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Understanding mammary gland functioning in livestock species using in-vitro mammary epithelial cells model -An overview

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ABSTRACT

Understanding the mechanisms of the development of the mammary gland can increase the efficiency of milk production, as well as improve animal health. Mammary epithelial cells (MEC) are the functional unit of the mammary gland. Although, there is a well-established MEC cell line, known as MAC-T, the use of a primary cell line is preferred because it more closely mimics an in vivo model. This review focuses on utilization of MECs as a potential in vitro model to better understand mammary gland functions in livestock species. Recently, considerable advances in three dimensional modeling of the mammary gland have been made with the used of extracellular matrix for the study of branching morphogenesis which may enable rapid advances in our understanding of mammary gland biology. Progress in the exploitation of MECs as in vitro model is more productive than ever, however further research is vital.

Indexing terms/Keywords

Mammary gland; mammary epithelial cell; cell culture.

Academic Discipline And Sub-Disciplines

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SUBJECT CLASSIFICATION

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Review article

INTRODUCTION

The mammary gland is a special organ that undergoes natural cycles of proliferation, differentiation and apoptosis, as well as remodeling, throughout life under the cyclical influences of multiple steroid and polypeptide hormones [1]. These developmental events are regulated in response to a precise interplay between epithelial cells and their surrounding microenvironments as well as outer environmental conditions [2,3]. In addition to the soluble factors recognized for their role in growth control, microenvironments are also comprised of multiple stromal cells as well as insoluble glycoproteins of extracellular matrix (ECM). Growing evidence has indicated an important role played by the stromal cells in regulating normal mammary tissue morphogenesis and their aberrant behavior during the progression of breast cancer [4,5]. However, an explanation for these processes at different levels of cell and tissue complexity remains sparse due to a lack of appropriate model systems for study.

Because of the commercial value of milk there is a great interest in understanding mechanisms involved in milk protein expression and udder resistance to pathogens which cause infectious agalactia or secretion of abnormal milk. Demanding transcriptomic studies investigating mechanisms influencing mammary gland metabolism usually involve in vivo experiments. Additionally, treatments in vivo can have systemic effects which make controlling the environment of epithelial cells in a predictable way very difficult [6] for this reasons adequate in vitro model mimicking the function of the mammary gland would be of great importance for the study of physiological, biochemical and immunologic functions of the mammary gland. Furthermore, there are almost no techniques that would allow the maintenance of organs ex vivo long enough to permit necessary molecular biological investigations. As such, an enormous potential exists in the use of three-dimensional (3-D) cell culture models as surrogates for tissues. In the recent years, mammary cell culture models were mainly used to study cell differentiation during lactation, innate immune response to infections and response to hormonal induction of lactogenesis in mammary epithelial cells (MECs).

Several ruminant immortalized cell lines such as MAC-T [7] and BME-UV [8] have been established by stable integration of the simian virus large T-antigen (SV40LtA). However, because of their low responsiveness to lactogenic hormones, transformed mammary cell lines were mainly used to study insulin growth factor 1 (IGF-1) metabolism [9]. It is still not clear how modifications in immortalized cell lines alter physiological pathways of transformed cells, therefore the use of primary cell lines is much more representative of the in vivo system maintaining organ-specific functions and signal transduction pathways [10].

CHARACTERIZATION OF MAMMARY EPITHELIAL CELLS (MECS)

Demanding transcriptomic studies in combination with challenging experiments in livestock animal species could be replaced by good in vitro models mimicking the function of ruminant mammary gland. When using tissue explants, however, it is inherently difficult to distinguish between primary mitogens and secondary regulators of mammary gland function/development. To circumvent most of these difficulties, emphasis has been placed on cell culture methodologies to study growth regulation, hormonal responsiveness, or biochemical properties of mammary epithelial cells (MEC). Previous works have led to the development of stable epithelial cell lines of bovine mammary gland [7,11,12]. The concentration of fetal bovine serum (FBS) in media had a strong effect on the proliferation of bovine mammary epithelial cells. When compared with 0% FBS treatment, bovine mammary epithelial cells grown in 5 to 10% serum underwent a 3-to 4-fold increase in cell number during the 12 d of culture [13]. Cloned bovine mammary epithelial cells also depended on FBS for proliferation and a marginal advantage could be attributed to the higher FBS concentration (10% FBS) [9]. Vimentin is the intermediate filament protein normally expressed by cells of mesenchymal origin. Vimentin is considered as a marker of myoepithelial cells [9]. However, cytokeratin 18 is one of the most common members of the intermediate filament gene family, and generally exists together with its filament partner keratin 8. It is expressed in single layer epithelial tissues of the body and is specific for epithelial cells [10]. Immunocytochemical staining of goat mammary tissue showed that vimentin was present in myoepithelial cells but not in epithelial cells. However, cytokeratin 18 was found in both epithelial and myoepithelial cells of the goat mammary gland [14]. Bovine myoepithelial cells were positive to anti-vimentin and negative to anti-cytokeratin-18 monoclonal antibody [15]. Primary bovine mammary epithelial cells with characteristic fibroblast morphology were positively-stained with anti-vimentin [15]. The different result is that vimentin was also seen in lactating bovine mammary gland epithelial cells grown without hormones; whereas, cultures grown in the presence of hormones expressed only cytokeratins, which are specific for epithelial cells [16]. Rose et al. [17] found monolayers of bovine mammary epithelial cells were stained positive for anti-pan-cytokeratin, anti-type VII cytokeratin than for vimentin. They suggested that the epithelial cells grown on plastic plates had some characteristics of myoepithelial cells for weak positive staining to vimentin.

To assess the differentiating capacity of buffalo MECs (BMECs) the cells were grown on attached collagen type I matrix. The morphological differentiation of BMECs to duct-like and acini-like structures on attached collagen gels provide evidence for their responses to microenvironment [11]. Different MEC lines from different species viz, bovine [6], caprine [9] and ovine [18], mouse [19], human [20] have been found to undergo collagen mediated morphological and functional differentiation resulting in duct and mammospheres. Integrins play a significant role in cell attachment, spreading and migration in vitro. Human normal MECs have been reported to form ridges and ball-like structure when collagen fibrils were added to mammary epithelial cells [21] which results due to integrin mediated morphogenesis.

Cytoskeleton expression is important in identifying epithelial cell lineage. Cytokeratins are intermediate filaments of epithelial cells and are important in defining the cell phenotype [22]. It has been reported that cytokeratin filaments appear as interconnected bundles in the cytoplasm. The cytokeratin network is denser around the nucleus, cytoplasmic vesicles and in the periphery of the cell where the filaments run parallel to the cell surface, which after several subcultures may

reduce to the area surrounding the nucleus [23]. Cytokeratin 18 is normally associated with simple epithelium and all luminal epithelial cells of human mammary gland. Positive reaction of BMECs with anti-cytokeratin 18 antibody indicated their luminal epithelial lineage.

Casein secretion is considered as an important feature of mammary epithelial cells. Differentiation of MECs is characterized by expression of milk protein such as b-casein, whey acidic protein and milk fat [24]. Milk fat droplets are secreted from MECs by a budding process in which droplets of triglyceride formed in the cytoplasm are gradually enveloped by a layer of apical plasma membrane called milk fat globule membrane (MFGM). Butyrophilin (BTN1A1) an acidic glycoprotein comprises over 40% by weight of total protein of bovine MFGM [25] and is specific to mammary tissue and only expressed at high levels on the apical surfaces of secretory MECs during lactation [26]. Expression of BTN1A1 and β-casein (CSN2) are considered to be the response of the mammary epithelial cells to hormonal induction [27].

HEAT STRESS STUDIES

Heat stress is a significant financial burden to animal agriculture in most areas of the world. During warm summer months milk production can decrease between 10 to 35% and this is a costly issue in dairy industry [28]. Environmental heat stress on livestock can affect reproductive efficiency, milk composition, milk yield, and animal health. It is not completely understood why stress has such adverse effects on dairy animals and has always been a major concern for livestock industry. So, mammary gland is used as a model to study the effect of heat stress for the identification of the underlying gene networks and molecular pathways associated with heat stress in our native livestock species. The effects of mild hyperthermia on bovine mammary epithelial cells and the results showed that the cell viability, ultrastructural features as well as mitochondrial function were significantly influenced by the mild heat treatment [29]. This depression in cell viability and occurrence of bovine mammary cell apoptosis and necrosis was induced through the mitochondrial-triggered cell death pathway, which further induces G2/M arrest by DNA damage. It has been long suggested that mitochondria may be directly involved in the heat-shock cellular response [30]. MECs are sensitive to environmental changes and reported to be used as in-vitro model for evaluating hyperthermia induced damage [29,31]. In addition, the MECs culture model has been used to study growth characteristics and cellular level changes during environmental heat stress [27, 29,32-34]. Further it is reported that the thermal stress induced the changes in gene expression along with the rapid regression of bovine mammary epithelial cells ductal structures [35]. The results to date indicate that a portion of the loss in milk yield during acute thermal stress is associated with direct effects of thermal stress on mammary epithelial cells. Overall, the transcriptome profile indicated down-regulation of genes involved in cell structure, metabolism, biosynthesis, and intracellular transport and of the up-regulated genes, the majority were involved in cellular repair, protein repair, and apoptosis after loss of thermotolerance. These data indicate that morphogenic activity in the mammary epithelium might depend upon the expression profile of a core set of genes, and that structural assembly might be controlled at the genomic level. So it was concluded that the acute heat stress of growing mammary epithelial cells directly reduces cellular growth and ductal branching and down regulated genes associated with protein synthesis and cellular metabolism. The mammary gland is a complex and highly specialized tissue with diverse physiological, biochemical and immunological functions.

CELL-CELL INTERACTION STUDIES

In 2D mammary epithelial model systems, glucocorticoids contribute to cell-cell interactions by inducing there organization of tight and adherens junctions leading to enhanced cell-cell adhesion and decreased paracellular permeability [36-37]. Essential cell-cell interactions occur via the epithelial junctional complex of tight junctions, adherens junctions, and desmosomes [38]. Debnath et al. [39] suggest that a primary event in acinus formation is establishment of epithelial cell polarity. The establishment of polarity in epithelial cells most probably goes hand-in-hand with the development of cell-cell contacts through apical junctions [38]. Firestone and coworkers [36-37, 40-41] suggest that in mammary tumor cell monolayers, glucocorticoids induce tight junction formation and cell polarity through a multi-step cascade involving early rapid stimulation of the transcriptional regulator, Id-1, and later induced recruitment of tight junction proteins, adherens junction proteins and Ras and PI3 kinase signaling proteins to the sites of cell-cell contact [40]. This parallels necessary down-regulation of fascin, an adherens junction-associating actin-bundling protein [37].This work suggests that glucocorticoids act to support acinus formation and may do this by regulating the expression of proteins required for cell-cell (ZO-1 and occludin) and ECM-cell contacts (β4 integrin). Milk protein expression is triggered by prolactin stimulation acting through the JAK1/STAT5 signaling pathway [42]. However, it has been shown that treatment with prolactin in isolation is insufficient to induce STAT5 signaling and β-casein expression in primary mammary epithelial cell cultures [43] and cell lines [44]. The biochemical signaling from the extracellular matrix, cell-cell signaling mechanisms coordinate to maintain epithelial polarity and differentiation. Gap-junction connexin expression was found to be localized to regions of cell membrane when CID-9 cells were grown on EHS-matrix in contrast to a plastic substrate where it remained cytosolic. Furthermore β-casein expression was dependent on gap-junction mediated inter-cellular signaling and was absent in the presence of functional blocking antibodies of β1 integrins, and inhibitors of cAMP [45]. Disrupting Rac1, a GTPase involved in cortical actin remodeling, resulted in the disorganization of laminin deposition by a kidney epithelial cell line (MDCK) and a resultant loss of polarity [46]. The surrounding stroma, which supports the glandular epithelium is more than just a passive supporting structure. It contains many cell types including fibroblasts, adipocytes and inflammatory cells that can influence the epithelium by releasing growth factors and cytokines or directly modulating the extracellular matrix in which the cells reside [47]. The gross influence of the stroma was highlighted in early heterotypic recombination experiments where microdissected mammary epithelia were recombined and cultured within salivary gland stroma [48]. The resulting epithelial tissue morphology closely resembled dichotomous branching patterns typical of salivary gland. Similar work recombining highly branched mammary epithelia with the epithelium-divested mammary fat pads of sparsely branched glands resulted in a sparsely branched morphology [49]. Despite the utility of cell lines, there is a pressing need

for better models of mammary gland development that recapitulate branching morphogenesis, lobuloalveolar development and stromal interactions. This is important for both studies of mammary gland biology and breast cancer.

THREE-DIMENSIONAL CULTURE

Growth of primary mammary cell cultures from lactating mammary gland on plastic usually results in loss of tissue specific functions. Cells in this state do not synthesize any of the milk components nor do they have the cellular response of in vivo cells [50]. In contrast to the limitations inherent to two-dimensional (2D) culture systems, many aspects of organization of mammary epithelial structures were recapitulated in vitro when primary mammary epithelial cells or established cell lines were exposed to a 3D physiological exogenous matrix, e.g. collagen, Matrigel [51-53]. Recently, the advent of three dimensional (3D) culture models has allowed investigators to make significant progress toward characterizing factors involved in the establishment and maintenance of epithelial architectures [51,54]. The growth of MECs on pre-formed extracellular matrices results in morphological differentiation as well as in synthesis of milk components [17]. Kabotyanski et al. [55] studied transcription of β-casein (CSN2) and suggested that the expression of CSN2 is induced synergistically by lactogenic hormones together with local growth factors, cell-cell and cell-substratum interactions. However, while the use of 3D culture systems has proven to be advantageous in the characterization of behavior of a single human mammary cell type (especially epithelial cells), these studies have largely ignored the fact that no epithelial cells exist as “isolated islands” in the mammary tissue in vivo [39]. Monolayer culture models are easy and convenient in vitro systems, however they do not recapitulate the glandular structure of epithelium in vivo, thus cannot provide the optimal system for studying the regulation of proliferation, polarization and differentiation of glandular epithelium. The development of three dimensional (3D) cell culture models has allowed investigators to make significant progress toward characterization of the factors involved in the establishment and maintenance of epithelial architecture. Three dimensional (3D) basement membrane cultures provide a unique opportunity to model the architecture of epithelium in vitro [38].Unlike monolayer cultures, mammary epithelial cells (MECs) grown in 3D recapitulate numerous features of glandular epithelium in vivo, including the formation of growth-arrested polarized acini with a hollow lumen and basal deposition of basement membrane components, such as collagen IV and laminin V [56-59]. The work by Delabarre et al. [60] showed that the primary bovine MECs cultured on EHS-matrix-coated inserts attached easily and rapidly reorganized the substratum into alveolar structures. The same cells seeded on type I collagen-coated inserts reorganized a typical epithelial cell pavement when cell seeding density was sufficient. Moreover they developed tight junctions, desmosomes and apical microvilli. Bovine BME-UV1 cells form 3D acinar structures mammo-spheres upon seeding on Matrigel. The same cell line plated on tissue culture dishes grows in monolayer forming a typical epithelial cobblestone structure. The bovine MECs undergo a series of proliferative and morphogenic events resulting in the formation of growth-arrested mammospheres, composed of a single layer of polarized epithelial cells, which remain in direct contact with basement membrane components, and surround a hollow lumen [61]. A similar rate of acinar structure development was observed in human and mouse mammary epithelial cell lines cultured on Matrigel: MCF-10A matured during 16-20 days, HC11 cells were grown for 10-12 days in 3D culture to form acini with polarized cells surrounding an empty lumen [62-63].

METABOLOMIC STUDIES

Mammary epithelial cells are being used for study of metabolomics of mammary gland. An understanding of the mechanism and regulation of metabolite uptake in the mammary gland is necessary to increase milk production in livestock. Recent studies [64- 67] have found that GLUT1 is most abundantly expressed in mammary glands and that the levels increase during pregnancy and peak during lactation, results that may be related to the abundant synthesis of proteins and lactose in breast tissue. GLUTs are expressed in every cell of the body and provide the metabolic energy and building blocks for the synthesis of biomolecules and control glucose utilization, glucose production and glucose sensing [68]. Glucose is of central metabolic importance in virtually all organisms, and is pivotal in lactating animals because glucose is the primary precursor of lactose synthesis, and lactose controls the milk volume by maintaining osmolarity of milk [69]. The increased glucose availability stimulated glucose uptake by the mammary epithelial cells. However, the high concentrations of glucose enhanced BMEC proliferation [70].The glucose availability did affect the glucose transport and utilization by the BMEC. The intermediary metabolites of glucose, such as glucose-6-phosphate and NADPH can actas survival factors [71-72], and cell growth is dependent on energy supply [73-74]. Aboagye and Bhujwalla [75] reported that choline phospholipid metabolite levels progressively increase in cultured HMECs as cells become more malignant. In the model system used here, phosphocholine levels and total choline containing phospholipid metabolite levels increased with progression from normal to immortalized to oncogene-transformed to tumor-derived cells. So it was propose that carcinogenesis in human breast epithelial cells results in progressive alteration of membrane choline phospholipid metabolism. TGF-β reversibly induced an alteration in the differentiation of normal mammary epithelial NMuMG cells from epithelial to fibroblastic phenotype. The change in cell morphology correlated with (a) decreased expression of the epithelial markers E-cadherin, ZO-1, and desmoplakin I and II; (b) increased expression of mesenchymal markers, such as fibronectin; and(c)a fibroblast-like reorganization of actin fibers. This phenotypic differentiation displays the hallmarks of an epithelial to mesenchymal transdifferentiation event [76]. Basement membrane extracellular matrix (ECM), but not fibronectin or collagen, was shown to suppress apoptosis of mammary epithelial cells in tissue culture and in vivo. The results suggested that ECM regulates apoptosis in mammary epithelial cells through an integrin-dependent negative regulation of ICE expression [77].

TRANSCRIPTIONAL STUDIES

Mammary epithelial cells are also utilized as model to understand the transcriptional changes occur during lactation cycle viz lactation and involution. Environmental stress causes several anomalies to the MECs such as inhibition of protein

synthesis, defects in protein structure and cytoskeleton deformation. These anomalies invoke large changes in gene transcription and protein synthesis which ultimately determine cell survival and acclimation or cell death. Collier et al., [32] studied the transcriptome profile in mammary epithelial cells indicating down regulation of genes involved in cell structure, metabolism, biosynthesis, and intracellular transport. HSPs regulate the cellular response to thermal stress and affect expression of a wide variety of genes and associated pathways. Transcriptome differences indicated the evidences for paracrine interactions between tissues in stimulation of IGF1 signaling pathway, stromal reaction, angiogenesis, neurogenesis, and immune response. Casey et al. [78] showed that GHR and PRLR transcripts possess higher expression in the epithelial compartment indicate the response of mammary gland towards systemic effects of respective hormones during this period of development. The epithelial cells were characterized with blocks that regulate protein synthesis, metabolism and secretion processes. Several transcription factors can regulate FA synthesis. One family of transcription factors designated sterol regulatory element binding proteins (SREBP), which belong to the basic helix-loop-helix leucine zipper family, are known to regulate FA synthesis [79]. Different SREBP isoforms have different roles in regulating lipid synthesis, with SREBP-1a being responsible for regulating both FA and cholesterol synthesis, whereas SREBP-1c and SREBP-2 contribute to the regulation of FA synthesis and cholesterol synthesis, respectively [80]. As a regulator of lipid synthesis, SREBP-1 has been studied extensively in different species [81]. In bovine mammary gland, SREBP-1c is one of the mechanisms regulating milk fat synthesis [82]. Immortalised bovine mammary epithelial cell line can be used as an in vitro screening system to identify superior transgenes, and to improve genomic modification technological research, thereby improving the efficiency of transgenic animal production [83]. Transcriptional analysis of multiple genes sheds light on the number of genes involved in the immortalization of BME65Cs cells. A significant change (but not loss) of p16INK4a and p53 were found in BME65Cs cells, suggesting that the inhibition of senescent-relevant pathways contributes to theBME65Cs cell line immortalisation. Cycles of growth, differentiation, and apoptosis characterize the fate of mammary epithelial cells throughout the life of the individual. These cellular processes are under the control of steroid and peptide hormones [84]. Our understanding of the regulation of proliferative, differentiating, and apoptotic processes has made great progress in the past years. Extracellular hormones, growth factors or cytokines relay their effects on the transcription of genes through the recognition of specific receptors and intracellular signaling molecules. Prolactin and its receptor are particularly important for mammary development. Prolactin plays a role in the morphological and biochemical differentiation of the epithelial cells during pregnancy and regulates milk protein synthesis during lactation. The investigations into the molecular events underlying this crosstalk have yielded unexpected insights. It has been demonstrated that Stat5 (signal transducer and activator of transcription [85] and the glucocorticoid receptor (GR), the downstream effectors of prolactin and glucocorticoid hormones interact and exhibit transcriptional synergy [86] and transcriptional repression [87]. Prolactin binds to the extracellular domain of the prolactin receptor (PRL-R) and causes its dimerisation [88-90] which activates the Stat5 which binds to DNA sites in the nucleus known as GAS elements and modulates the activity of target genes, e.g. the β-casein gene [91-92].

CONCLUSION

MEC culture is a valuable tool for various crucial studies regarding transcriptional and proteomics relevance of cellular behavior, interaction, involution and pathology. Many challenges remain to be overcome if mammary epithelial cells are to advance significantly beyond the use as in vitro versatile models for mammary gland functioning. However, recent advances have no doubt presented researchers with novel alternatives that have furthered our understanding of how tissue structures arise in three dimensions. Perhaps ultimately such models could be tailored to provide environments typical of the virginal, gestational and involuting gland, so enhancing our understanding of disease processes in livestock.

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