Late Gestational Hyperprolactinemia Increased Binucleation of Epithelial Cells in the Mammary Glands of Pigs Across Development

By

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ABSTRACT

The mammary gland (MG) is a remarkable tissue that undergoes a number of stages of epithelial growth and differentiation and is regulated by hormones such as prolactin (PRL). Recent research has found that binucleated cells (BNC) in the MG of mice form at the time of late gestation and remain until the termination of lactation, although their functional purpose is unknown. In order to improve the understanding of their development pattern, we aimed to quantify the abundance of BNC in the MG of primiparous pigs (n=4) from late gestation day 90 through lactation day 21. Further, we used hyperprolactinemic primiparous gilts (n=4) treated with the dopamine antagonist domperidone (DOM) to test the latter's effect on the abundance of BNC within the MG during the same period. The cellular architecture was assessed by three-dimensional confocal microscopy and immunofluorescent staining. Our results indicate that BNC abundance increased during the gestation-lactation window to reach maximal levels on day 21 of lactation. We also found that DOM-treated gilts had a significantly greater incidence of BNC compared to controls ($p < .05$) and that on lactation day 21 there was a statistically greater number of BNCs in DOM-treated sows ($p < .05$). These findings demonstrate that not only do BNCs exist in the porcine MG but, that they may play a functional role during lactogenesis.

LIST OF ABBREVIATIONS

°C	degrees Celsius
β-catenin	catenin beta-1
2D	three-dimensional
3D	three-dimensional
µm	micrometer
ANOVA	analysis of variance
ARUKA	aurora kinase-A
BNC	binucleated cell
CLSM	Confocal laser scanning microscopy
CON	control
D	day
D_2	dopamine receptor
DA	dopamine
DAPI	4′,6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
DOM	domperidone
E	estrogen
ECM	extracellular matrix
EDTA	ethylenediamine tetra-acetate
EGF	epidermal growth factor
ER	estrogen receptor
FFPE	formalin-fixed, paraffin-embedded
FUnGI	fructose, urea, and glycerol for imaging
g	gram
h	hour
H&E	hematoxylin and eosin
iDISCO	three-dimensional imaging of solvent-cleared organs
IF	immunofluorescence
IHC	immunohistochemistry
JAK-STAT	Janus kinase signal transducer
kg	kilogram
KO	knockout
MEC	mammary epithelial cell
MET	metoclopramide
MFP	mammary fat pad
mg	milligram
MG	mammary gland
min	minute
ml	milliliter
MNC	mononucleated cell
P	progesterone
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline + tween
PLK-1	polo-like kinase-1

PR	progesterone receptor
PRL	prolactin
PRLR	prolactin receptor
RI	refractive index
RNA	ribonucleic acid
TE	tris-ethylenediaminetetraacetic acid
TEB	terminal end bud
TDLU	terminal ductal lobular unit
WGA	wheat germ agglutinin

CHAPTER 1: LITERATURE REVIEW

OVERVIEW OF THE MAMMARY GLAND

Mammary Gland Background

The mammary gland (MG) is a highly evolved organ only found in mammals (Khan and Sajjad, 2021) that produces nutritious fluid from complicated cutaneous glands in order to feed the young. Generally speaking, the MG in males is rudimentary whereas in females its development is more advanced through its coordination with the reproductive system (Oftedal, 2002). Studying the development of the MG has led to a better understanding of what regulates its growth and functionality, including cellular proliferation and differentiation, tissue remodeling and polarity, branching morphogenesis, and regulated involution. Many of these mechanisms are also perturbed in breast cancers (Inman et al., 2015), a disease most frequently diagnosed in women (Schams et al., 2003). MG research may lead to novel discoveries to better detect, prevent, and treat breast cancer.

The study of MG biology also extends to the dairy industry which plays a vital part in global food systems and rural area sustainability (IDF Factsheet, February 2013). The dairy industry contributes to the economy of communities, regions, and countries (IDF Factsheet, February 2013), and has grown to become a $600 billion industry in the US.

Different animal models are used for breast research, as gland properties and functionalities vary depending on the species. The pig MG, for example, is commonly used to model the human breast, as it shares many of the same functional and morphological characteristics (Rowson-Hodel et al., 2015).

Mammary gland structure

The MG is both developmentally and anatomically unique, as seen in Figure 1A. There are a variety of different proliferative and morphologic stages of development that lead to the production and secretion of milk for offspring through complex biological processes (Macias and Hinck, 2012). The MG itself can be divided into three main parts, the skin, the parenchyma, and the stroma (Khan and Sajjad, 2021). The skin-associated nipple or teat is comprised of longitudinal smooth-muscle fibers which help offspring to attach themselves. The nipple also has a high concentration of nerves that help with milk letdown through tactile stimulation.

The functional parenchyma comprises glandular tissue that constitutes most of the MG in the lactating state. Functional parenchyma contains a complex, yet organized assembly of branching ducts and terminal secretory lobules, all of which develop before and during puberty (Khan and Sajjad, 2021). Most of these structures are radially symmetrical, where ducts also form milk reservoirs to aid with suckling through lactiferous sinuses or cisterns depending on species.

Lastly, both the fibrous and fatty stroma play structural and connective roles by providing a framework to support the teats and functional parenchyma. Parenchymal composition is unique for every species; for instance, ruminants have more rope-like interlobular connective tissue with a higher concentration of fibroblastic connective tissue in their mammary fat pad (MFP) than other species. Fibrous stroma comprises suspensory ligaments that support the mass of the MG as a whole as it fills with milk (Khan and Sajjad, 2021). Suspensory ligaments separate lobes, and in humans, these ligaments suspend the gland from the pectoral fascia. Heifers have a lateral, a deep lateral, and a median suspensory ligament that attach the udder to the body. Even though the composition varies depending on species, the fatty stroma of the MGs makes up the majority

of its volume prior to gestation and contains a range of extracellular matrix proteins that organize stromal architecture (Alowami et al., 2003).

Cell types in the mammary gland

As with other gland types, the MG is comprised of multiple cell types, as seen in Figure 1B, including epithelial, adipose, vascular endothelial, connective tissue, immune, and lymphatic cells. Each performs a specific role during MG development and function, where their cooperation supports proper organ functionality. The details of each of these unique features will be discussed below.

Epithelial cells

The mammary epithelium is made up of a tissue bilayer of two distinct cell types organized into an inner layer of luminal cells and an outer layer of basal cells (Cristea and Polyak, 2018; Macias and Hinck, 2012). The basal epithelium includes myoepithelial cells that are in contact with the basement membrane and pluripotent stem cells. Myoepithelial cells express cytokeratins 5 and 14, as well as smooth muscle actin, which facilitates their contraction during lactation (Rowson-Hodel et al., 2015). Additionally, myoepithelial cells produce a range of molecules that support MG development and function by maintaining tissue polarity (Gudjonsson et al., 2002) and luminal cell survival (Boudreau et al., 1995). The apically oriented luminal epithelium lines ducts and secretory alveoli, and expresses cytokeratins 8 and 18 (Inman et al., 2015). Some of these cells are positive for estrogen receptors (ERs) and progesterone receptors (PRs) and have been described as “hormone sensing,” where both receptors are critical

signaling molecules that regulate lactogenesis, mammary epithelial cell (MEC) proliferation, and involution.

The extracellular matrix (ECM) is a complex network of proteins and other macromolecules that gives structure to cells and tissues in the body. In the MG, the ECM regulates gland growth during embryonic development, postnatal ductal growth, and branching morphogenesis (Kass et al., 2007). As such, it plays a direct role in MEC differentiation through ECM-MEC interactions. Integrins, dystroglycan, discoidin domain receptor 1 tyrosine kinases, and syndecans are among the variety of transmembrane receptors that mediate these interactions (Kass et al., 2007). β1 integrin regulates pregnancy-induced alveolar morphogenesis (Kass et al., 2007).

Binucleated cells vs. mononucleated cells

Mammary epithelial cells have been presumed to be mononucleated cells (MNCs). The belief was that like any normal-functioning MNC, MECs progress through all different stages of the cell cycle in order to renew themselves or promote MG development. During late gestation and early lactation, the MECs in the mammary parenchyma were thought to proliferate rapidly through DNA replication in order to populate the MG in preparation for lactation (Banerjee et al., 1971). The idea that mammary epithelial cells proliferate was supported by observations that many MECs initiated DNA synthesis and increased DNA content. Knight *et al.* reported that mammary DNA and RNA in rats increased from day 2 to day 14 of lactation (Knight et al., 1984), while Banerjee *et al.* showed that total mammary tissue DNA in mice increased significantly from day 15 of gestation to day 6 of lactation (Banerjee et al., 1971). This increase in DNA synthesis facilitates gland development by altering the shape of MECs, their response to

hormonal and growth factor signals (Kass et al., 2007), and their differentiation. However, DNA synthesis does not necessarily constitute cell proliferation (212).

Using scRNA-sequencing of mammary epithelium, Pat *et al.* demonstrated that prior to puberty the MEC populations are mostly homogenous (Pal et al., 2017). However, this homogeneity is not conserved over MG development during pregnancy and lactation, which suggests that not all MECs are identical. During lactation, there is a varied expression of mRNA for milk protein genes and vascular endothelial growth factors between MECs (McCracken et al., 1994; Molenaar et al., 2003; Singh et al., 2017) and alveoli (Goldhar et al., 2005) respectively. Variation in MEC populations could indicate a functional advantage that may be not be manageable by homogenous MEC populations (Dueck et al., 2016).

A striking discovery was recently made by Rios *et al.* who found that a significant population of binucleated MECs exists in the MGs of late pregnant and early lactating mammals. These binucleated cells (BNC) form during this period due to failed cytokinesis, which is likely regulated by the mitotic kinases aurora kinase-A (AURKA) and polo-like kinase-1 (PLK-1) (Rios et al., 2016). The authors found that both kinases play an crucial role for BNC formation at the lactogenic switch. At the switch to lactation, complete deletion of the AURKA gene suppressed BNC formation and milk synthesis, and inhibition of PLK-1 prevented cytokinesis (Rios et al., 2016).

These authors used high-resolution 3D confocal microscopy as well as membrane and nuclear immunofluorescence staining to quantify and locate BNCs in the MG. Their results revealed that 50-60% of the MECs in lactating mice, 30% of MECs in wallabies, and 40% of MECs in cows were binucleated (Rios et al., 2016). In contrast, they found that myoepithelial

cells comprising the sheath surrounding alveolar luminal cells remained mononucleated at all stages of development (Rios et al., 2016).

Flow cytometric analysis of a variety of cellular subsets showed that the proportion of ploidy in luminal cells increased during late pregnancy (Rios et al., 2016). The number of polyploid alveolar luminal cells in a pregnant mouse increased from 17% on day 16.5 of pregnancy to 50% on day 18.5 (Rios et al., 2016).

Another discovery regarding these BNCs was that they had substantially greater cellular volumes compared to MNCs, as determined using software tools Imaris (surface module) and FIJI (binary segmentation masks (Rios et al., 2016). The analysis revealed that while total cellular volume did increase, the size of individual nuclei was comparable across both cell types (Rios et al., 2016).

For the longest time it was thought that long-term lactation competence required the division of MEC through mitosis during lactogenesis (Lockwood et al., 1967). However, this assumption appears to not be the case. The recent discovery of BNC during the developmental window of late pregnancy to lactation raises the question of whether BNCs have a functional advantage over MNCs in terms of their lactational capacity.

Although BNCs are commonly associated with cancer, they exist in other organs including the liver, salivary glands, the heart, and the endometrium (Grizzi and Chiriva-Internati, 2007). Binucleation can also be caused by several mechanisms including failure of cytokinesis, post-mitotic fusion, and fusion of two nuclei after cytokinesis (Grizzi and Chiriva-Internati, 2007). Little is currently known about the potential advantages or disadvantages BNCs might have over MNCs.

Adipocytes

Adipocytes make up a large proportion of the MFP for adult females and are local regulators of epithelial cell growth (Hovey et al., 1999). Adipocytes play a crucial role during different stages of MG function and development through the secretion of vascular endothelial growth factors (VEGFs), which regulate angiogenesis in the MG (Hovey et al., 2001a). There are major morphologic changes in adipocytes within the MG during the transitions between pregnancy, lactation, and involution. In rats, the cross-sectional area of adipocytes increased by 20% from the time of nulliparity to day 11 of gestation, where they maintained their size until day 20 (Pujol et al., 2006). Elias *et al.* confirmed this result and proposed that parenchymal enlargement is responsible for MG growth during pregnancy (Elias et al., 1973). In fact, adipocytes from pregnant female mice doubled their lipogenic capacity compared to nulliparous females (Bartley et al., 1981). Lactogenesis is a metabolically demanding process, so it is no surprise that adipocytes have a reduced lipid content during later gestation and early lactation (Matsumoto et al., 1995a; Hovey and Aimo, 2010; Gregor et al., 2013). After parturition, adipocytes in the MG of mice have a multilocular appearance, consistent with there being lactation-induced lipolysis in adipocytes within the MG during lactation (Elias et al., 1973; Matsumoto et al., 1995a). Following weaning and the start of MG involution, adipocytes in the MG of rodents begin to replenish their lipid stores and return to their original unilocular appearance within a four-day period (Elias et al., 1973; Hovey and Aimo, 2010). Changes in adipocyte morphology reflect the important role they play in the tissue remodeling and structural regulation of the MG (Hovey and Aimo, 2010).

Fibroblasts

Fibroblasts work in a conjoined effort with adipocytes to modulate MG formation and development. While both of these cell types help advance epithelial cell expansion into the MFP, fibroblasts may also serve other functions. For example, mammary stromal fibroblasts have close proximity to the basal side of the epithelial branches (Neville et al., 1989; Muschler and Streuli, 2010), which facilitates their bi-directional communication with the growing epithelium. Molecules such as growth factors, proteases, and other elements (Howard and Lu, 2014; Inman et al., 2015) instruct the epithelium, where they not only support epithelial morphogenesis and survival but, also the homeostasis of the ECM (Liu et al., 2012; Lühr et al., 2012; Wang and Kaplan, 2012). The main types of fibroblasts in the mammary gland are lobular and interlobular fibroblasts, where their relative abundance differs depending on species (Morsing et al., 2016). Lobular fibroblasts share similarities to bone marrow-derived mesenchymal stem cells by helping maintain the growth and morphogenesis of epithelial progenitors (Morsing et al., 2016). Interlobular fibroblasts are located outside of lobules (often referred to in certain species as terminal duct lobular units - TDLUs) which segregates them from direct contact with the adipose tissue (Parmar and Cunha, 2004). Interlobular fibroblasts have stromal collagen fibers that are denser than those associated with intralobular fibroblasts, which can present an elevated risk for developing breast carcinomas (Berry et al., 2003a; Martin and Boyd, 2008). The expression profile of interlobular fibroblasts is similar to that of tumor stroma that can be characterized by an accumulation of breast cancer-associated fibroblasts in the TDLUs (Morsing et al., 2016).

Vascular and immune cells

Angiogenesis and lymphangiogenesis in the MG occur alongside the development of the mammary epithelial branches during early development and puberty (Inman et al., 2015). The MG develops an extensive vascular network comprised of vascular endothelial cells and mammary vascular endothelial cells. Mammary vascular endothelial cells regulate endothelial cell proliferation and differentiation in the MG as well as lymphocytes and inflammatory responses in the lymphatic network of the MFP (Inman et al., 2015). Immune cells such as macrophages and eosinophils are vital for branching morphogenesis, particularly by mediating invasion of the MFP by terminal end buds (TEB) (Gouon-Evans et al., 2000). Using mice homozygous for a null mutation in the CSF1 (Csfm(op)/Csfm(op)) gene, a key growth factor for macrophages, Gouon-Evans *et al*. found that in the absence of CSF1 there was impaired TEB formation, epithelial expansion, and associated branching of the MG. Similar results were found in the absence of eosinophils, as regulated by eotaxin (Gouon-Evans et al., 2000).

A. **B.**

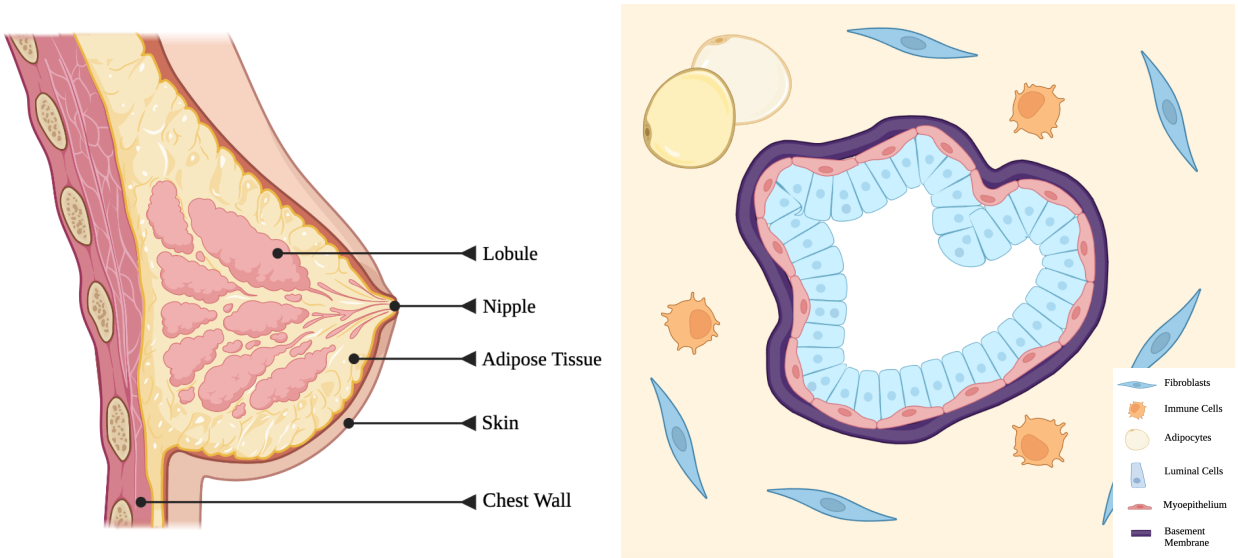

Figure 1: Breast anatomy and mammary gland structure. (A) Breast anatomy represents different components of a human breast and (B) the mammary gland structure illustrating the epithelium surrounded by major stromal cell types.

Mammary gland development

Humans, pigs, rodents, and cows all have estrous or menstrual cycles that regulate MG growth and function. Hormonal changes during puberty, pregnancy, lactation, and later involution (Inman et al., 2015) alter MG structure and function in order for it to successfully synthesize and secrete milk. Estrogen (E) and progesterone (P) are two of the major circulating hormones responsible for the development and maintenance of alveoli and TEB. The MG of different species progresses through development at distinct rates. The MG of female mice undergoes isometric growth prior to puberty and allometric growth after the onset of puberty (Filgo et al., 2016).

Allometric growth of the heifer MG is initiated prior to puberty (Sejrsen et al., 1982) and continues until sexual maturity. During gestation the MG undergoes complete lobuloalveolar growth and differentiation, expanding further into the MFP through dichotomous branching and increasing epithelial density. Around the time of parturition, there are increased circulating levels of E and associated decreases in the levels of P. The onset of lactation is initiated in response to a decline in circulating P around the time of parturition, concurrent with increased levels of E. The role of these hormones is discussed later. After weaning the MG goes through a period in which lactation terminates, milk is removed, and epithelial structures involute. During involution, many signaling pathways are required to remodel the gland back into its pre-pregnancy configuration (Macias and Hinck, 2012).

Embryonic development

The MG arises from the epidermis, forms during embryogenesis and is said to have evolutionary origins stemming from a modified sweat gland (Kass et al., 2007). The ectoderm is responsible for the development of primary morphological structures of the MG through later differentiation. At first, the ectoderm begins thickening ventrolaterally through cell migration to form two bands at the ventral aspect of the Wolfian ridge (Kass et al., 2007). This ridge is a longitudinal bilateral section found on either side of the trunk of some embryos, between the fore and hind limb buds. As ectodermal cells condense further, lines form and then separate into small hillocks. that are characterized as raised areas on the ectodermal layer that will later develop into the glands themselves. For mice, within a 24-36 h period, the two symmetrically located mammary lines then resolve into multiple pairs of mammary placodes that fall along the ventral–lateral border of the embryo itself (Kass et al., 2007). In the mouse, there are ten placodes. The placodes are thickened ectodermal cells compared to the surrounding epidermis and are columnar in structure (Kass et al., 2007). The mesenchyme then progresses through dramatic morphological changes by condensing around the developing placodes to form the nipple region. Each placode will then invaginate into the underlying dermal mesenchyme at the site of future galactophores to form mammary buds, then forming the rudimentary ductal structure of the MG (Macias and Hinck, 2012). A galactophore is the external opening of an MG duct located at the teat or nipple. The then-developed bud of epithelial cells will push further into the underlying mesenchyme using cell division. Condensed mesoderm surrounding the bud regions will later become the stroma of the MG.

Neonatal and pubertal development

At birth, the basic branched structure of the MG begins its next stage of development when the mammary ducts progress through isometric growth, which matches it to the rest of the body (Howlin et al., 2006). Around the time of puberty, allometric growth is then initiated by hormonal signaling and the mammary rudiment will further branch into the adipose-filled MFP that aids in the formation of the ductal network. Ductal structures formed in the MFP of different species have distinct characteristics such as branching complexity and their relative diameter. In female humans, lipid accumulation in adipocytes of the MFP does not occur until puberty, when it causes morphologic development to begin in an adipose tissue that is less dense. The development of the ductal network benefits later alveolar formation capacity and subsequent milk production during pregnancy and lactation (Howlin et al., 2006).

Allometric growth includes the formation of TEBs that are highly mitotic structures located at the end of elongating lactiferous ducts and that help direct and expand the ducts into the MFP, increasing epithelial density within. These TEBs are comprised of parenchymal tissue and are the main components involved in ductal elongation and formation during puberty. There are unique cell types that give rise to TEBs during puberty (Inman et al., 2015) that ramify into the MFP where they continue to extend until they reach the outer limits of the MFP, at which point they terminate outward growth and regress slightly (Ball, 1998; Brisken, 2002). Terminal end buds are comprised of a single outside layer of undifferentiated cap cells and inner layers of body cells. Cap cells located at the end of the TEB are what make it highly prolific. Cap cells directly contact the stroma through a thin basal lamina (Inman et al., 2015), and its trailing edge differentiates into myoepithelial cells creating a thicker basal lamina (Williams and Daniel, 1983;

Margot and Bonnie, 2000). The inner layer body cells create a trailing hollow lumen by regulating central body cell apoptosis and differentiation into outer luminal epithelial cells (Humphreys et al., 1996; Hennighausen and Robinson, 2005). End bud expansion gives rise to the ductal epithelium later in the adult MG (Hennighausen and Robinson, 2005). Bifurcation of the TEB yields a branching pattern within the epithelium and gives rise to various cell populations. Lateral branches extending from the original duct will then form subtending ducts and alveolar sprouts that expand into the surrounding MFP (Brisken, 2002). The mammary parenchyma becomes fully embedded within the MFP throughout the various estrous cycles. Terminal end buds are regulated by both E and prolactin (PRL) hormones, as discussed later. Following pubertal growth, the parenchyma remains relatively quiescent until conception (Tucker, 1987).

The MG in humans and pigs has a more complex development during puberty than is seen in the mouse. Not only does the parenchyma have TEBs, but they also have TDLUs. Terminal ductal lobular units are derived from TEBs but are unique in morphology and cellularity. In the human MG TDLUs can be further categorized based on size, with TDLU-1 being their most primitive form and TDLU-4 fully developed (Jose Russo et al., 2001). TDLU-4s are most prevalent in the MG during pregnancy.

During pregnancy, the MG undergoes massive amounts of tissue remodeling (Oakes et al., 2006). Luminal epithelial cells rapidly expand to form alveoli that are lined with cells capable of milk secretion at the time of parturition (Inman et al., 2015). During alveolar morphogenesis the MG undergoes epithelial cell proliferation increasing both its surface area and density. Alveoli are sites of milk synthesis and storage that develop from ductal branches that have spread throughout the MG (Akers, 2016). After mid-pregnancy, cell differentiation in the

alveoli of mice increases as the gland enters the secretory initiation phase (Richert et al., 2000). Each alveolus is comprised of two types of cells, luminal cells, and external basal cells that make up contractile myoepithelial cells surrounding each alveolus in a basket-like configuration (Hennighausen and Robinson, 2005; Oakes et al., 2006). As parturition nears, tight junctions between alveolar cells close, and colostrum proteins move into the alveolar lumen in preparation for the secretory activation phase (Nguyen et al., 2001).

Lactation development

During the period of labor and lactation, the MG proceeds through further growth and cellular differentiation. Throughout lactation, the glandular portion of the breast is populated by epithelial structures with few signs of stroma (Pillay and Davis, 2022). Lactogenesis must occur before the onset of lactation (Vonderhaar and Topper, 1973). Increased stimulation by insulin (INS) and PRL then prepares the MG for lactogenesis by causing MECs to polarize and initiate the synthesis of milk. Lactogenesis happens in two stages known as lactogenesis I and lactogenesis II (Neville et al., 2002). Lactogenesis I is initiated around mid-pregnancy and includes mammary differentiation and the ability to synthesize certain milk components (Neville et al., 2002). Progesterone and PRL are requisite hormones during both stages. High levels of P from the placenta inhibit further differentiation (Pillay and Davis, 2022) and prevent milk secretion (Neville et al., 2002). Lactogenesis II, also known as secretory activation, begins at the onset of parturition and is characterized by declining levels of circulating P (Neville and Morton, 2001) and rising levels of PRL (Neville et al., 2002). During lactogenesis II there is an increase in the expression of milk protein genes by MEC, closure of tight junctions between alveolar cells, and movement of lipid droplets and casein micelles into alveoli (Neville et al., 2002).

Colostrum and milk are secreted into the lumen and stored (Demirci et al., 2018). In goats, the milk production capacity of MECs remains high until peak lactation (Knight and Peaker, 1984). Milk secretory products within the alveolar lumen are subsequently forced towards the nipple and ejected by contracting myoepithelial cells stimulated by oxytocin. Oxytocin is a small nine-amino acid hormone and neurotransmitter that is released from the posterior pituitary gland and facilitates childbirth and breastfeeding (Magon and Kalra, 2011). Total milk production is proportional to alveolar capacity, frequency of contraction, and success of milk evacuation (Auldist et al., 2000). Continuous lactation is maintained by regular milk removal, stimulation of the nipple (Pillay and Davis, 2022), and combined PRL and oxytocin levels (Neville et al., 2002). At the cessation of lactation, the MG begins to involute, coincident with the deprivation of lactogenic hormones, and apoptotic signaling that aids with tissue remodeling (Pillay and Davis, 2022).

ENDOCRINE CONTROL OF MAMMARY GLAND DEVELOPMENT

The MG goes through dynamic changes throughout the lifecycle of a female, during which time much of its development is regulated by hormones and growth factors. The initial formation of the MG occurs during embryogenesis, as promoted by signals from the mesenchyme. During puberty and adulthood, circulating hormones released from the pituitary gland and ovaries modulate its epithelial expansion and differentiation (Arendt and Kuperwasser, 2015). At this time, the MG advances through cyclic proliferation and apoptosis and is influenced by the estrous/menstrual cycles (Potten et al., 1988). The parenchyma configures ductal branches and alveolar buds in the MFP via hormonal changes during estrous (Arendt and Kuperwasser, 2015). Full MG development occurs during pregnancy, after which luminal

epithelial cells synthesize milk under the influence of PRL (Arendt and Kuperwasser, 2015). A variety of hormones play a role during pregnancy; however, most changes are regulated by levels of estrogen and progesterone.

Prolactin secretion and its receptor

Prolactin is mostly synthesized by the pituitary gland, but it is also produced by the central nervous system, the immune system, the uterus, and the MG itself. Synthesis and secretion of PRL are controlled by the hypothalamus as well as through the actions of dopamine. Lactotroph-derived PRL is secreted in a range of posttranslational forms, differing in their size, electrical activity, signal response time, basal hormone release, and morphology (Horseman, 1999).

Dopamine and estrogen are two main regulators of PRL secretion. In mammals, circulating PRL is maintained at low levels by the suppressive effect of dopamine (DA) inhibiting factors, which bind to type 2 dopamine receptors (D_2) on lactotrophs. As a clear illustration of this suppression, ectopic pituitary tissue will secrete copious amounts of PRL if DA inhibition is lost. Hyperpolarization of lactotrophs mediates DA inhibition (Andrews, 2005), which is the chemical property many drug designers utilize when developing DA inhibiting drugs.

Prolactin receptors (PRLR) on the surface of target cells activate signaling cascades that will lead to downstream gene transcription via the Janus kinase signal transducer (JAK-STAT) pathway. Initiation of this signaling occurs when PRL binds to PRLR on the cell membrane causing PRLR-ligand molecule dimerization (Gorvin, 2015).

Prolactin regulates a wide range of physiological functions including growth, reproduction, and osmoregulation. One of the primary functions of PRL is to work with other hormones in an effort to promote milk production and MG development through MEC activity (Nagasawa et al., 1986).

Prolactin in the Mammary Gland

Kacsóh *et al.* showed that the MG also can synthesize and secrete PRL (Kacsoh et al., 1993). Locally synthesized PRL from the MG, and in the circulation from the pituitary, can be transported across the mammary epithelium into the alveolar lumen by the circulatory system, leading to the final concentration of PRL in milk (Ben-Jonathan et al., 1996). Neonatal digestion then utilizes the high concentration of PRL found in milk for the maturation of its hypothalamic neuroendocrine system (Kacsoh et al., 1993).

In the MG, PRL and placental lactogens promote lobular budding during organogenesis, epithelial cell proliferation and expansion into the MFP during pregnancy, and cellular differentiation during lactation. Lactation initiates after parturition once both P and placental lactogen levels drop. Plaut *et el.* demonstrated that the high levels of PRL in the plasma of sows immediately decreased following parturition and with the onset of lactation (Plaut et al., 1989).

The PRL receptor is important for MEC maturation, MG development, lactation, and reproduction. Akers *et al.* (Akers et al., 1981) showed that induced hyperprolactinemia, through PRL supplementation during pregnancy, could increase milk secretion during lactation. Hyperprolactinemia also increases PRLR mRNA, which the synthesis of PRL depends on (Farmer et al., 1999). Using PRLR knockout (PRLRKO) mice, Ormandy *et al.* found that subjects heterozygous for PRLR had less ductal development in the MG, more underdeveloped

branching, and less mammary tissue than wild-type female mice (Ormandy et al., 1997). Further, the majority of homozygous PRLRKO mice were unable to reproduce and had abnormal ovulation cycles leading to increased infertility (Bole-Feysot et al., 1998; Kelly et al., 2002).

Prolactin and PRLR concentrations correlate with MG development and lactation and can be manipulated using dopamine-related drugs (Farmer et al., 2000; Farmer and Palin, 2005). For instance, Farmer and Petitclerc used bromocriptine, a dopamine D_2 receptor agonist, to inhibit PRL secretion. Using this approach, these authors found that PRL exposure between days 70 and 110 of gestation in gilts is critical for MG development (Farmer et al., 2000; Farmer and Petitclerc, 2003).

Supplemental PRL post-partum did not have any effect on milk yield in cows (Plaut et al., 1987). Using bromocriptine, Akers *et al.* found that cows having suppressed serum PRL levels subsequently had decreased milk yield, while cows supplemented with PRL prior to parturition had greater milk secretion during lactation (Akers et al., 1981). Using sows, VanKlompenberg *et al.* demonstrated the induction of hyperprolactinemia using domperidone (DOM), a dopamine antagonist, enhanced lactogenesis, amplified secretory activity, and increased piglet growth (VanKlompenberg et al., 2013). Notably, this hyperprolactinemia had no effect on mammary epithelial cell proliferation (VanKlompenberg et al., 2013). These findings were also corroborated by Mathews *et al.* using metoclopramide, another dopamine antagonist (Mathews et al., 2021). An interesting experimental observation made by Gaugler *et al.* (Gaugler et al., 1984) and VanKlompenberg *et al.* (VanKlompenberg et al., 2013) was that photoperiods can also influence mammary growth and lactation. Prolactin concentrations and gestation-associated mammary growth were suboptimal during shortening photoperiods in the

fall months (VanKlompenberg et al., 2013), where piglets born then tended to be smaller at the time of weaning (Gaugler et al., 1984).

Progesterone and estrogen secretion and their receptors

Estrogen is a steroid hormone that has influence over many systems including reproductive organ development (Osborne et al., 2000). While both males and females produce this hormone, females utilize it much more and therefore synthesize more of it. In the female, E regulates the reproductive system and supports the development of female characteristics such as the MGs. Estrogen in females is primarily produced by ovarian follicles (Senger, 1997) but is also synthesized by the liver, adrenal glands, and MG (Osborne et al., 2000). The MG responds to E in order to help develop and also aid the termination of lactation after weaning. Like PRL, estrogen levels fluctuate with the menstrual cycle and reproductive stage of a female.

Estrogen receptors are ligand-activated transcription factors that reside in the nucleus of cells when active. Around 75% of breast cancers express ERs (Pritchard et al., 2013; Blok et al., 2015) and in most of these cases, E promotes the growth of breast cancer cells. A common practice in treating breast cancers via endocrine therapy is to inhibit estrogen functionality by using ER antagonists such as fulvestrant (Ciruelos et al., 2014), tamoxifen, or aromatase inhibitors like anastrozole, and letrozole. The ER activates or inhibits gene expression due to coactivator and corepressor proteins (Shang et al., 2000; Stashi et al., 2014). Estrogen receptor activity can also be influenced by its phosphorylation state (Lannigan, 2003).

Estrogen is an important hormone in the female reproductive system; however, it is mostly recognized for its collaborative role with progesterone (P). Progesterone is an endogenous steroid hormone that also occurs in females and males but is primarily known for its

role in female reproduction (Cable et al., 2022). Progesterone is produced by the adrenal cortex and gonads but is also secreted by the ovarian corpus luteum and placenta during different stages of pregnancy (180). A major role for P is seen with the reproductive system during gestation. A primary function of P is to regulate low levels of vascular tone in the myometrium and maintain normal amounts of inflammatory mediators in the uterine cavity (Cable et al., 2022). Lack of P can lead to a weakened immune response, infertility, endometrial hyperplasia, and a heightened risk of endometrial intraepithelial neoplasia. On the other hand, excessive amounts of P have been known to increase the risk for granulosa cell tumors and breast cancer (Schubert et al., 2014).

Like receptors for many steroid hormones, the P receptor (PR) is located in the nucleus of the cell and regulates networks of target gene expression through binding to its corresponding hormone (Grimm et al., 2016). Progesterone receptor specifically is a regulator of tissues involved in reproduction (Grimm et al., 2016), which includes stages of development and proliferation during gestation, lactation, and involution. Similar to the structure of ER, PR includes a DNA and regulatory binding domain that is distinct from its hormone-binding domain. Transcription is initiated by a receptor-ligand molecule binding to the target promoter region via a P-response element (Grimm et al., 2016). Progesterone receptor-A and PR-B are the two main isoforms of the receptor, with the only small difference being that PR-A is missing the first 164 amino acids found in PR-B (Wen et al., 1994). In humans, the PR-A (hPR-A) functions as a transcriptional repressor of the progesterone-responsive promoter, and hPR-B is a transcriptional activator (Wen et al., 1994). Haslam *et al.* used immunohistochemistry (IHC) to prove that the mouse MG is more abundant for PR-B than PR-A (Aupperlee et al., 2005). Schams *et al.*

confirmed that the PR-B isoform is more abundant during gestation and parturition using western blot, suggesting that PR-B plays a key role in pregnancy and lactation (Schams et al., 2003).

Progesterone and estrogen in the mammary gland

A study by Woodward *et al.* found that MEC proliferation in ovariectomized heifers was enhanced via treatment with E (Woodward et al., 1993). Estrogen in the MG induces MEC proliferation by utilization of ERs. In fact, the frequency of ER-positive cells correlates directly with the developmental stage of the MG in mice (Haslam, 1988). Mueller *et al.* found that adult mice require ER in both stromal and MECs for complete MG development (Mueller et al., 2002). In an alternative knockout study conducted by Bocchinfuso *et al.,* ERKO mice lacking ER-α had decreased ductal growth and alveolar development in the MG (Bocchinfuso and Korach, 1997). Combined, these data highlight the critical role of E during MG development and function.

Progesterone regulates alveolar development and promotes ductal side branching (Nandi, 1958). Using PRKO mice, Lydon *et al.* showed that without PRs, the mouse MG had incomplete development due to impaired alveolar budding and branching (Lydon et al., 1995). The need for PRs furthers the idea that they play a crucial regulating role in MG morphology. Additionally, Brisken *et al.* found that a paracrine mechanism exists in order to help with PR regulation (Brisken et al., 1998). Estrogen and P with their corresponding receptors have essential roles in MG development during the reproductive cycle.

As mentioned above, E stimulates PR transcription which increases the effects of P on MEC development (RC et al., 1998). High levels of P can however be used to lower E in the circulatory system, thereby inhibiting PR transcription (G et al., 1999). Estrogen and P

cooperatively MG development and MEC proliferation. Bocchinfuso *et al.* found that E alone provided minimal mammary duct elongation, however, when combined with P, it promoted significantly increased levels of lobuloalveolar development (Bocchinfuso et al., 2000). Woodward *et al.* similarly found that MEC proliferation underwent an intermediate response when E and P were administered together (Woodward et al., 1993). Hovey *et al.* further found that in ovariectomized mice, MEC proliferation in the MG was lower when E, P, or PRL were administered independently compared to when administered in certain combinations. Interestingly, when P and PRL were given together, there was a 400-fold increase in MEC division compared to saline-treated females (Hovey et al., 2001).

STUDYING THE MAMMARY GLAND OVERVIEW

To better understand the form and function of the MG, a variety of techniques have been developed. Techniques include, but are not limited to, a range of imaging techniques, histology, and *in vivo* and *in vitro* studies. Non-invasive breast imaging can be used to diagnose mammary cancers and encompasses techniques such as x-ray mammography, ultrasound, and magnetic resonance (MR) mammography (Popiel et al., 2012). Other imaging and clearing protocols on fixed or live MG tissue can facilitate the visualization of its complex cellular structures, morphology, and development. Visualization can be exceptionally challenging, as much of the MG epithelium is encased within a stromal matrix (Lloyd-Lewis, 2020). Histological staining can help better distinguish cellular constituents and is advantageous when studying the MG.

Genetic manipulation in the mammary gland

Genetic engineering utilizes artificial selection, artificial insemination, in vitro fertilization, cloning, and genetic manipulation in order to advance the understanding of gene function and organization. Useful human medical applications have come from recombinant DNA techniques, especially in the context of the MG. The genetically engineered mouse model in particular has been useful in researching the importance of specific gene products to MG development and lactation (Hadsell, 2004). Too many genes play a role in MG form and functionality to list here, representing the importance of genetic engineering to the field. As briefly mentioned previously, Rios *et al.* found that AURKA and PLK-1 play important roles in the binucleated epithelial cell phenomenon found around the time of the MG lactation switch (Rios et al., 2016). Deleting the AURKA gene, using WAP-icre, inhibited the production of BNCs, leading to a five-fold decrease in cells with 4N DNA. Additionally, the treated mice had a subsequent decrease in milk protein synthesis and severe stunting of pups (Rios et al., 2016). A fluorescence-activated cell sorting analysis showed that >98% of binucleated cells expressed Elf5. The Elf5 promoter is known to be highly expressed in luminal cells of pregnant and lactating MGs and is a transcriptional regulator of secretory alveolar cells (Oakes et al., 2008). By using a doxycycline-inducible cre, under the control of the Elf5 promoter, Rios temporally deleted AURKA in late pregnancy versus early lactation (Rios et al., 2016). The resulting dams showed impaired lactation and a decrease in BNCs, where up to 20-30% of litters died around day 10 in response to insufficient milk production by AURKA-deficient mothers (Rios et al., 2016).

Histology in the context of the mammary gland

Histology is the microscopic study of cells and tissues through staining and sectioning methods. The histology field has advanced throughout the years to improve its chemical, molecular biology assays, and immunological techniques. Today it is extensively used in the medical field as well as in research to study diseases in different systems of the body. The four basic types of tissues in every organ includes epithelial, connective, muscle, and nervous tissue. The distinction between organ tissue formations makes them unique and alludes to their respective function.

The five key stages of histology are fixation, processing, embedding, sectioning, and staining. Each stage can be done in a variety of ways depending on the desired purpose of the study. Sectioning for instance is performed on either paraffin-embedded or frozen tissue and can generate either thick or thin sections. While thin sections are often used, thick sections conserve more of the tissue morphology.

Mammary gland staining

Staining is a crucial step used to visualize cellular characteristics and tissue morphology. Staining can mark cells, flag nucleic acids, and highlight proteins. Hematoxylin and eosin (H&E) is one of the principal stain combinations used in histology for the purpose of determining tissue types and morphological changes. H&E staining is a popular technique as it works across a variety of fixatives and highlights crucial tissue characteristics (Popiel et al., 2012). Hematoxylin stains nucleic acids with a deep blue color, while eosin stains the cytoplasm, extracellular matrix,

and proteins nonspecifically with a pink hue (Fischer et al., 2008). Many other staining techniques exist that are used for more in-depth tissue analysis.

Immunohistochemistry is one such technique that utilizes antigen-antibody interactions to detect target protein expression, distribution, and concentration. The two main forms of IHC and parallel tissue processing are IHC in formalin-fixed, paraffin-embedded (FFPE), and IHC in frozen sections. The fixation process preserves histologically relevant morphology and antigenicity of target molecules. Standard fixatives include aldehydes, alcohols, and acetones. There is no standard fixation protocol for all target molecules, as each antigen requires different fixatives or fixation times in order to best conserve antibody binding proficiency (Duraiyan et al., 2012). Formalin fixation is commonly used for preserving the morphology of FFPE tissue. However, formalin fixation can sometimes negatively impact IHC staining by preventing antibody-target binding due to crosslinking between amino acids within the target antigen and surrounding proteins of the target antigen (Duraiyan et al., 2012). Crosslinks mask epitopes on an antibody and impede antigen binding. Heat-induced antigen retrieval can recover these masked epitopes by promoting their availability and revealing immunophenotypes (Duraiyan et al., 2012). A blocking buffer comprised of unrelated proteins or other compounds can prevent the non-specific binding of antibodies to further enhance antibody-target detection. After initial section preparation dependent on the IHC protocol, the tissue is then incubated with primary antibodies against antigens of interest. Using either direct or indirect detection, target expression can then be analyzed. Direct detection is when a primary antibody is labeled with a fluorescent

or enzymatic label. Indirect detection relies on the addition of a labeled secondary or tertiary antibody in order to better amplify the signal.

There are a variety of markers used in IHC that aid in the identification of target biomolecules. Immunofluorescence (IF) is a common technique that utilizes fluorochromes conjugated with antibodies in order to visualize target molecules. Fluorochromes provide clear contrast for detection, high specificity, sensitivity, and multiple staining capabilities (Zaqout et al., 2020). Immunofluorescence has the added aesthetic appeal of producing colorful staining. Types of IF labeling include enzymatic labeling, chemical labeling, protein labeling, and genetic labeling used to generate fluorescent signals from a wide variety of fluorophores emitting across the visible and near-infrared spectrum.

In the MG, cell membrane and nuclear IF staining allow for the detection of epithelial structures embedded in the surrounding adipose tissue of the MFP. Immunofluorescence aids with cell identification, quantification, and histological examination during later imaging and analysis (Zaqout et al., 2020). Various membrane targets such as catenin beta-1 (β-catenin), epithelial cadherin (E-cadherin), actin filaments (F-actin), and wheat germ agglutinin (WGA) are commonly used for localizing and studying the epithelial membranes within the MG. β-catenin is a dual-function protein encoded by the human CTNNB1 gene that is involved in cadherin-based cell-cell adhesion and regulation. E-cadherin is another important surface molecule for cell-cell adhesion of epithelial tissue in most organs. F-actin is a cytoskeletal component found in the cytoplasm and cell membrane as well and is part of the contractile apparatus of cells (González-Gutiérrez et al., 2020). Lastly, WGA is a binding protein typically bound to carbohydrates that can also be located at the cell membrane of mammalian cells. One

widely-used nuclear stain is 4′,6-diamidino-2-phenylindole (DAPI). DAPI binds to adenine–thymine-rich regions in DNA and is used to determine the number of nuclei and assess cell morphology.

Tissue thickness

Much of the IF staining for these molecules is affected by dilution of targeting antibodies, incubation periods, washing steps, and tissue composition. Tissue thickness is a major variable when it comes to IF staining, where tissue sections can range from a few microns (μm) up to cm-scale, the latter using optical-clearing-based methods (Lloyd-Lewis et al., 2016). Histological steps typically require much longer incubation times for thicker tissue to allow for full sample permeabilization. In the context of the MG, whole-mount sections are useful in order to see its complex three-dimensional (3D) structure (Tolg et al., 2018). Thick sections can reveal epithelial ductal networks of the MG and coupled with proper staining provide a window into its development and function. Typically, thicker samples are not transparent and are challenging to visualize using a standard microscope. Before the invention of 3D imaging, the solution was to section tissue into thinner slices of around 10 μm each and combine these to create 3D data (Lloyd-Lewis et al., 2016). However, this method is not only labor-intensive but does not always provide the desired information. Confocal and multiphoton microscopy to image thicker tissue allows for the study of the complex natural structure of the MG.

Confocal microscopy

Confocal laser scanning microscopy (CLSM) is an important method for visualizing the 3D structure of the MG. Confocal microscopy works by capturing a series of two-dimensional (2D) images at different depths of a sample and reconstructing a 3D image, a process known as optical sectioning. Instead of evenly flooding a specimen with light, a CLSM uses point illumination to focus a small beam of excitation light one narrow depth level at a time (Elliott, 2020). Specified fluorescent dyes absorb the excitation light based on their respective absorption coefficients and emit light of a longer wavelength due to the process's incurred energy loss (Dempsey et al., 2011) to be detected by the photodetector of the microscope. The brightness of the fluorescence depends on the dye itself as well as the surrounding molecules.

A CLSM provides a high-resolution 3D image of the intact tissue but the quality is contingent on tissue preparation. Excitation light penetration and emission vary due to tissue thickness and staining. A typical tissue sample is comprised of an abundance of components including water, embedded cellular structures, lipids, and proteins (Dempsey et al., 2011). Each of these elements differs in their interactions with light penetration through the tissue, particularly with refractive index (RI), which is the ratio between the velocity of light in a vacuum compared to its velocity through a medium. Transparency of tissue is decreased based on RI differences between molecular components. Light penetration and overall clarity for the means of imaging can be enhanced by tissue clearing. The goal of tissue clearing is to make large, fixed biological samples transparent (Lloyd-Lewis et al., 2016) by homogenizing the RI of a sample and removing obstructive components. Multiple clearing methods exist that address the issues that arise with deep-tissue imaging. These techniques rely on organic solvent-based or hydrophilic reagent-based clearing solutions to homogenize RIs within the tissue. Some methods include prior hydrogel embedding to stabilize cellular structures.

The MG in particular is a lipid-rich and optically opaque organ, which makes visualization of the mammary epithelial tree at single-cell resolution a challenge. There are five main tissue clearing techniques used for the MG: Three-dimensional imaging of solvent-cleared organs (iDISCO), passive clarity technique (PACT), clear unobstructed brain imaging cocktails (CUBIC), see deep brain (SeeDB), and fructose, urea, and glycerol for imaging (FUnGI) (Lloyd-Lewis et al., 2016; Rios et al., 2016). Solvent-based clearing such as iDISCO provides the highest level of tissue transparency (Richardson and Lichtman, 2015; Tainaka et al., 2016), although it also leads to significant shrinkage of the tissue sample and a rapid decline in endogenous fluorescence signals (Ertürk et al., 2012; Pan et al., 2016). Lloyd-Lewis *et al.* found that SeeDB was the optimal protocol for clearing the MG of mice compared to iDISCO, PACT, and CUBIC, although they noticed some tissue size reduction during its lengthy five-day protocol (Lloyd-Lewis et al., 2016). FUnGI is another fructose-based clearing solution like SeeDB but only requires a two-hour incubation period. Rios *et al*. found that it produced high levels of optical transparency, morphology preservation, quality of whole-mount immunostaining, and suitability for subsequent rehydration and 2D analyses (Lloyd-Lewis et al., 2016; Rios et al., 2016). Overall, it seems that the optimal clearing protocol for the MG is yet to be established and has received very little use and application for studying the MG of species outside of commonly-used rodents.

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CHAPTER 2: LATE GESTATIONAL HYPERPROLACTINEMIA INCREASES BINUCLEATION OF EPITHELIAL CELLS IN THE MAMMARY GLANDS OF PIGS ACROSS DEVELOPMENT

ABSTRACT

The mammary gland (MG) is a remarkable tissue that undergoes a number of stages of epithelial growth and differentiation and is regulated by hormones such as prolactin (PRL). Recent research has found that binucleated cells (BNC) in the MG of mice form at the time of late gestation and remain until the termination of lactation, although their functional purpose is unknown. In order to improve the understanding of their development pattern, we aimed to quantify the abundance of BNC in the MG of primiparous pigs (n=4) from late gestation day 90 through lactation day 21. Further, we used hyperprolactinemic primiparous gilts (n=4) treated with the dopamine antagonist domperidone (DOM) to test the latter's effect on the abundance of BNC within the MG during the same period. The cellular architecture was assessed by three-dimensional confocal microscopy and immunofluorescent staining. Our results indicate that BNC abundance increased during the gestation-lactation window to reach maximal levels on day 21 of lactation. We also found that DOM-treated gilts had a significantly greater incidence of BNC compared to controls ($p < .05$) and that on lactation day 21 there was a statistically greater number of BNCs in DOM-treated sows ($p < .05$). These findings demonstrate that not only do BNCs exist in the porcine MG but, that they may play a functional role during lactogenesis.

INTRODUCTION

Milk underlies the economics of animal production and the dairy industry. A successful lactation reflects many variables, where its optimization can improve growth rates and survivability of mammalian offspring as well as increase milk yield. The synthesis of milk by the mammary gland (MG) depends on the activity of mammary epithelial cells (MEC) that arrange as alveoli during late gestation, in preparation for the subsequent secretory phase. During this phase, the MEC and alveoli are particularly responsive to a changing endocrine environment. Among the combination of hormones that direct gestation and lactation, prolactin (PRL) is often considered the most important for both MG growth and differentiation.

Prolactin synthesis and secretion are under hypothalamic control via the local synthesis and release of dopamine (DA) (Horseman, 1999). After its secretion, PRL binds PRL receptors (PRLR) on the cell surface of tissues like the MG epithelium. Dopamine inhibiting factors maintain low levels of circulating PRL prior to parturition by binding to type 2 dopamine receptors (D_2) on lactotrophs. Prolactin is crucial for milk production in pigs, where its circulating levels rise significantly prior to parturition (DeHoff et al., 1986). Highlighting the importance of this phase, Farmer *et al.* suppressed PRL secretion in late gestating gilts which led to a reduction in the mass of the epithelial parenchyma and as well as a decline in the concentration of the MG (Trott et al., 2009). To mirror these findings, VanKlompenberg *et al* administered domperidone (DOM), a dopamine antagonist, to late gestation primiparous female pigs from day 90-110 to induce hyperprolactinemia (VanKlompenberg et al., 2013). Those authors showed that elevated PRL levels during gestation increased alveolar cross-sectional area and the presence of lipid droplets. During lactation, they saw increased milk yield and greater subsequent piglet growth by 21% compared to control sows (VanKlompenberg et al., 2013).

Mathews *et al.* found a similar increase in the milk production of primiparous sows administered metoclopramide to induce hyperprolactinemia during the same period of late gestation (Mathews et al., 2021). While VanKlompenberg *et al.* reported no increase in epithelial cell proliferation in DOM-treated sows, those authors did find that hyperprolactinemia during late gestation had a positive carryover effect into lactation on the expression of several milk-related genes, including β-casein, acetyl CoA carboxylase-α, lipoprotein lipase, α-lactalbumin, and glucose transporter, weeks. The mechanisms by which PRL coordinates epithelial growth and differentiation during these different stages of lactogenesis remain unclear.

In a recent publication, Rios *et al.* provided potential insights to this question by demonstrating that a significant proportion of secretory alveolar cells in the MG during late pregnancy and early lactation are binucleated (Rios et al., 2016). Binucleated cells (BNC) have long been associated with various cancer types and arise due to failed cytokinesis, post-mitotic fusion, and fusion of two nuclei after cytokinesis (Grizzi and Chiriva-Internati, 2007). Binucleation is a common occurrence in organs such as the liver, salivary glands, heart, and endometrium (219), which supports there being a functional reason for their development. Rios *et al.* reported an abundance of BNC in the lactating MG, including the demonstration that 50-60% of the MECs in mice, 30% of MECs in wallabies, and 40% of MECs in cows were binucleated (Rios et al., 2016). *Rios et al* also performed flow cytometric analysis of different cellular subsets and found that ploidy in MEC increased during late pregnancy (Rios et al., 2016). This demonstrated induction of BNC in the gland during late gestation aligns with an earlier study by Smith and Vonderhaar who suggested this possibility when they found increased DNA synthesis during late pregnancy without evidence of single-cell duplication (Smith and Vonderhaar, 1981). The presence of aurora kinase-A (AURKA) during the lactogenic switch

appears to mediate the formation of BNCs, where inhibition of AURKA disrupted the synthesis of BNCs and suppressed milk production. These BNCs were also found to have a greater cell volume compared to MNCs, suggesting an important role during lactogenesis (Rios et al., 2016).

Given the relationship between late-gestational hyperprolactinemia and the subsequent ability of MEC to synthesize and secrete milk, we hypothesized that DOM-induced hyperprolactinemia during the PRL-sensitive window of late gestation would increase the subsequent abundance of BNCs in the MG. Our data show that DOM-treated sows had significantly more BNCs in their MG in late lactation, where their abundance increased over time during lactation, which mirrored the increased milk output by the MGs (VanKlompenberg et al., 2013). Further research will help explain the functional purpose of BNCs in the MG.

MATERIALS AND METHODS

Mouse tissue

Samples of MGs from lactating mice were as described in Berryhill *et al* (Berryhill et al., 2021). Animals, diets, and all experiments were approved by the University of California, Davis Institutional Animal Care, and Use Committee. Balb/cJ mice (Jackson Laboratories, Bar Harbor, ME) had *ad libitum* access to food and water and were housed with a 14-hour light to 10-hour dark cycle. Mice were fed the AIN93G control diet (Harlan Laboratories, Indianapolis, IN, 15% fat supplied primarily as soybean oil). At eight weeks old, female mice were mated and assigned the control diet found in Table 1 of the Berryhill *et al.* study (Berryhill et al., 2021). The first day of gestation was defined as the day of detection of a seminal plug (day 1 of gestation). All female mice were first parity and were culled on day 1 of lactation. Litter size was standardized to n = 6 pups.

Pig tissue

Tissues from sows used in this work were previously collected by VanKlompenberg *et al.* (VanKlompenberg et al., 2013). In that study, pregnant Yorkshire-Hampshire crossbred primiparous gilts were randomly assigned to receive either no treatment (CON) or domperidone (DOM) from 90 to 110 days of gestation. Gilts were weighed on days 90, 96, 100, 105, and 110 of gestation in order to adjust the appropriate dose of DOM treatments. Treated gilts received Equidone (11% wt/vol DOM, 0.4 mg/kg BW; Equitox Pharma, Central, SC) in their feed, whereas CON gilts received no supplement. The dose of DOM had been previously documented to induce hyperprolactinemia in humans (Wan et al., 2008). Samples from eight sows were selected for the present study, of which 4 were CON-sows (two from Fall and two from Winter),

and 4 were DOM-treated sows (two from Fall and two from Winter). All gilts were fed twice daily and had *ad libitum* access to water. Pregnant females were housed separately in gestation pens throughout their treatment periods and then were moved to individual farrowing crates on day 111 of gestation until their piglets were weaned on day 21 of lactation. The day of parturition was designated day 1 of lactation.

Serial biopsies of the MG were performed with a vacuum-assisted approach (VanKlompenberg et al., 2012) on days 90, 100, and 110 of gestation and on days 2 and 21 of lactation. All biopsies at a given time point were from the same gland position, and sampling across time followed a sequential pattern that moved contralaterally and then diagonally forward starting from the right-most posterior gland of the mammary chain. Tissues taken during each biopsy were fixed in 10% formalin overnight at 4°C.

Experiment 1: Comparing the ability of iDISCO and FUnGI to clear mammary tissues

iDISCO Clearing

The iDISCO clearing protocol was performed as previously described (Renier et al., 2014). Briefly, sections of formalin-fixed paraffin-embedded (FFPE) sow mammary gland ranging in thickness from 4μm to 100μm were first stained with H&E prior to iDISCO clearing. Tissues were dehydrated in graded methanols and then submerged in a 66% dichloromethane and 33% methanol solution at room temperature for 3 hours on a rocking platform. Samples were then submerged in 100% dichloromethane on a rocking platform (2 x 15 minutes). Lastly, each section was incubated in dibenzyl ether in a closed airtight container before being mounted on a Fisherbrand Superfrost Plus Microscope Slide. Mounted sections were then imaged with a standard brightfield microscope.

FUnGI Clearing

We cleared tissue with a protocol referred to as fructose, urea, and glycerol for imaging (FUnGI) clearing was performed using the published protocol previously optimized by Rios *et al.* (Rios et al., 2019). Briefly, the FUnGI clearing agent comprised 50% glycerol (vol/vol), 2.5 M fructose, 2.5 M urea, 10.6 mM Tris Base, 1 mM EDTA, and had an RI of 1.46 at room temperature. The FFPE sections of sow MG (ranging from 4μm to 100μm thickness) were first stained with DAPI (blue), WGA (red), and for β-catenin (green). Samples were then incubated with 100-300μL of FUnGI clearing solution at room temperature for two hours. The FUnGI solution was then removed and reapplied prior to coverslipping. Each coverslip was sealed with clear nail polish and dried overnight at room temperature prior to imaging.

Experiment 2: Optimization of Antigen Retrieval

Antigen retrieval - 9 pH TE Buffer

Tris-ethylenediaminetetraacetic acid (TE) buffer (pH 9) was comprised of Tris, ethylenediaminetetraacetic acid (EDTA), H_2O, and 1M HCl in order to adjust the pH to 9.0. After deparaffinization, each section was steamed in 97°C TE buffer within Coplin jars for 40 minutes prior to being cooled in a water bath at room temperature for 10 minutes. After antigen retrieval, each section was processed through immunofluorescent staining.

Antigen retrieval - 6 pH Citrate Buffer

Citrate buffer (pH 6) was comprised of 0.0825 sodium citrate, 0.0175 M citric acid, and H_2O, and was adjusted to a pH of 6.0 using 1M HCl. Sections mounted on slides were first

deparaffinized prior to antigen retrieval. Each slide was then steamed at 97°C in a Coplin jar filled with citrate buffer for 40 minutes before being cooled in a room temperature water bath for 10 minutes. After antigen retrieval, each section was processed through immunofluorescent staining.

Immunofluorescence protocol

Mammary gland biopsy samples were fixed in 4% paraformaldehyde, dehydrated, and then embedded in paraffin blocks. Each block was sectioned at 30-µm thickness onto Fisherbrand Superfrost Plus Microscope Slides. Sections were then deparaffinized using xylenes and dehydrated through graded ethanols. Tissue sections were then pretreated with Triton X-100 in phosphate-buffered saline. Each section then underwent antigen retrieval for 40 minutes by steaming in Coplin jars containing TE Buffer pH 9.0. Following antigen retrieval, 10% horse serum was applied to each section for 1 hour at room temperature to reduce non-specific binding of antibodies.

After blocking, each section was incubated with rabbit anti-E-cadherin IgG (1:100, Cell Signaling Technology Inc.) in 10% horse serum overnight at 4°C. Sections were then rinsed in PBS-Tween20 before an AlexaFluor 488 donkey anti-rabbit IgG secondary antibody (1:500, Jackson ImmunoResearch Laboratories, Inc.) and an Alexa Fluor 594 conjugate of wheat germ agglutinin (WGA) (1:1200, Biotium Inc.) were applied overnight at 4°C. Tissues were rinsed in PBS-Tween 20 and then incubated with DAPI in PBS (1:1000) for 20 minutes at room temperature. After rinsing in PBS-Tween20, FUnGI clearing solution was applied for 2 hours at room temperature. Prior to imaging FUnGI solution was then reapplied, coverslipped, and sealed with nail polish.

Confocal imaging

All images of immunofluorescently labeled 30-µm-thick tissue sections were acquired using a Leica TCS SP8 inverted confocal microscope with a 40x/1.3 oil (HC PL APO CS2) objective lens. Laser power and gain on the microscope were adjusted manually to optimize fluorescence intensity and to reduce photobleaching for each of the fluorophores AF-488 (green), AF-594 (red), and DAPI (blue). Photodetector bandwidth for each fluorophore was modified to minimize any background signal. A step size of 0.3µm, line averaging of 2, and a resolution of 3000µm x 3000µm were kept constant for all images captured for the purpose of quantification. Imaging was then performed from the top to the bottom of the epithelial structures (typically 30µm) for each sample. Captured images were then filtered for background signal with Huygens Professional deconvolution software (Scientific Volume Imaging, Netherlands). Deconvolution was used on all images to improve image quality.

Quantification of cells

Image reconstructions were generated using Bitplane Imaris image management software (Oxford Instruments, United Kingdom). Each file was individually edited for optimal brightness and contrast and was then quantified for BNC cells using ImageJ (LOCI, University of Wisconsin). The DAPI (blue) layer of stained nuclei was displayed in gray to provide better contrast for counting. Images for each 30-µm section were first separated into 15-µm sub-planes. Counting was then performed by focusing through each sub-plane starting from the middle. Only BNCs and MNCs were counted using the multi-point tool if their entire cell surface was visible in the sub-plane. The area of each image was fully quantified and typically contained between

100-500 cells. Files were saved under number IDs taken from a random number generator so that the single evaluator was blinded to the treatment and development period of the sample. Values were logged in an Excel spreadsheet against information for sow ID, development period, and treatment. The same CON and DOM-treated sows were used for analysis across gestation days 90 (n = 3; n = 3), 100 (n = 3; n = 3), and 110 (n = 4; n = 4), as well as lactation days 2 (n = 4; n = 4), and 21 (n = 4; n = 4). Each 30-μm section was imaged at either two or three randomly selected fields.

Statistics

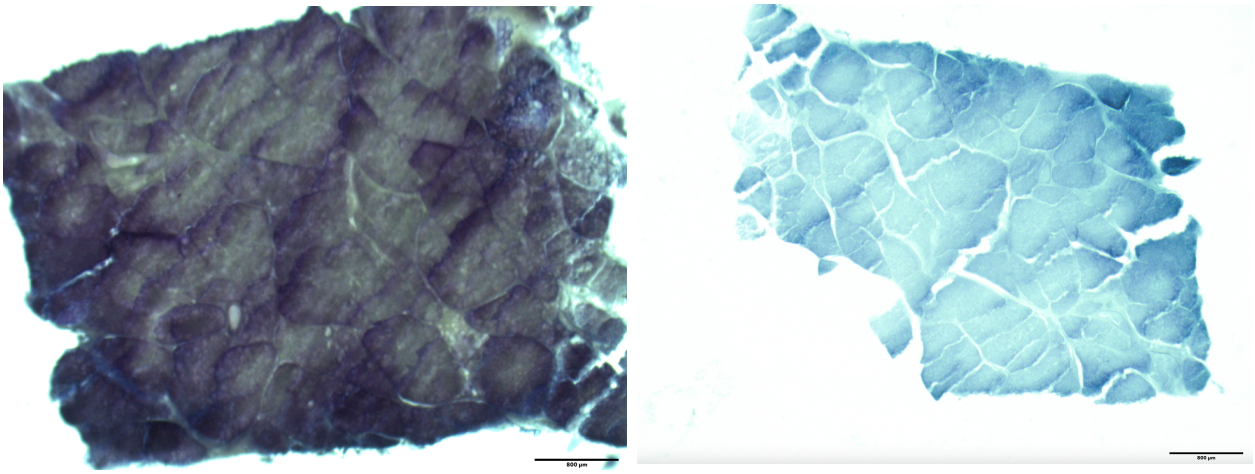
The data was log-transformed to address unequal variance, prior to analysis by ANOVA for the main effects of time, treatment, and their interaction. Statistical difference was declared at $p < .05$. Individual means comparisons were by t-test.

RESULTS

Comparison of clearing capability of iDISCO and FUnGI

As a first step toward 3D imaging of mammary tissue in thick sections, we compared the ability of iDISCO and FUnGI clearing protocols to improve the image quality for immunostaining of porcine MG tissue. Clearing in general made tissue more transparent and allowed for deeper visualization. As shown in Figures 2A and B, tissue was nicely cleared using the iDISCO protocol, providing fewer visual obstructions caused by background artifacts most likely due to mismatched RIs. However, there was substantial tissue loss during clearing as well as noticeable structural changes in the tissue itself. There were multiple advantages afforded by the FUnGI clearing solution, including a faster protocol compared to the full-day protocol of iDISCO. Additionally, FUNGI avoided the use of organic solvents that can quench fluorescence (Rios et al., 2019). Tissue cleared with FUnGI solution, as shown in Figures 2C and D, was optimal for preserving tissue morphology as well as clearing itself. The cleared epithelium was noticeably more translucent, allowing for visualization of its ductal networks within the section. After comparing samples assessed for clearing time, tissue preservation, and clarity, we concluded that the FUnGI clearing protocol was best suited for our applications.

A.



B.

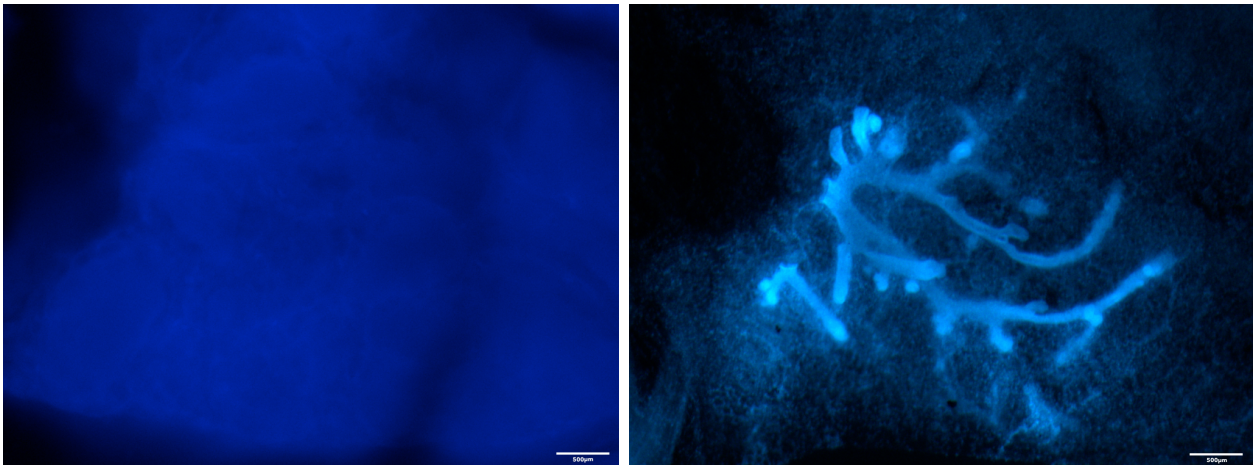


Figure 2: Clearing of porcine mammary tissue using iDISCO and FUnGI protocols. Mammary gland section (approximately 100µm) stained with hematoxylin and eosin (A) prior to the iDISCO protocol and (B) after iDISCO clearing. The sample was imaged using a brightfield dissecting microscope and was noticeably more transparent. The section did not maintain structural integrity, lost a small portion of its original tissue, and shrank in size. Another section of sow mammary tissue (~100µm) was stained for DAPI and β-catenin and imaged (C) prior to the FUnGI protocol and (D) after clearing. This section visibly maintained its structure after clearing and allowed for visualization throughout the different layers of tissue. The ductal network can be seen within the section and had a strong fluorescent signal.

Comparison of antigen retrieval capability of citrate buffer and TE-buffer

In an effort to enhance the signal intensity of fluorescently labeled targets we tested two methods of heat-induced antigen retrieval using citrate buffer, pH 6, and Tris-EDTA, pH 9. While both buffers achieved some degree of antigen retrieval, as seen in Figures 3A and B, the TE-buffer was more effective for "unmasking" protein epitopes for the antibodies used. The section in Figure 3A underwent antigen retrieval using citrate buffer (pH 6.0) while the section in Figure 3B was retrieved using TE buffer (pH 9.0). Overall, the fluorescence signal that was captured after antigen retrieval with TE buffer was more intense and clearer.

A. **B.**

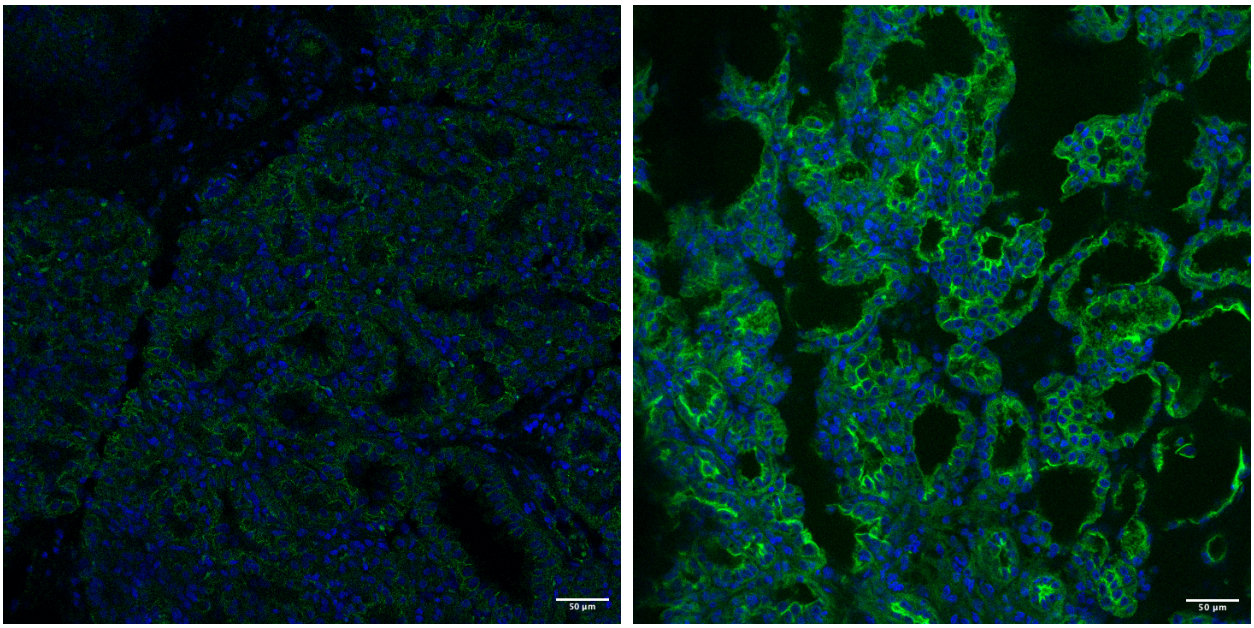


Figure 3: Comparison of antigen retrieval using either citrate buffer pH 6 or TE-Buffer pH 9. Heat-induced antigen retrieval was performed on a 10µm section of sow mammary glands using (A) pH 6 citrate buffer and (B) pH 9 tris-ethylenediaminetetraacetic acid (TE) buffer, respectively. Both sections underwent antigen retrieval at 97°C in their respective buffers for 40 minutes and were then stained with DAPI (blue) and β-catenin (green). After the immunofluorescence protocol, each section was imaged using ultraviolet and green fluorescent protein filters and superimposed in Adobe Photoshop to generate the multicolored images. Both DAPI and β-catenin were visible in both treatments, however, the mammary gland section that underwent antigen retrieval in TE buffer had a higher staining intensity and antibody penetration.

Cells were initially stained for E-cadherin (green) and DAPI (blue) but did not provide a full outline of the cell membrane, as represented in Figure 4A. While the basal portions of each luminal cell were visible the apical regions were missing. We concluded that in order to properly characterize each cell an apical membrane stain was critical. Separately, others have published that staining for WGA was an effective method for detecting the apical cell surface because of its ability to bind murine molecules (Valdizan et al., 1992). We implemented an Alexa Fluor 594 conjugate of WGA with our normal IF staining protocol, which provided a complete outline of each luminal cell Figure 4B. Only minimal non-specific staining appeared when staining for WGA.

A. **B.**

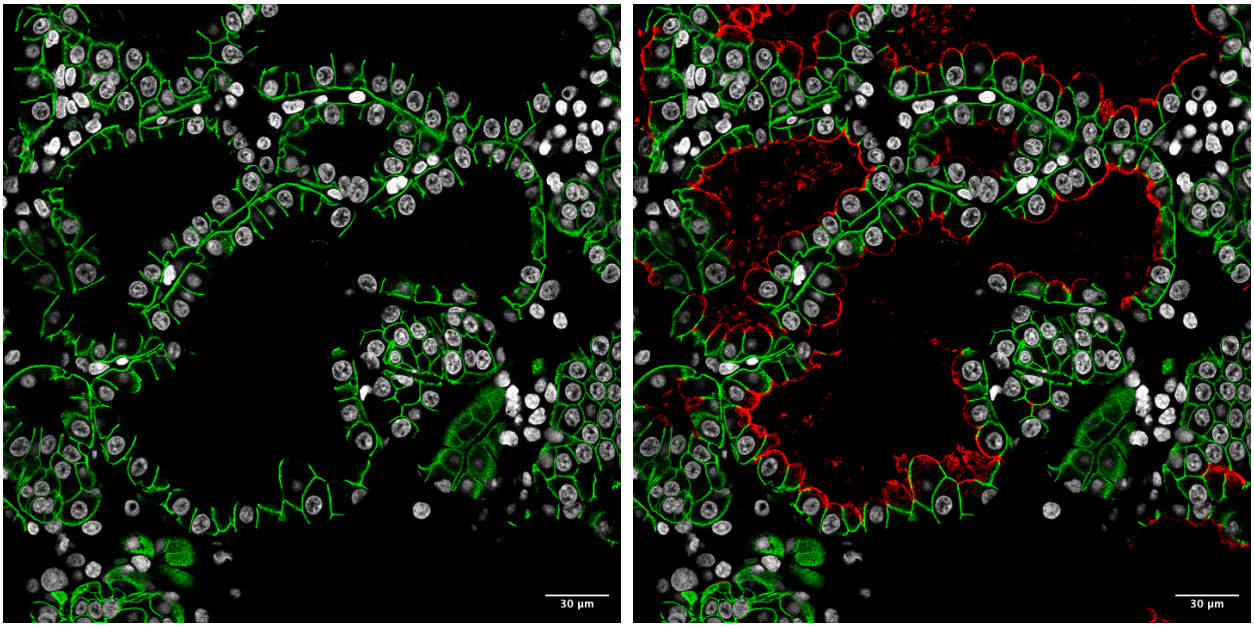


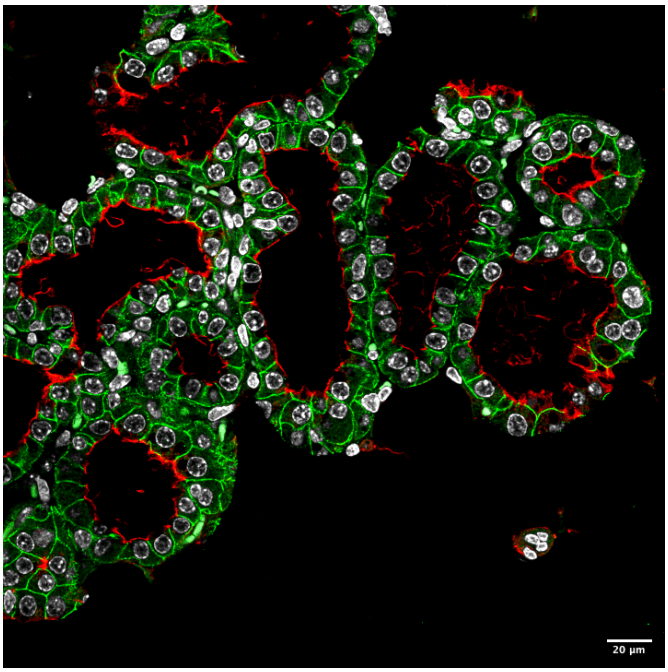
Figure 4: Membrane staining of the porcine mammary gland (30µm) comparing staining with A) E-cadherin alone (green) or B) E-cadherin (green) + WGA (red). The section underwent the immunofluorescence staining protocol for E-cadherin (green), wheat germ agglutinin (WGA; red), and DAPI (white). The images were captured on a Leica confocal microscope at 40X magnification and show the same single z-stack layer of thickness of around 0.3 µm. Colored channels were overlayed using ImageJ and represent how the apical membrane stained with WGA (red) can provide a full staining circumference for each luminal cell.

Binucleation of mammary epithelial cells

After optimizing the immunofluorescent protocol, we used confocal microscopy to visualize murine and porcine mammary tissues of varying thicknesses. A preliminary analysis revealed that various antibodies could not be successfully localized with any consistency when sections were 100μm thick. Additionally, sections greater than 50μm did not adhere properly to slides and were often lost during various steps of the protocol. We found that sections averaging around 30μm thickness were best suited for BNC quantification given they were ideal for reagent penetration and provided adequate depth for multiple layers of total cell imaging.

Given the results of Rios, we first examined sections of the mammary glands from day 1 lactation of Balb/c female mice (n = 4), as seen in Figure 5A. Immunofluorescent staining and cell quantification were performed in order to validate their findings and to substantiate our own methods aimed at detecting BNC in porcine MG tissue. As shown in Figure 5B, 23.8% of the luminal epithelial cells were binucleated. Given that BNCs have not been identified in the pig MG previously before for the pig MG, we first determined their presence in biopsies of MG tissue from 4 sows across different states of first parity pregnancy and lactation. We also examined the incidence of BNC in sows across the same period that had been treated with DOM during late gestation in order to test the effect of late-gestational hyperprolactinemia.

A.



B.

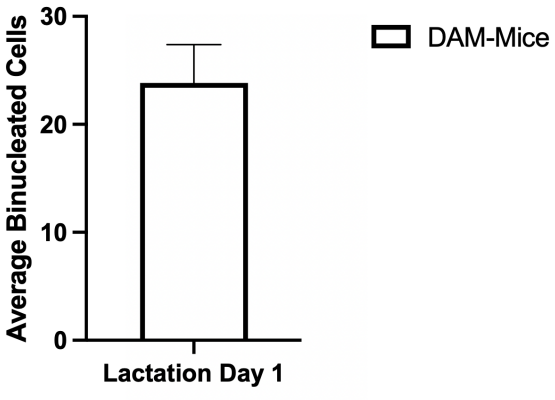


Figure 5: Stained 30µm section of Balb/c mouse mammary gland with a graph representing the average binucleated cell percent of all tested mice. (A) A representative section of lactating day 1 mouse mammary gland was stained for E-cadherin (green), wheat germ agglutinin (WGA; red), and DAPI (white) (n = 4 mice). The image was captured using a Leica confocal microscope at 40X magnification and shows multiple alveoli. (B) The average binucleated percent for the mammary gland of all mice was 23.8%.

Data collected from CON sows provided a baseline for the BNC population across late gestation and lactation. Figure 6 depicts representative stained sections of CON and DOM-treated sow MGs across the study period and revealed an increase of the alveolar diameter of DOM sows by D100 of pregnancy. Throughout the development window of D90 to L21 of both treatment groups, the binucleated cell population increased continuously. Figure 7 shows the averaged BNC percentage data across late gestation and early lactation for CON and DOM sows. Statistical analysis revealed that DOM treatment across all time points had a significant effect compared to CON ($p < .05$). Additionally, we found that period of development had a

significant main effect ($p < .0001$) and that there was a positive effect of DOM on the number of BNCs in the mammary gland ($p < .0001$), although there was no significant interaction found between treatments. Lactation day 21 had a statistically greater number of BNCs in DOM-treated sows (t-test, $p < .05$), having an average BNC percent of 14.2% compared to CON sows of only 8.1%.

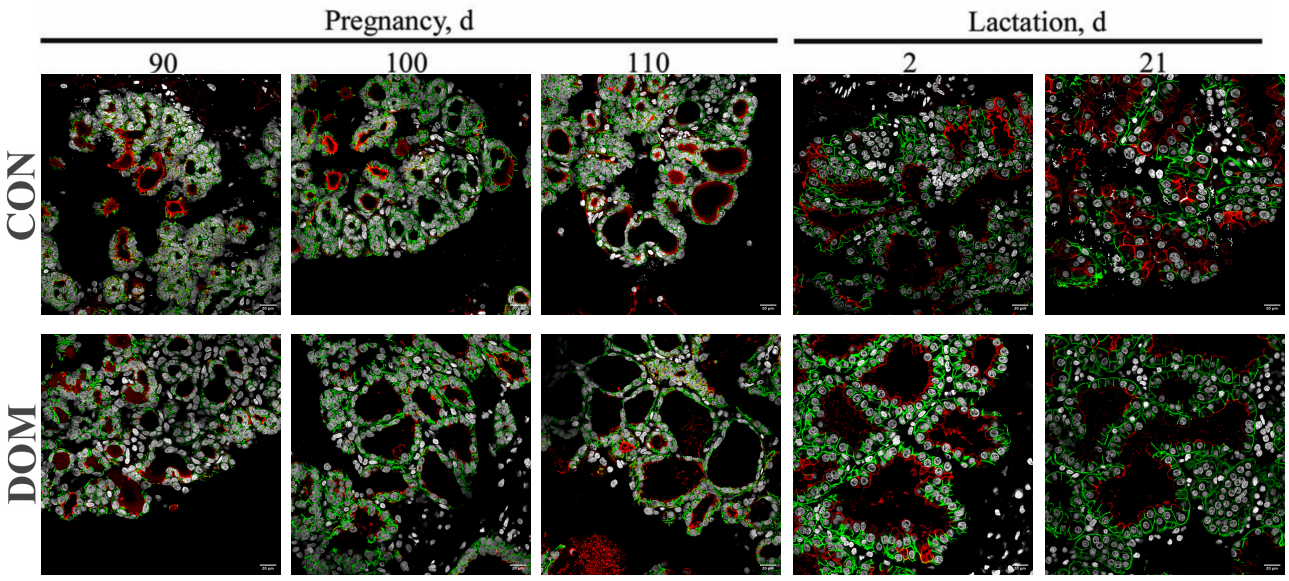


Figure 6: Temporal changes in mammary gland histology of control sows (CON) and domperidone-treated (DOM) sows across late gestation and lactation. Both untreated and DOM-treated sows were biopsied on day 90, day 100, and 110 of gestation and on lactation day 2 and day 21. The top row is of a representative immunofluorescent stain of the mammary gland tissue of an untreated control sow stained for E-cadherin (green), wheat germ agglutinin (WGA; red), and DAPI (white). The bottom row is a representative immunoflourescent stain of mammary gland tissue from a DOM-treated sow using E-cadherin (green), WGA (red), and DAPI (white). The alveoli and individual luminal cells were visibly larger in size and volume respectively for DOM-treated sows compared to untreated sows. Both control and DOM sows showed an increase in overall alveolar size over the course of development.

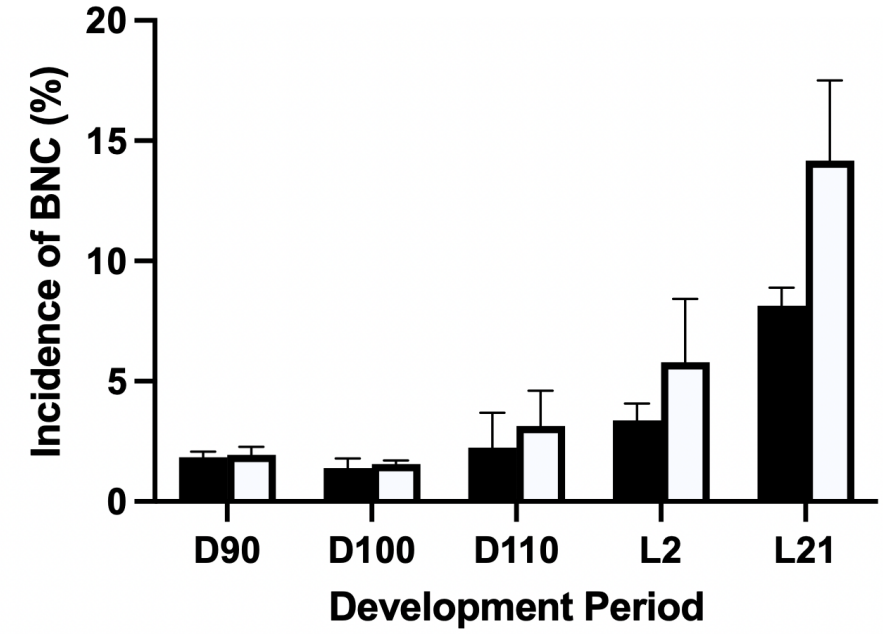


Figure 7: Graph of the abundance of binucleated cells in the mammary glands of control (CON) and domperidone-treated (DOM) sows across late gestation and lactation. Tissue samples were collected via mammary gland biopsy on days (D) 90, 100, and 110 of gestation and on days 2 and 21 of lactation (L; n = 3 sows for D90 and D100, n = 4 sows for D110, L2, and L21). Binucleated (BNC) and mononucleated cells were individually counted and recorded using the multi-point tool in ImageJ. A total of 144 15μm sections were quantified for control (black bars) and DOM-treated sows (white bars) across their respective development periods (84 for CON; 60 for DOM-treated). The graph represents the average percent BNCs counted in all samples. There was a significant effect of DOM-treatment across all time points compared to CON ($p < .05$) as well as a significant main effect of time ($p < .0001$). Additionally, there was a positive effect of DOM on the number of BNCs in the mammary gland ($p < .0001$). On L21 there was a statistically greater number of BNCs in DOM-treated sows (t-test, $p < .05$).

DISCUSSION

Our findings reveal that the incidence of BNC in the MG of primiparous pigs increases during the course of lactation, where the presence of BNCs in the MG of pigs had not been previously described. Notably, there was a stage-dependent change in BNC incidence, which was maximal at L21, and which corresponds to the peak of lactation in pigs. While the presence of BNC in the MG of pigs is consistent with their demonstrated presence in mice, cows, wallabies, and humans, there appears to be a distinction in the timing of their appearance and regulation. Rios *et al.* found that in the lactating human, seal, and wallaby MGs, binucleated cells comprised ~30% of all observed luminal cells, whereas 40% were observed in bovine mammary tissue (Rios et al., 2016). Binucleated cells in the MG of mice arose during late gestation at D18.5 and by L4 50% of the luminal cells contained 4N DNA content (Rios et al., 2016). This timeline, however, was different in the pig MG model. We found that binucleation did not noticeably rise from late gestation day 110 to early lactation day 2. Rather, BNCs increased significantly by later lactation on L21. While the timeline may differ from other mammals, the increase of BNCs in the porcine MG mimics its increase in milk production throughout lactation supporting the notion that they play a functional role in MG development and lactation.

A longstanding belief has been that DNA synthesis in the MG was a precursor for cell replication. However, a body of evidence suggests otherwise. Banerjee *et al.* discovered that during early lactation in the mouse the abundance of ^{3}H-thymidine labeled cells in the MG increased while the mitotic index remained constant. Those authors concluded that the augmentation of DNA synthesis may not have represented cell duplication, but rather may have reflected partial replication of the cellular genome, which has been referred to as metabolic DNA synthesis (Banerjee et al., 1971). This mechanism was proposed by Pelc *et al.* as a means to

enhance gene amplification to support high rates of RNA and protein synthesis in metabolically active tissues (Pelc, 1968). In the context of the MG, milk synthesis is occurring during this stage of early lactation. Smith and Vonderhaar also submitted that DNA synthesis might lead to polyploidy within cells as an explanation for their observation of increased DNA synthesis without the duplication of cells (Smith and Vonderhaar, 1981). The authors proposed that traversal of the cell cycle is not required for functional differentiation of the MG (Smith and Vonderhaar, 1981). All of these observations left some gaps as to why there was little cell proliferation after the documented high levels of DNA synthesis. The revelation of BNCs in the MG by Rios *et al.* suggested that the conserved abundance of BNCs across different species during gestation and lactation might offer an explanation.

The function of BNCs in the MG remains unclear, although it is tempting to speculate on their potential advantages over MNCs. Binucleation has been documented in other organs, where polyploidy may confer a variety of advantages that depend on the function of the organ (Brodsky and Uryvaeva, 1977). For example, the liver has a high propensity for binucleation with approximately 40% of its hepatocytes being BNCs (Seddik Hammad et al., 2014). The initial rise of one tetraploid hepatocyte with two diploid nuclei is caused by incomplete cytokinesis (Guidotti et al., 2003), an event that usually occurs during acute and chronic liver damage (Seddik Hammad et al., 2014). Generally speaking, BNCs in the liver are considered terminally differentiated, however, binucleated hepatocytes retain the ability to undergo future DNA replication with complete cytokinesis (Wang et al., 2017). Terminal differentiation of BNCs suggests that certain types of organ growth can be accomplished with little need for cellular proliferation.

Cardiomyocytes within the heart also have binucleation (Anatskaya and Vinogradov, 2007), where they may optimize cellular response and improve cell survival under stressful conditions or under high physiological tension (Anatskaya and Vinogradov, 2007). Another theory proposes that binucleation in the heart helps meet the high metabolic demand of cardiomyocytes by producing twice the amount of RNA to help synthesize proteins (Ahuja et al., 2007). The MG has a similar need to synthesize proteins for lactogenesis.

As piglets continue to grow throughout lactation, milk synthesis increases to meet their changing demand. VanKlompenberg *et al.* found that hyperprolactinemia during late gestation led to higher milk production of gilts during later lactation days 14 and 21 and subsequently increased the body weights of their piglets (VanKlompenberg et al., 2013). Mathews *et al.* presented similar results, finding that piglets nursing from MET-treated sows consumed more milk compared to controls during the same period (Mathews et al., 2021). Binucleated cells in DOM-treated sows showed a similar pattern, increasing their presence in the MG towards later lactation. These findings suggest that BNCs may play a role in prolactin-stimulated milk production, perhaps by an epigenetic mechanism. Indeed, reverse transcriptase quantitative PCR analysis revealed that DOM-treated sows on L2 and L21 had significantly greater, or close to significantly greater, increases in mRNA expression for β-casein, acetyl CoA carboxylase-α, lipoprotein lipase, α-lactalbumin, and glucose transporter 1, all of which are essential for milk synthesis (VanKlompenberg et al., 2013). Given that an increased number of BNCs was found in DOM-treated sows during L2 and significantly during L21, these findings suggest that BNCs may help to enhance lactogenesis and ensuing lactation. Because BNCs have twice the genomic template, it is possible that they provide a transcriptional advantage when it comes to a synthetically active MG.

In conclusion, this work expands on the previously understood importance of PRL for the MG (Farmer and Petitclerc, 2003). More importantly, however, our related findings implicate that BNCs are a regulated occurrence and are important for MG development and lactation. The larger cytoplasmic areas of BNCs compared to MNCs (Rios et al., 2016) increase available milk storage, and apical secretion regions, and may offer greater growth proficiency of the MG itself.

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