ABSTRACT: Mammary development proceeds from an aggregation of cells in the ventral ectoderm to the establishment of an elaborate tree of alveoli, ducts, and cisternae. However, despite abundant data on endocrine regulation of ruminant mammary growth, we know comparatively little about cell lineages, expression of differentiation markers, and plasticity in mammary cell phenotype. Histologic analyses have revealed cell populations with distinct histochemical profiles, but functional assessment of cell populations during development has been limited to analysis of proliferation and frequency estimations of morphotypes. The lack of transplantation models, limited availability of validated antibodies with reactivity to bovine antigens, and similar technical challenges have generally hindered the pace of discovery, but the application of new technologies such as laser microdissection, transcriptional profiling, and multispectral image analysis are yielding important clues into bovine mammary cell ontogeny and developmental regulation. Our analyses have shown that prepubertal ovariectomy affects epithelial architecture, increases the proportion of cells expressing the estrogen receptor, and increases myoepithelial cell development, all concomitant with a dramatic reduction in the mass of parenchymal tissue. Our observations point to a dual role for ovarian secretions in the control of not only the rate of epithelial development, but also the nature of the parenchymal development. The balance of stimulus and inhibition pathways cooperatively regulates mammary growth. The increased reliance on objective staining analyses and quantitative approaches will ensure broader repeatability, application, and extension of the findings regarding the impact of the ovary and other regulatory entities and factors. Advances in understanding the ontogeny of mammary epithelial cells, coupled with established and increasing knowledge of endocrine factors affecting mammary development, may yield intervention strategies to improve dairy profitability.

Keywords: mammary gland, myoepithelial, ovariectomy, parenchymal development

INTRODUCTION

No symposium or paper series arranged in honor of the lifetime research contributions of H. A. Tucker would be complete without consideration of fundamental aspects of bovine mammary development. Early work by Tucker and colleagues (Sinha and Tucker, 1969) helped to transition the entire lactation physiology field and promote the adoption of modern research techniques to solve pressing questions of mammary development and physiology. A complete account of all the historical literature relating to bovine mammary development is beyond the scope of one article, so readers are directed to some of the still pertinent reviews penned by Tucker (1981) and others (e.g., Akers, 1990, 2002; Sejrsen et al., 2000). Instead, the discussion presented here will highlight the present state of knowledge relating to development of the bovine mammary lumenal and myoepithelial cell lineages and


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future areas for emphasis in training mammary physiology researchers.

OVERVIEW OF PREPUBERTAL RUMINANT MAMMARY HISTOLOGY

At birth, the bovine mammary parenchyma consists of a rudimentary duct network connected to a small cisternal cavity. In contrast to the long, infrequently branching ducts of the prepubertal murine mammary gland (Williams and Daniel, 1983), the ruminant mammary parenchyma develops as a compact, highly arborescent parenchymal mass (Wallace, 1953; Capuco et al., 2002). In the prepubertal murine mammary gland, ducts frequently extend several thousand microns without branching. In the ruminant mammary gland, ductal elongation is accomplished through the growth, development, and subsequent extension of highly arborescent terminal ductal units (TDU). As illustrated by our previous work on bovine mammary development (Capuco et al., 2002), ruminant mammary TDU consist initially of solid chords of epithelial cells that penetrate into the mammary stroma. As the solid chord of epithelial cells extends into the mammary stroma, lateral outgrowths emerge at closely spaced intervals. Eventually, 5 to 10 separate ductule outgrowths are arranged around the central epithelial chord. Lumenal spaces within the TDU are minimal. The cells of the ductule are arrayed in a radially arranged fashion and meet in the center to form a series of interrupted microlumena along the midline of the ductule. The walls of the ductules are stratified into 2 to 4 cell layers. The basal cell layer is populated by undifferentiated myoepithelial precursor cells that form an almost continuous stratum upon which 1 to 3 layers of epithelial cells are positioned. A small population of the cells span the entire distance from the underlying myoepithelial precursors to the midline (i.e., from the basement membrane to the central longitudinal axis) of the ductule. The lateral outgrowths of the TDU terminate as blind-ended ducts. The distal extremity of the ductules remains covered with myoepithelial precursors, although there are occasional discontinuities in the layer and cells that span the distance from the basement membrane to the ductal lumen can be observed (Safayi et al., 2011a,b).

Proliferation of epithelial cells establishes a cellular framework of the nascent TDU as a compact branching structure with minimal lumenal space. Once the TDU architecture is well established, lumen formation occurs as the apical cell surfaces along the midline of the ductules begin to separate. Lumenal spaces formed by the separation of apposed epithelial layers then begin to expand through undefined mechanisms. New TDU are formed as outgrowths from these small newly developed ducts while lumenal spaces enlarge and ductal remodeling occurs. A relatively dense investment of collagenous connective tissue surrounds the entire TDU throughout all stages of parenchymal growth and development. Ruminant mammary TDU very rarely come into direct contact with adipocytes.

Analysis of prepubertal ruminant mammary tissues reveals a curious pattern of cell proliferation. Contrary to what might be expected, cells in the distal portions of the ductules in a TDU do not seem to proliferate at a substantially greater rate than other cells in the TDU. Three-dimensional reconstructions and analysis of serial histologic sections provide evidence that proliferating cells are well distributed throughout the TDU, but are less frequent in the subtending duct (Capuco et al., 2002). Because the TDU is a complex structure, it may be understandable that there is proliferation along the ductules and at branch points, not just at the distal end. In contrast, the majority of proliferative cell activity in the murine mammary gland is localized in the leading edge of the terminal end-bud (Williams and Daniel, 1983). On a slightly larger scale, we have previously noted that TDU in the peripheral regions of the parenchymal mass had greater labeling indices than those in internal regions of the parenchyma [i.e., closer to the gland cistern (Ellis et al., 2000; Ellis and Capuco, 2002)], indicating that parenchymal development may be regulated in multiple ways to control overall glandular development.

THE CHALLENGE OF DEFINING MAMMARY PARENCHYMAL CELL POPULATIONS

Data on the development of specific epithelial cell lineages within the bovine mammary gland are very limited. Our previous report (Ellis and Capuco, 2002) documents the appearance, morphology, and histochemical staining characteristics of parenchymal cells during the prepubertal development period. The staining methods used in the prior studies highlight nuclear morphology and general protein accumulation within the cell that is correlated with development of organelles and cytoskeletal elements. In combination with bromodeoxyuridine labeling, the histochemical staining patterns allowed for the quantification of proliferation among different classes of cells. As in murine systems (Chepko and Smith, 1997), the lighter staining cells were the primary proliferative cell population. Unfortunately, the interoperator repeatability of such subjective staining classification was limited, and we adopted and developed staining techniques with better potential for standardization. Our recent work with immunohistochemical and immunofluorescent staining of myoepithelial markers provides a more objective and repeatable approach to the anal-
alysis of parenchymal cell lineages (Safayi et al., 2012). However, it is important to note that interpretation of immunohistochemical data should include consideration of all elements in sample preparation and analysis.

As an example, the use of plastic resins provides for excellent morphological analyses at the light or electron microscopy levels (e.g., Akers and Heald, 1978; Cerri and Sasso-Cerri, 2003); however, these resins are not readily amenable to many immunolabeling protocols (Korn and Ellis, 2009). Some of our initial analysis of prepubertal mammary development relied on glycol methacrylate and similar resins for histologic embedding of tissue (Ellis et al., 1998; Ellis and Capuco, 2002). Whereas these antibodies and protocols worked quite well for bromodeoxyuridine staining to characterize prepubertal cell populations (Ellis and Capuco, 2002), paraffin sections were used for other immunohistochemical characterizations (Capuco et al., 2002). In some instances, an antibody may appear to provide reasonable labeling within plastic-embedded tissue, but further analysis reveals an underlying lack of sensitivity. For instance, immunohistochemical staining of smooth muscle actin (SMA) in glycol methacrylate embedded tissues revealed a striking difference in the appearance, distribution, and differentiation of myoepithelial cells after prepubertal heifers were ovariectomized (Ballagh et al., 2008). Validation of our conclusions was provided by reference to internal positive control tissues, namely, vascular smooth muscle. In intact animals, essentially no myoepithelial staining was observed, but vascular smooth muscle staining was abundant, specific, and consistent. Immunofluorescent analyses of paraffin-embedded tissue sections from the same animals provided for greater sensitivity of detection and revealed expression of SMA in basal parenchymal cells from intact animals. The apparent contrast in conclusions serves as a case study in the need for careful selection of analytical methods and approaches. The general conclusion from our earlier report, namely, that that ovariectomy results in increased myoepithelial development, is still valid but should be tempered by the additional insight from more recent studies. Our recent report (Safayi et al., 2012) provides additional supporting data and extends our observations to provide a detailed account of myoepithelial changes that attend ovariectomy in prepubertal heifers.

A nomenclature for basal and luminal layers is routinely applied in other model systems (e.g., human and mouse), but indiscriminate use of the terms may disguise the informative subtleties in comparative histology. As an example, bovine mammary epithelium from intact animals in an allometric growth phase has a more pronounced stratification than the simple terms basal and luminal would suggest. During the prepubertal period, an embedded cell layer is readily evident, and there are 3 or more clearly distinguishable strata. As we have noted previously (Ellis and Capuco, 2002), most of the embedded cells touch neither the basement membrane nor the luminal spaces. However, the embedded layer does express steroid receptors (Capuco et al., 2002; Berry et al., 2003) and may thus play a pivotal role in regulating morphogenesis.

**GENERAL ASPECTS OF MYOEPITHELIAL DEVELOPMENT**

Progressive differentiation of myoepithelial cells has been studied primarily in murine model systems. Radnor (1972) provided an extensive histologic analysis of perinatal mammary development in rats, including a morphologic assessment of myoepithelial differentiation. At birth, murine mammary tissues were disorganized. Within the first 2 wk, cells began to orient themselves, with flattened cells indicative of the myoepithelial phenotype appearing in the basal stratum. Radnor (1972) noted changes in the orientation of cell nuclei as the developing myoepithelial cells became flattened between the luminal cells and the basement membrane. Increased cytoplasmic staining intensity and the development of extended cytoplasmic processes accompanied the observed changes in myoepithelial development. Between 8 and 70 d of age, myoepithelial differentiation was reportedly more related to position within the gland than to chronologic age. Based upon the observations of mitoses, cell shape changes, and the appearance of cellular organelles, Radnor (1972) proposed that luminal cells serve as precursors to the myoepithelial population. Williams and Daniel (1983) subsequently noted that the distal ends of murine terminal end buds (i.e., the structure responsible for growth of the ductal system and penetration into the surrounding mammary fat pad) were devoid of myoepithelial cells. Myoepithelial cells developed along the flanks of the terminal end buds and formed a continuous sheath around the subtending duct.

Early work by Dulbecco et al. (1983) led to the identification of cytokeratin markers specifically expressed in myoepithelial cells. There are many reports in the scientific literature indicating that myoepithelial cells express cytokeratins 5 and 14, and to a lesser extent, cytokeratin 17. Luminal epithelial cells express cytokeratins 8 and 18, so the 2 strata can be distinguished on the basis of their molecular phenotype. Mature myoepithelial cells are also known to express other characteristic proteins that relate to their contractile properties, including SMA, calponin, and smooth muscle heavy myosin chain (Adriance et al., 2005). Specific adhesion molecules, such as P-cadherin and β1-integrin, are expressed by mature myoepithelial cells, as are proteins related to the formation of desmosomal and hemi-des-
mosomal attachments. Myoepithelial cells are known to contribute to the formation and stabilization of the basement membrane through expression and secretion of laminin and collagen IV. Myoepithelial cells express neutral endopeptidase, which is also known as cluster of differentiation marker 10 (CD10) and common acute lymphoblastic leukemia antigen (CALLA; Kalof et al., 2004) and specific protease inhibitors, such as mammary serine protease inhibitor (maspin; Zhang et al., 1999), as well as receptors for oxytocin, epidermal growth factor, and hepatocyte growth factor and fibroblast growth factors (Deugnier et al., 1995; Yamaji et al., 2006).

Deugnier et al. (1995) also performed a detailed immunofluorescent assessment of myoepithelial differentiation in the rat mammary gland and noted that SMA and smooth muscle heavy myosin chain were expressed in basal mammary cells at birth. Interestingly, other molecules required for contractility (e.g., calponin, vinculin) were not expressed until 1.5 to 3 wk of age. Furthermore, the expression of β₁-integrin in the basal layer became markedly more intense after 1.5 wk, possibly indicating that the developing myoepithelial cells began to adhere more strongly to the stromal tissues. As in salivary myoepithelial cells (Ogawa, 2003), the expression of SMA appears to be an early marker of mammary myoepithelial differentiation, but functional development of contractility in rodents is likely delayed until at least a few weeks after birth.

**PREPUBERTAL BOVINE MYOEPITHELIAL DEVELOPMENT**

Research specifically related to bovine myoepithelial cells has largely focused on the contractile behavior acquired when the cells fully differentiate (Bruckmaier and Blum, 1998). Information is available to explain how myoepithelial cells respond to oxytocin or epinephrine during lactation (Akers, 2002; Bruckmaier, 2005). However, surprisingly little attention has been paid to earlier stages in the differentiation of bovine mammary myoepithelial cells, especially during prepubertal mamogenesis. Capuco et al. (2002) noted that the classically flattened morphology of basal myoepithelial cells was largely absent in heifers at 3 mo of age, that staining for smooth muscle actin appeared weak and discontinuous, and limited ultrastructural analyses failed to provide evidence for contractile fibers. Others (Hellmén and Isaksson, 1997) have assessed myoepithelial markers in tissue from a pair of lactating cows, but the myoepithelial cells are already well developed in lactating animals.

In addition to better characterizing the expression of SMA, our investigations of myoepithelial cells in tissue sections from prepubertal heifers and re-analysis of previously published work (Berry et al., 2003) have revealed that reduced or absent ovarian secretions produce consistent changes in the architecture of the mammary parenchyma. In the presence of decreased concentrations of systemic estrogens, the bovine mammary epithelium generally has only 2 layers: the basal layer of (pre)myoepithelial cells and the suprabasal layer of luminal cells. In addition to reduced epithelial stratification, ductal lumens are markedly dilated in the mammary gland of ovariectomized heifers. In ovariectomized heifers, myoepithelial cells also tend to have a more elongated appearance with extended cell processes and an increased interdigitation with stromal tissues (Figure 1). The expression of progesterone receptor may also be reduced to essentially undetectable levels in ovariectomized heifers, depending on the age of the animal and the interval between surgery and sampling (S. Ellis, R. M. Akers, A. V. Capuco, and S. Safayi, unpublished data). These characteristic alterations produce a recognizable “ground state” of mammary parenchymal histology. In mice, myoepithelial cells are known to affect epithelial polarity and stratification (Gudjonsson et al., 2005), so our observations are consistent with literature reports for murine species.

**MYOEPITHELIAL EFFECTS ON LUMENAL CELLS**

Understanding the relationships between different cell types will be a critical factor in understanding bovine mammary development. The inverse relationship observed between myoepithelial development and growth of mammary parenchyma in heifers that were ovariectomized at d 40 leads to the intriguing hypothesis that myoepithelial cells are capable of inhibiting parenchymal development. Our hypothesis is still preliminary and only supported by correlative evidence to date. Part of the challenge in determining a functional role for myoepithelial cells lies in the many functions that may affect morphogenesis. Contractility of myoepithelial cells has been extensively documented, but researchers are actively investigating the involvement of other aspects of myoepithelial cell physiology in the regulation of parenchymal development and even tumorigenesis. Williams and Daniel (1983) reported a thickening of the basal lamina associated with myoepithelial cell differentiation. Myoepithelial cells are now known to produce extracellular matrix molecules, including collagen IV and laminin (Gudjonsson et al., 2002). Myoepithelial cells also secrete protease inhibitors (e.g., maspin) and express endopeptidases (CD10/CALLA; Kalof et al., 2004) that degrade target molecules capable of stimulating parenchymal cell proliferation and angiogenesis. Mammary serine protease inhibitor also interacts with extracellular matrix molecules, including collagen type
I, in ways that help to stabilize epithelial structures (Zhang et al., 1999; Khalkhali-Ellis, 2006). Endothelin-1 acts as an autocrine or paracrine growth factor and is degraded by CD10 (Kajiyama et al., 2005), so there is a possibility that myoepithelial cells act not only to inhibit parenchymal cell proliferation, but may also modulate angiogenic processes operating in the adjacent stroma.

Man et al. (2003) observed focal disruption of myoepithelial layers associated with metastatic processes and with tumor progression. Defects in myoepithelial development or survival that caused exposure of luminal epithelial cells to the basement membrane apparently led to activation of an inflammatory response and paracrine stimulation of parenchymal cell proliferation, but may also modulate angiogenic processes operating in the adjacent stroma.

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BOVINE MAMMARY STEM CELLS

The historical data relating to bovine mammary stem cells have been reviewed previously (Capuco and Ellis, 2005), and subsequent advances have been limited. To date, there are still no verified markers of mammary stem cells for any species. Despite the bewildering array of potential markers, none have been reliably specific for identifying stem cells within the mammary gland. We speculate that parenchymal development in ovariectomized heifers is inhibited by the development of a continuous myoepithelial cell layer, coupled with the attendant changes in epithelial stratification and physical separation of progenitor cells from the basement membrane.
2009). Utilizing laser microdissection, label-retaining epithelial cells have been isolated, and based on their molecular profiles, potential makers have been identified (Choudhary et al., 2011), but further testing is still in progress.

Some of the most convincing data related to the isolation and propagation of mammary stem cells for any species was generated by largely excluding other cell types and working with the residual uncharacterized epithelial cell populations (Stingl et al., 2006; Shackleton et al., 2006). These investigators had the advantage of working with murine species, for which cell transplantation and gland repopulating assays are practical. Although a cleared fat pad technique has been demonstrated for ruminant species (Hovey et al., 2000), utilization of the technique has been very limited due to expense and the constraints imposed by a lack of in-bred or immunologically deficient strains. Others have reported in vitro studies to show that colony-forming units are readily isolated from primary bovine tissues and can develop as xenografts in immunocompromised hosts (Martignani et al., 2009). Such studies support the concept that bovine mammary stem cells do exist and can contribute to multiple lineages, but the reports are not yet extensive enough to provide for definitive analyses. Likewise, Motyl et al. (2011) have described the gene expression of bovine mammary cells isolated on the basis of stem cell antigen-1 (SCA-1) expression. However, as the figures presented in the report demonstrate, SCA-1 expression is not solely restricted to mammary epithelial cells, so the conclusion that SCA-1 defines a stem cell pool may be unwarranted until the contributions of cell populations within the SCA-1 isolate can be ascertained.

OTHER MAMMARY CELL TYPES

Regardless of species, parenchymal development involves the interaction of epithelial cells and stromal tissues. Data from murine systems demonstrate that many cell types within the mammary stroma, including fibrocytes and fibroblasts, adipocytes, eosinophils, macrophages, and mast cells, play important roles in prepubertal mammary development (Gouon-Evans et al., 2000). Several lines of evidence demonstrate that pro-inflammatory migratory leukocyte cell populations in the stromal tissues are required for mammary development. The interaction of innate immune system components in developmental systems has been highlighted recently by Watson (2009) and will prove an interesting topic for future studies in bovine mammary tissue. Genetic models with reduced macrophage or eosinophil cell populations have diminished parenchymal development (Gouon-Evans et al., 2000; Gyorki et al., 2009). It is particularly striking that depletion of mast cells can so dramatically affect mammary development in rodent models (Lilla and Werb, 2010) because they are frequently observed in bovine mammary tissue sections close to TDU and interspersed among alveoli (S. Ellis, unpublished data). No data are presently available to describe the effect of similar cell depletion studies in ruminant systems, but the use of inert liposomes with sequestered cytotoxins to ablate phagocytic cells (Gyorki et al., 2009) may have some promise in cattle, where models of genetic deficiency in immune cell development are limited.

TRAINING THE NEXT GENERATION

One of the goals for the honorary symposia was to define areas of opportunity and training for future generations of lactation physiologists. In a 1981 review, Tucker highlighted both the challenges and opportunities of training students for a career in lactation physiology by including discussion of research areas that included molecular biology, hormone receptors, harvesting of milk, endocrine-nutrient metabolism, genetic basis of hormonal control of lactation, and environmental physiology (Tucker, 1981). Many of the areas highlighted in the early 1980s remain active and increasingly important areas of research. Molecular biology in particular has proven to be both a monumental boon and an ever-increasing challenge to lactation physiologists. When Tucker penned his 1981 review, PCR had not been invented, the prospect of sequencing even one complete genome was almost laughable, and the idea of sequencing thousands of individual animal genomes and transcriptomes was preposterous. The technologic advances of the day included RIA, receptor assays, electron microscopy, and cell culture. Since then, advances in enzyme and reporter reagents, computing technology, and detector sensitivity have opened untold vistas for further exploration. Micro-RNA molecules, not even discovered until 1993 (Lee et al., 1993), are now seen as central regulators of cell physiology, and advances in optical microscopy have shattered seemingly intractable resolution limits. Electron microscopy has seen similar technical advances for both scanning and transmission microscopes, including the advent of focused ion beam systems to allow selective dissection and visualization of cell structures with unprecedented precision.

With specific reference to the challenge of understanding the development and differentiation of bovine mammary epithelial cell lineages, we believe it will be especially important to instruct future trainees in data management skills. As an example, microscopists since the age of van Leeuwenhoek have been frustrated in their attempts to objectively describe and analyze their work. The advent of photographic techniques and digital imaging have greatly simplified the process, but subjective determinations and assurances are still widely used as the basis for image interpretation. However, the early
work of Hu (1962) made a quantitative analysis of image moments and features a real possibility. Later improvements by Teague (1980) led to the development of Zernike Moments, a series of orthogonal polynomials that can describe planar object shape information with high fidelity in a scale-, orientation-, and rotation-independent fashion. In combination with sensitive imaging devices and modern computers, the Zernike polynomials can be used to add informative and objective shape information to histologic analyses without the requirement for tedious and time-consuming manual feature measurement. The availability of open-source image analysis tools makes such technologies readily accessible to laboratory groups throughout the research community [e.g., CellProfiler and CellProfiler Analyst; http://www.cellprofiler.org (see Kamentsky et al., 2011)]. Image analysis software can now vastly accelerate the process of data extraction, but as with many bioinformatic tools, the yield of data is simply enormous. Data management and analysis will become increasingly important as studies that used to yield a few values for the percentages of proliferating cells suddenly return millions of correlated data points to describe the size, shape, texture, protein expression, and physical associations of thousands of cells. Data from high-throughput combinatorial chemistry studies now routinely include hundreds of observations on millions of individual cells, and similar outputs from histologic sections and automated microscopy systems seem very promising. Given the pace of technological advances, the importance of training future scientists to independently manage large volumes of data cannot be overstated.

SUMMARY AND CONCLUSIONS

It has been noted that even with the years of research and untold hours of study, trial, and error, we still cannot fully or reliably replicate the success that Mother Nature routinely has in promoting the development and differentiation of the mammary gland. To quote Tucker more directly, “Viva la mom!” (Tucker, 2000). Lactation physiologists will increasingly have to integrate a wealth of experimental data with improved management strategies and therapeutic interventions to further improve upon the noteworthy successes that the last decades of mammary research have yielded. Developing such therapeutic interventions will require a more comprehensive understanding of the mammary cell lineages and developmental regulators. Whether future therapies to manipulate myoepithelial physiology are possible or useful remains to be seen. Nevertheless, an improved awareness of mammary cell lineages and techniques to assess parenchymal morphogenesis is clearly warranted.

LITERATURE CITED


