding influence on the process appears to be the pregnancy status of the animal when milk removal is terminated (45).

In nonpregnant mice, forced weaning results in the initiation of apoptosis of mammary epithelial cells within 24 h and irreversible mammary involution within 3 days. Extensive tissue remodeling occurs, during which there is a loss of alveolar structure. The net result is that the histological appearance of the involuted mammary gland is similar to that of a virgin mouse. Likewise, mammary involution in sows proceeds rapidly after piglets are weaned. The mass and DNA content of porcine mammary glands decline to approximately 30% of their prior levels by 7 days postweaning and essentially remain at that level until the next pregnancy (46). Thus, mammary involution is rapid and extensive in nonpregnant rodents and sows.

In contrast to most species, current management of dairy cows and goats results in an overlap of lactation and pregnancy such that these animals typically are pregnant when milking is terminated during late lactation. Thus, when milk stasis occurs, the mammogenic and lactogenic stimulation of pregnancy opposes the stimuli for mammary involution. As described more fully below, processes of both mammary growth and involution occur during this nonlactating (dry) period between successive lactations; we have referred to the events during this period as regenerative involution. Previous studies have demonstrated the importance of a dry period to maximize milk yield in subsequent lactation for cows (47–49) and goats (50).

During lactation, the number of mammary epithelial cells gradually declines to approximately 50% of the number initially present at the onset of lactation (31). The number of cells must therefore be restored fully prior to the next lactation, lest that lactation be compromised. Parameters of mammary growth were evaluated in multiparous cows that were managed for a traditional 60-day dry period and in cows that were milked continuously during the prepartum period (51). There was no demonstrable loss of mammary cells (DNA) during the dry period. Instead, the total number of mammary epithelial cells increased proportionally with advancing pregnancy for both dry and lactating cows. However, DNA synthesis was approximately 50% greater in dry cows than in lactating cows, which indicates that this increased DNA synthesis in dry cows was for replacement of existing mammary cells rather than for accretion of additional cells. Furthermore, the cells synthesizing DNA were primarily (> 90%) epithelial. Thus, a dry period may be important for replacing senescent epithelial cells prior to the next lactation. Although the number of mammary epithelial cells at parturition did not differ between cows that experienced a dry period and those that were milked continuously, the epithelial cells in mammary glands of dry cows were produced more recently. The concept of cell turnover is illustrated in Figure 2b.

Although extensive mammary cell loss does not occur during a typical bovine dry period, extensive tissue remodeling does occur, and this includes changes in cell populations, alveolar structure, and synthesis of extracellular matrix (51–53). Others have demonstrated that apoptotic cell death occurs after milk stasis in the bovine mammary gland (54, 55), and conversely increased
milking frequency decreases apoptotic rate (56). There is an increase in both the apoptotic index and the Ki-67 (nuclear proliferation marker)-labeling index during the dry period (57). This is consistent with extensive cell turnover during the dry period, as processes of cell proliferation and apoptotic cell death were both increased following dry-off.

Increased mammary cell turnover during the dry period likely is a consequence of concomitant pregnancy and milk stasis, because the mammogenic effects of pregnancy tend to counterbalance the apoptotic effects of milk stasis by promoting cell proliferation and inhibiting apoptosis. In this regard, the events occurring during the dry period differ significantly from mammary involution as studied frequently in rodent models, in which mammary involution is rapid and extensive (Figure 2c). However, when mice are in the last trimester of pregnancy at the time of weaning, alveolar integrity is maintained, mammary apoptosis is reduced, and cell proliferation is enhanced relative to that in nonpregnant cohorts (58). Consequently, we refer to the dry period as a period of regenerative involution to describe more fully the processes of cell renewal and tissue remodeling that occur following milk stasis with concomitant pregnancy (41).

We hypothesized that the dry period is critical for replacing progenitor cells, which have a limited life span and are responsible for expanding and maintaining the number of mammary secretory cells (45). Pertinent data in support of this hypothesis are provided by several rodent and cow studies. A decline in the proportion of epithelial cells that are putative stem/progenitor cells (based on morphological criteria) has been reported during extended lactation in mice, along with evidence for oxidative damage to mitochondrial DNA and protein (28). Thus, the progression of lactation may induce damage to epithelial cells that impairs synthetic capacity and decreases tissue-regenerative capacity, i.e., senescence. Similarly, a decline in mammary RNA synthesis, total RNA, and RNA/DNA ratio supports loss of epithelial function during extended lactation in mice and rats (59). Further, mammary glands of rats that were not permitted a dry period had fewer cells during the middle of the ensuing lactation than did glands of rats that were permitted a dry period of optimal length, although cell number did not differ at onset of that second lactation (60). If replacement of senescent cells is a critical event during the dry period, lack of a dry period of sufficient length to allow replacement may decrease the persistency of the ensuing lactation. Finally, morphological evaluation of bovine mammary tissue at the onset of lactation, following a prepartum period during which mammary glands were milked continuously or permitted a dry period, showed that tissues from continuously milked quarters contained regions of diminished secretory activity, inactive cells, and apoptotic cells, which indicates reduced cell replacement (61).

Although there is considerable interest in reevaluating the length of the dry period to maximize the profitability of dairy farms, recent studies have confirmed the importance of a dry period for maximizing milk production in the next lactation (61–63). These studies have evaluated the possibility of eliminating or shortening the dry period when dry-period management of dairy cows incorporates treatment with bST and/or increased milking frequency. When cows were in third or subsequent gestation, treatment with bST through the prepartum period permitted continuous milking without altering milk production (62). However, a dry period between first and second lactations was essential and could not be circumvented or shortened by treatment with bST or by increasing milking frequency during the second lactation (61, 63). The need for continued body and mammary growth in young cows may explain their sensitivity to any management program that reduces dry-period length. In this regard, it is instructive to consider the potential energy demands of mammary growth and turnover during the dry period. During the 60 days prior to parturition, the mass of the mammary parenchyma increases by approximately 8.6 kg (51). Assuming that the calf weighs 33 kg at birth and that
65% of the growth occurs during the last two months of gestation, calf mass increases by approximately 21.5 kg during the dry period. Without considering the amount of cell turnover in the mammary gland, mammary growth is 40% of calf growth. If one considers that the DNA synthesis in a dry gland is 1.8 times that in a continuously milked gland, then the effective mammary mass (accrued + replaced) is 15.5 kg, or approximately 70% of calf growth. Therefore, energy demands for mammary growth and turnover undoubtedly are considerable. Beneficial effects of bST supplementation during the dry period have been reported and may result because somatotropin promotes the partitioning of energy toward the mammary gland (64) and also appears to promote mammary cell turnover (31). However, the energy demands for mammary growth, mammary cell turnover, and fetal growth may exceed the capacity of a young and growing cow that is also lactating during the prepartum period.

MAMMARY STEM CELLS AND PROGENITOR CELLS

Definitions and Characteristics

MaSC provide for net growth, renewal, and turnover of mammary epithelial cells and are therefore potential targets for strategies to increase production efficiency of domesticated species that are used for harvesting milk, meat, and fiber. From a biomedical perspective, the breast cancer stem cell hypothesis and the need to understand the biology and functions of MaSC during normal tissue development and homeostasis have spurred recent interest in the biology of MaSC (65, 66).

The two major categories of stem cells are embryonic stem cells and adult stem cells, which differ with regard to their tissues of origin and ability to generate multiple cell lineages. Embryonic stem cells have been isolated from the epiblast, or inner cell mass, of the blastocyst (67, 68) and are termed pluripotent because they are capable of generating cells of all three germ layers of the organism—ectoderm, mesoderm, and endoderm—and hence all tissues of the adult organism. (The fertilized egg and the progeny of its first several divisions are said to be totipotent, or omnipotent, because they are capable of generating all embryonic and extraembryonic cell types necessary to produce a viable organism.) Adult or somatic stem cells (e.g., MaSC) are found in the tissues of adult organisms, and their regenerative potentials, under normal circumstances, are more limited than those of embryonic stem cells. These cells are said to be multipotent in that they are capable of forming the differentiated cell types of related lineage found within the tissue in which they reside. Another term, “distributed stem cells,” has been introduced recently to describe adult stem cells and to emphasize that these cells retain a restricted version of the developmental potential of the inner cell mass or epiblast in a distributed, but tissue-specific, pattern (69). We will continue to use the terms adult or somatic stem cells. Additionally, we will use the term MaSC in its conventional context to describe stem cells for the epithelial component of the mammary gland. However, note that there are also mesenchymal stem cells within the mammary gland that provide for the stromal (fat pad/connective tissue) lineages of this organ.

Adult stem cells are responsible for the growth, differentiation, and maintenance of the tissues that they inhabit (70–72). Proliferation of stem cells can involve either symmetric or asymmetric cell division. When a stem cell undergoes symmetric division, it gives rise to two daughter stem cells and expands the stem cell population. When a stem cell undergoes asymmetric division, it gives rise to a stem cell (self-renewal) and a progenitor cell. A progenitor cell has more restricted renewal capacity than a stem cell and typically possesses a more restricted differentiation potential. Whereas a somatic stem cell is functionally viable for the life span of
the organism, progenitor cells have limited proliferation capacity and eventually undergo senescence. Another feature of cell lineage in a variety of tissues is the greater proliferative activity of progenitor cells than of the parental stem cell. Stem cells frequently are relatively inactive, and the proliferative activity of the tissue is met by regulation of the number of actively proliferating progenitor cells (73). Because of the need to expand both the MaSC and the progenitor cell compartments in proportion to duct growth, MaSC are likely to be relatively proliferative during periods of ductal morphogenesis. This certainly appears to be the case in murine mammary gland (74), which has served as the predominant model for MaSC research.

Mouse Mammary Stem Cells

Advancements in the study of MaSC have depended largely upon the development of a technique for implantation of mammary tissue fragments or cells into a cleared fat pad (epithelium-ablated fat pad) of syngeneic recipient mice (3, 75, 76). Early studies showed that MaSC, which were capable of repopulating the cleared mammary fat pad after repeated serial transplantations (i.e., they possessed tissue-regenerating and self-renewal capacities), were present throughout the mammary tree and the mammary cycle (77). Transplantation studies employing limiting dilutions of enzymatically dispersed mammary epithelial cells also provided evidence for progenitor cells with lobule-limited or duct-limited regenerating capacity (78). Finally, the capacity of a single genetically marked epithelial cell (79), or a cell isolated by multiparameter cell-sorting techniques (80), to reconstitute the mammary epithelium of a cleared mammary fat pad provides convincing evidence for the presence of MaSC.

Initial attempts to characterize MaSC were based on morphological criteria. Adult stem cells are characteristically undifferentiated cells that are discernible using electron or bright field microscopy. Histologic analyses of multiple stages of mammary gland development in mice and rats have suggested that a population of pale staining cells, present during multiple stages of mammary development, may serve as MaSC (81). These pale or light cells have been described in mammary tissue from all species examined, including humans (82), mice (77), rats (81), goats (83), sheep (84), and cattle (85–87). Based on ultrastructural analyses, Chepko & Smith (81) hypothesized that small, light-staining cells (SLC; small round cells possessing light cytoplasmic staining and large spherical nuclei) within the mammary parenchyma act as multipotent MaSC. This assessment was based upon the mitotic competence, cytologic features, transition species, spatial distribution, and frequency of the SLC relative to other mammary epithelial cell types. A subset of SLC were proposed to act as stem cells, whereas undifferentiated large light cells act as transit-amplifying cells, which accounts for patches of these cells in rapidly proliferating mammary epithelia (during pregnancy and early lactation). Similar patches also were noted for label-retaining epithelial cells (LREC, putative stem cells discussed subsequently) and their progeny, which have been referred to as stem cell transitional units (88).

One method used to identify MaSC is based upon the capacity of these cells to retain BrdU-labeled DNA for an extended period (74, 89, 90). Retention of labeled DNA may be attributed to the ability of stem cells to retain the parental DNA strand during asymmetric cell division (91) or may be quiescence of the stem cell population so that the DNA label is not diluted by frequent cell divisions (92). The high proliferation index of LREC in mammary glands of rapidly proliferating, estrogen-treated mice indicates that mammary LREC retain their labeled DNA by asymmetric distribution of DNA strands (74). However, during periods of low mammary proliferation, quiescence of the stem cell population may be an attribute that accounts for retention of labeled DNA. In mice, LREC are found in populations of sorted cells that possess an increased capacity to regenerate the mammary epithelium upon transplantation into the cleared mammary
fat pad (80, 89), which supports LREC’s stem cell character. The LREC includes cells that are both positive and negative (66%) for ESR1 and PGR (74).

Based on fluorescence-activated cell sorting (FACS) with multiple biomarkers and use of mammary transplantation to evaluate multilineage potency, Shackleton, Stingl, and colleagues obtained and characterized a population of cells from enzymatically dispersed mammary tissue that was enriched for MaSC (80, 93). Critical to the success of this approach was the use of markers (Lin⁻) to deplete the population of hematopoietic (CD45 and TER119) and endothelial cells (CD31), as well as markers to select epithelial cells that expressed β₁- or α₆-integrin (CD29 or CD49f, respectively) and heat-stable antigen (CD24). Selection based upon integrin expression likely selected for cells in a basal location. The sorted cells with the greatest regeneration potential were ESR1⁻ (94) and expressed CD24, but at low levels (95, 96).

The cellular hierarchy of mammary epithelium was investigated most recently using a lineage-tracing approach (97). This investigation provided evidence for multipotent MaSC that populate epithelial lineages during fetal and prepubertal development but also for long-lived, unipotent myoepithelial and luminal stem cells. These latter cells may be the predominant source for cell renewal capacity in the mature mammal, with the unipotent luminal stem cells having the ability to expand and differentiate during recurrent cycles of pregnancy. Lineage tracking was enabled by cre-mediated activation of the fluorescent reporter gene (YFP); the specificity of the promoters driving the reporter gene is essential, and it is uncertain whether the basal cell promoters that were used marked the multipotent MaSC in these lineages. In contrast, the s-SHIP promoter has been used to mark multipotent MaSC in TEBs (cap cells) and alveolar buds (98), and other in situ localization techniques indicate the presence of multipotent MaSC in the adult murine mammary gland (74, 99). The roles of unipotent and multipotent MaSC in maintaining homeostasis in mammary tissues of adult mammals remains to be determined fully.

The Mammary Stem Cell Niche

Morphological evidence suggests that MaSC in mature virgin mice are basally located within the mammary epithelium, typically underlain by cytoplasmic extensions of myoepithelial and luminal cells and near ESR1⁺ epithelial cells (100, 101). The microenvironment in which a stem cell resides is commonly referred to as the stem cell niche, through which signals from nearby cells and the stromal matrix appear to regulate stem cell activity. There is strong evidence that stem cell activity in the mammary gland is largely dictated by the stem cell niche. Indeed, the normal lineages of neural stem cells (102) and spermatogonia (103) are redirected when placed in a mammary environment, which permits them to repopulate a cleared mammary fat pad. Beyond its utility as a marker for FACS enrichment of MaSC, deletion of β₁-integrin from basal (keratin 5-positive) cells abrogates the regenerative potential of murine MaSC (104), which emphasizes the importance of epithelial interactions with the extracellular matrix. Although expression of ESR1 by mammary epithelia is necessary for ductal growth, genetically marked ESR1-null mammary epithelial cells can effectively regenerate the mammary tree when mixed initially with wild-type epithelial cells (105). Thus, the stem cell niche, cell-cell interactions, and cell-matrix interactions are important for imparting and regulating MaSC function.

As discussed earlier, duct morphology and murine stroma differ from those in many other species, most notably human and bovine. This has made transplantation of human or bovine cells into the cleared mammary fat pad of immunodeficient mice an ineffective model to assess the regenerative capacity of mammary cell populations. Consequently, alternative approaches have been used. These include in vitro (106–109) and in vivo (110, 111) methods to assess lineage potency. Although valuable information has been gained from these approaches, none truly
recapitulate the regenerative capacity of native MaSC. Because functions of MaSC are influenced strongly by the stem cell niche, cell-cell interactions, and cell-matrix interactions, MaSC function may be severely compromised in these systems. Transplantation studies may induce progenitors to differentiate into lineages to which they do not contribute under normal physiological conditions.

**Human Mammary Stem Cells**

To investigate human MaSC, Dontu and colleagues (106) used an in vitro, nonadherent culture system that results in the formation of mammospheres, which are enriched for MaSC and progenitor cells. Increased aldehyde dehydrogenase (ALDH)–1 activity was associated with a population of human primary mammary epithelial cells that exhibited stem cell properties and were ESR1– (112, 113). The regenerative capacity of a population of human mammary epithelial cells, sorted based on expression of CD49þ and EpCamlow, was demonstrated by implanting them under the kidney capsule of estrogen/progesterone-treated mice within a collagen matrix also containing human fibroblasts (114).

Evaluation of the expression of cell-lineage markers and in vitro propagation studies in human breast tissue from reduction mammoplasty led to the conclusion that a regional stem cell lineage is present in the human breast, wherein MaSC are present in mammary ducts and lobule progenitor cells are prevalent in the terminal ductal lobules (115). Similarly, genetic marking has been used to identify putative activated MaSC in alveolar buds of the mouse (98). The latter work suggests the presence of multipotent, though dormant, MaSC in mature alveoli. The distribution and function of MaSC in mature mammals remain to be elucidated fully.

**Bovine Mammary Stem Cells**

Initial investigations of bovine MaSC/progenitors were based upon morphological criteria (86). Lightly staining cells comprised 10% of the total epithelial cell population but accounted for most of the cell proliferation, and all proliferating cells were ESR1– (12). These results strongly support the concept that lightly staining mammary parenchymal cells comprise the primary proliferative cell population, and we suggest that the most undifferentiated population of these cells likely contains MaSC or primitive progenitor cells. However, because this population accounts for approximately 10% of mammary epithelial cells prepubertally, it clearly contains more than MaSC.

Subsequent studies have characterized putative MaSC/progenitor cells based on long-term retention of labeled DNA. LREC in mammary epithelium of calves were localized in the basal layer or in the embedded (LRECe) layers between the basal and luminal cells of a multilayered epithelium (90,116). These LREC were present in quantities (90) consistent with the prevalence of stem cells in mouse mammary gland (79, 80, 93). The proliferation rate of these cells was such that 5.4% of LREC were Ki-67þ (90) and 14% were PCNAþ (116), which is consistent with the Ki-67 labeling index of rapidly proliferating mammary epithelia (5–8%) in calves at the same stage of mammary development (117). The LREC in bovine mammary gland represented a mixed population, with regard not only to locality within the mammary epithelium but also to expression of ESR1 (90, 116). Similarly, LREC in mouse mammary gland represented a mixed population of ESR1þ and ESR1– cells (74). In prepubertal bovine mammary gland, LREC that were near the basement membrane were ESR1– and were hypothesized to be MaSC, whereas the LREC that were located in the embedded layer(s) of the mammary epithelium were a mixed population of ESR1þ and ESR1– cells and were hypothesized to be progenitor cells (90, 116). The estrogen receptor
status of MaSC is of considerable interest because of the importance of estrogens for MaSC function, mammary ductal growth, and tumorigenesis. MaSC of mouse and human are ESR1− (16, 94, 96, 118). To fully populate a cleared fat pad, a single ER− stem cell presumably must undergo asymmetric division to provide a population of ER+ progenitors, which in turn regulates proliferation of MaSC (118, 119).

Cell-sorting techniques have also been applied to suspensions of bovine mammary cells in an attempt to evaluate progenitor cell lineages and to enrich for MaSC. Motyl and colleagues (87) isolated and evaluated gene expression in a population of mammary cells that were isolated by SCA1 expression and showed upregulation of genes that are characteristic of hematopoietic cells. The authors suggested that MaSC are composed of SCA1+ hematopoietic cells that have taken residence in the MaSC niche. However, because accompanying micrographs showed clearly that most SCA1+ cells were in the mammary stroma, and methods to enrich for mammary epithelial cells were not employed, the gene expression profile likely cannot be attributed to MaSC. Additionally, previous research indicates that although hematopoietic stem cells may populate the stroma of many organs, it is highly unlikely that these cells will populate the parenchymal tissue or the MaSC niche (120). Finally, expression of SCA1 does not coincide with the highest MaSC activity in sorted cells (80).

Martignani et al. (111) used ALDH activity as a selection criterion for cell sorting. They then used in vitro colony-forming assays to show the existence of bipotent and lineage-restricted progenitors (luminal = CK18+, myoepithelial = CK14+) and demonstrated that cells with low ALDH activity were capable of regenerating structures of mammary epithelium within collagen gels implanted beneath the kidney capsule of immunodeficient mice, whereas cells with high ALDH activity were luminal progenitors. This study not only provided data about characteristics of bovine bipotent progenitor cells but also validated a means to assess this potency.

Most recently, Rauner & Barash (108) used multiparameter FACS to obtain four populations of cells from enzymatically dissociated bovine mammary tissue. Adapting methods used for sorting murine mammary cells (80, 93), they sorted Lin− cells based on expression of cell surface markers CD24 and CD49f. Sorted and nonsorted cells were evaluated by differentiation potential in vitro using colony-forming and mammosphere assays. This important study confirmed many of the general aspects of MaSC/progenitor cells arising from mouse and human studies. The four populations attained were: (a) putative bovine MaSC (CD24medCD49fpos) that were bipotent (myoepithelial and luminal) and possessed a high growth rate; (b) basal bipotent progenitors [CD24negCD49fpos] with medium growth rate, which, along with reduced sphere-generating potential, distinguished them from putative MaSC; (c) luminal unipotent progenitors [CD24highCD49fneg] possessing low growth rate; and (d) luminal unipotent cells [CD24medCD49fneg] with little or no proliferative activity that express differentiation markers such as ESR1, PGR, and GATA. Rauner & Barash (108) notably reported that, although putative MaSC typically possessed little or no ALDH activity, as reported previously (111), a small proportion (0.35% of total viable cells) expressed high ALDH activity, which they hypothesized to represent a highly enriched MaSC population. Furthermore, although ALDH1+ cells were localized in the stroma, ALDH activity (based on activity of living cells for the fluorogenic ALDH substrate, aldefluor) in epithelial cells was associated with stem cell character. Differences between immunohistochemical staining for ALDH1 and ALDH activity may be attributed to antibody specificity. Additionally, a recent study using ALDH1 knockout mice indicated not only that ALDH1+ cells were nonessential for hematopoietic stem cell function but also that ALDH1 deficiency does not affect aldefluor staining (121). This suggests that ALDH activity is maintained by other ALDH isoforms (121), such as those detected in the bovine mammary gland and expressed at increased levels in putative MaSC (122).
based upon results from murine and human studies and illustrates the likely relationship of
progenitors that have been identified in the bovine mammary gland.

The power of applying FACS technology to stem cell research is that sorted populations are
viable and can be used to assess their regenerative capabilities, which define and distinguish stem
cells and progenitor cells. Rauner & Barash (108) found that, in contrast to human and mouse,
sorted bovine mammary epithelial cells were not capable of forming true mammospheres but did
form spheres that allowed for estimation of their differentiation potential. This may owe to
inhibition of cell-cell interactions by the binding of the antibodies used for sorting to cell surface
proteins. It must be recognized that cell-cell interactions are essential for expression of the
regenerative attributes of stem and progenitor cells within a given tissue. This is clearly illus-
trated by the ability of nonmammary cells to populate the MaSC niche and become reprog-
rammed to fulfill the duties of endogenous MaSC (123).

In addition to issues of the isolation and characterization of MaSC and progenitors from
a mixed suspension of mammary cells, all previous studies have evaluated MaSC after removing
them from their stem cell niche, i.e., the microenvironment consisting of surrounding cells, sig-
naling molecules, and noncellular components that supports stem cell function and survival. We
recently took an approach that retains histological information by characterizing gene expression
in putative MaSC/progenitors (LREC) directly after their in situ excision, by laser microdissection,
from the mammary epithelium (122). It must be recognized that to identify putative MaSC and
progenitor cells by long-term retention of DNA label is to select the cells based upon their life

**Figure 3**
Schematic representation of a proposed mammary epithelial hierarchy based upon data from mouse.
Multipotent, long-lived mammary stem cells (MaSC) give rise to a bipotent progenitor capable of generating
cells in the luminal and myoepithelial lineages. MaSC also give rise to short-lived, pregnancy-identified
mammary epithelial cells (PI-MEC). Recent lineage-tracing studies also suggest that unipotent luminal and
myoepithelial progenitors provide for much of the repopulating activity in an adult animal. Characteristics
(red) and presumed location of cell populations sorted from bovine mammary tissue are depicted.
history (i.e., the extent of label retention represents an integration of the cell’s past proliferation and differentiation events). Consequently, selecting putative MaSC and progenitor cells based on label retention likely represents an enrichment for these cell populations. LREC and neighboring epithelial control (non-LREC) cells were excised from two locations: the basal layer and embedded layers of the mammary epithelium. We hypothesized that basal LREC were enriched for MaSC, whereas embedded LREC were enriched for more committed progenitor cells, and that by comparing the transcriptomes of these cells with neighboring control cells we would obtain molecular profiles and biomarkers for MaSC and progenitor cells. Low expression of ESR1 and high expression of ALDH3B1 in basal LREC were consistent with stem cell character. We found high expression of NR5A2, a pluripotency transcription factor (124), and little or no expression of XIST, X chromosome inactivation factor (125, 126), in basal LREC. Comparisons between basal and embedded LREC showed downregulation of cell survival and proliferation factors (IGF2, HSPB6, LAMC1), NR5A2, and nestin (stem cell marker) in embedded LREC. Our data support the hypothesis that LRECs are enriched for MaSC and progenitor cells; basal LREC are enriched for progenitors with more stemness features (putative MaSC), and embedded LREC are enriched for more committed progenitor cells. The data provide molecular signatures that yield potential markers for MaSC and progenitor cells. Such markers will permit tracking of MaSC function as affected by physiological state or treatment.

Insights into the biology of stem cells will be gained through further confirmation of candidate MaSC markers. Such confirmation requires an evaluation of the self-renewal and differentiation potential of cells expressing these markers. Identification of appropriate biomarkers will provide a means to identify MaSC and will facilitate our understanding of MaSC functions in mammary development, homeostasis, and cancer. Specific cell-surface markers will provide a means for future isolation of MaSC and investigations of their biology. This review has not discussed investigations of MaSC in domesticated species other than the bovine. For information regarding recent attempts to identify MaSC in other agricultural species, we refer the reader to recent publications (127–129).

Modulating Mammary Stem Cells and Progenitors

The activity of MaSC and progenitor cells changes with physiological state to meet organ demands for growth and cell turnover. During early neonatal development, MaSC activity allows for the rapid growth and elongation of mammary ducts that are characteristic of this phase, whereas during an established lactation (with the exception of early lactation in litter-bearing species), epithelial cells proliferate at a very low rate (31). In mice, there clearly are changes in the population of mammary progenitor cells during mammary development and differentiation. For instance, there is a large increase in the number of progenitor cells in the murine mammary gland during pregnancy; these parity-identified mammary epithelial cells are present in the glands of virgin animals and can be expanded by progesterone treatment (18, 130, 131). In addition to the likely involvement of deregulated MaSC in the pathology of breast cancer, the idea that MaSC function is influenced by intramammary infection remains a possibility. Mesenchymal stem cell function is compromised by Mycobacterium tuberculosis infection (132). Whether MaSC function is altered during mastitis, particularly by chronic infections, remains to be investigated.

Many genetic pathways have been implicated as regulators of MaSC proliferation and differentiation. These have included Wnt, NOTCH, Hedgehog, BRCA1, p21, p63, Pten, and p53 (106, 113, 133, 134). Alteration of regulatory pathways by transgenic technology has been used to evaluate the effect on MaSC. For example, downregulation of NOTCH signaling in MaSC causes expansion of the MaSC population, whereas upregulation leads to expansion
of luminal progenitors (135), and knockout of p53 leads to expansion of MaSC by promotion of symmetric division (136).

Sherley and colleagues (138–140) pioneered methods to promote the expansion of somatic stem cells by a nontransgenic approach. In vitro experiments with rat hepatocytes (137, 138) and with hair follicle stem cells, which can also renew the epidermis and other components of the skin (139), indicated that p53 promotes asymmetric proliferation of somatic stem cells through downregulation of inosine-5′-monophosphate dehydrogenase (IMPDH), the rate-limiting enzyme for guanine nucleotide synthesis. A decrease in guanine ribonucleotide concentrations in these in vitro model systems promotes the nonrandom segregation of sister chromatids characteristic of asymmetric division of many somatic stem cells (140). Precursors of guanine nucleotide synthesis, such as xanthosine or inosine (purine nucleosides), bypass IMPDH-mediated guanine ribonucleotide synthesis and increase guanine ribonucleotide concentrations in the cell, which thereby promotes symmetric division and expands the stem cell population (139, 141).

Similarly, xanthosine treatment expands the population of putative bovine MaSC. As anticipated, in vitro treatment of primary mammary epithelial cells with xanthosine promotes symmetrical division and increases the number of putative MaSC (142). Most importantly, application of this approach to in vivo treatment of bovine mammary epithelial cells has proved effective. Infusion of xanthosine through the teat canal and into the mammary gland induces an apparent expansion of the MaSC population (116). This effect was evidenced by an increase in the number of LREC and an increase in tissue telomerase activity. Studies are in progress to evaluate the influence of intramammary infusions of xanthosine or inosine on milk yield and efficiency of milk production in dairy cows. Most recently, this approach has been used to enhance milk yield of transgenic goats, which were characterized by poor milk yield and persistency. At the author’s suggestion, colleagues used a protocol designed to expand the MaSC population in three lactating transgenic goats by infusing inosine into the mammary gland after each milking on days 5–7 of lactation (143). The treatment resulted in a 62% increase in milk production (Figure 4), which emphasizes the potential benefits that may be garnered by in vivo modulation of MaSC/progenitor cell function.

**CONCLUDING REMARKS**

In this review, we provide an overview of mammary development and mammary cell renewal. Although these issues clearly impact milk yield for dairy production, they are also key to providing sufficient quantity and quality of milk to suckling livestock. Activation of MaSC and progenitor cell pools through natural processes (e.g., pregnancy) obviously is critical to lactation, but exogenous manipulations (e.g., xanthosine or inosine infusions) may play an increasing role in dairy production settings. Additional discovery research is needed to better characterize mammary stem cells in cattle and other domesticated species so that management decisions can be informed by an accurate and more complete understanding of mammary physiology. This research will be fostered by the development of additional and specific markers for MaSC and progenitor cells. Although an assortment of biomarkers has been used to sort and enrich for these, a specific marker or set of markers that permits the identification of progenitor cells in situ has yet to be identified. Such markers would greatly advance our understanding and our ability to evaluate the impact of treatment targeted to enhance the activity of MaSC and progenitors.

Lessons from the comparative analysis of MaSC physiology will be especially critical in advancing understanding of mammary function, especially in light of the experimental and financial challenges associated with large-animal physiology research. The ultimate goal of such studies will be to understand the fundamental biology of the mammary gland so that production efficiencies...
can be maximized without compromising animal health or product quality. Beyond the immediate sphere of animal agriculture, comparative studies will better help answer fundamental questions about mammary gland biology and tumorigenesis. Advances in cell characterization provided by FACS and laser microdissection, coupled with high-throughput bioinformatic analyses, undoubtedly will provide additional approaches to modulate mammary development and productivity.

**DISCLOSURE STATEMENT**

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LITERATURE CITED


