Establishment and Characterization of Organoids from Mammary Tissue of Lactating Dairy Cows

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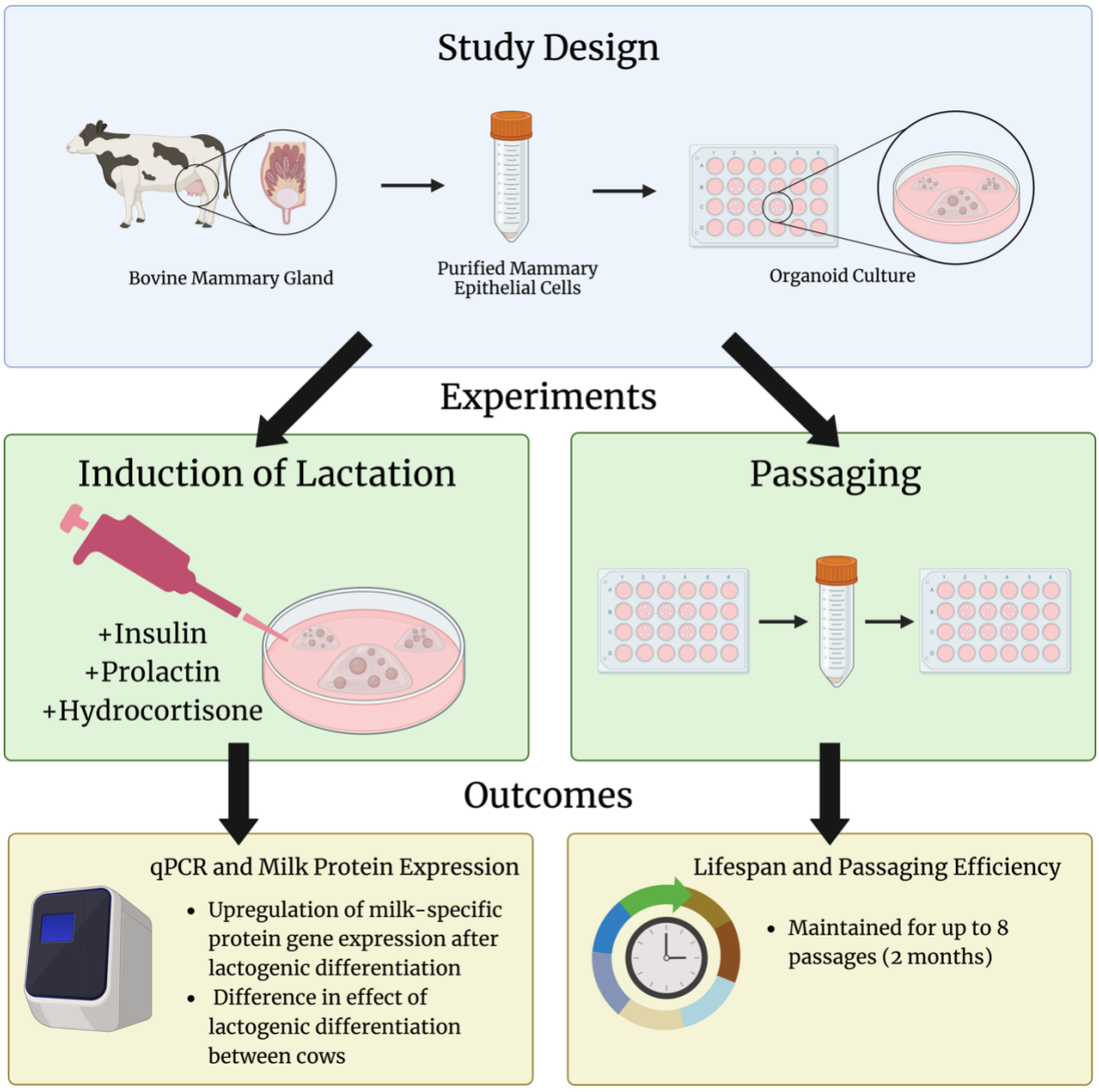
TABLE OF CONTENTS

Graphical Abstract



Abstract (English)

Dairy products are a staple of the human diet. However, milk production has a detrimental impact on the environment, thus necessitating research aimed at mitigating this impact by maximizing the efficiency by which cows transform feed into milk. The mechanisms of milk synthesis are not completely understood, possibly due in part to the lack of cellular models that structurally and functionally resemble the *in vivo* mammary gland. Therefore, the objective of this study was to establish and characterize mammary organoids from lactating dairy cows. Epithelial cells were isolated from the mammary glands of 3 lactating Holstein cows. Cells were grown in 2-dimensional culture, then seeded and expanded in 3-dimensional culture for 7 days. Organoids were treated for 4 days with either basal media or media containing lactogenic hormones. Gels were imaged throughout expansion and differentiation to analyze the formation and morphology of organoid structures. mRNA was isolated and relative gene expression was measured for milk-specific proteins α-S1 casein, β casein, κ casein, and ELF5 using quantitative real-time PCR. We detected the formation of spherical organoids within 2 days of initial seeding. We observed a significant upregulation in α-S1 casein, β casein, and κ casein gene expression after lactogenic differentiation was induced. Additionally, we observed a significant difference between cows in the effect of lactogenic differentiation on α-S1 casein, β casein, and ELF5 gene expression. Organoids were maintained for up to 2 months or 8 weekly passages. This culture method provides a novel 3-dimensional *in vitro* model of bovine lactation. The upregulation of milk-specific proteins after treatment with lactogenic hormones suggests that the functional capacity of the mammary gland is maintained. The variability in the response to lactogenic differentiation between cows suggests that individual characteristics of the cow *in vivo* are

maintained in organoids. Taken together, these results suggest that bovine mammary organoids should be considered as a culture model for future lactation studies.

Abstract (French)

Les produits laitiers sont un élément de base de l'alimentation humaine. Cependant, la production de lait a un impact négatif sur l'environnement, ce qui nécessite des études visant à atténuer cet impact en maximisant l'efficacité avec laquelle les vaches transforment l'alimentation en lait. Cependant, les mécanismes de synthèse du lait ne sont pas entièrement compris, peut-être à cause du manque de modèles cellulaires qui ressemblent structurellement et fonctionnellement à la glande mammaire in vivo. L'objectif de cette étude était donc d'établir et de caractériser des organoïdes mammaires provenant de vaches laitières en lactation. Des cellules épithéliales ont été isolées des glandes mammaires de trois vaches Holstein en lactation. Les cellules ont été cultivées en culture bidimensionnelle, puis ensemencées et expansées en culture tridimensionnelle pendant 7 jours. Les organoïdes ont été traités pendant 4 jours avec un milieu basal ou un milieu contenant des hormones lactogènes. Les gels ont été imagés tout au long de l'expansion et de la différenciation afin d'analyser la formation et la morphologie des structures organoïdes. L'ARNm a été isolé et l'expression relative des gènes a été mesurée pour les protéines spécifiques du lait: la caséine α-S1, la caséine β, la caséine κ et ELF5 en utilisant la PCR quantitative en temps réel. Nous avons détecté la formation d'organoïdes sphériques dans les 2 jours suivant l'ensemencement initial. Nous avons observé une régulation positive significative de l'expression des gènes de l'α-S1 caséine, de la β caséine, et de la κ caséine après l'induction de la différenciation lactogénique. De plus, nous avons observé une différence significative entre les vaches dans l'effet de la différenciation lactogène sur l'expression des gènes de l'α-S1 caséine, de la β caséine, et de l'ELF5. Les organoïdes ont été maintenus jusqu'à 2 mois ou 8 passages hebdomadaires. Cette méthode de culture fournit un nouveau modèle in vitro tridimensionnel de la lactation bovine. La régulation positive des protéines spécifiques du

lait après le traitement avec des hormones lactogènes suggère que la capacité fonctionnelle de la glande mammaire est maintenue. La variabilité de la réponse à la différenciation lactogène entre les vaches suggère que les caractéristiques individuelles de la vache *in vivo* sont maintenues dans les organoïdes. L'ensemble de ces résultats suggère que les organoïdes mammaires bovins devrait être considérée pour les futures études sur la lactation.

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Contribution of Authors

The authors involved in the research described in this thesis and their contributions are as follows:

Perri WIATRAK organized documentation of research findings and experimental blinding. She assisted in tissue collection. She collected and analyzed microscopic and gel images. She organized primer validation and qPCR experiments. She prepared the figures and wrote all chapters of this thesis. She revised all chapters of this thesis under the supervision of Dr. Sergio BURGOS.

Dr. Sergio BURGOS supervised the project. He conceived the project and designed the experiments. He administered treatment to organoids and isolated RNA. He performed the statistical analyses of qPCR results. He assisted the candidate with proofreading, reviewing, and processing all chapters of this thesis for publication. He obtained funding for this project.

Taïs VERANES, an undergraduate honors student, performed qPCR experiments with assistance from Perri WIATRAK.

Tatyana KUSTOVA is the laboratory technician who maintained and passaged 2-dimensional cell cultures and organoids. She prepared culture media and stock solutions.

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Meaning
GHG	Greenhouse gas
PRL	Prolactin
JAK2	Janus tyrosine kinase 2
MGF/STAT	Mammary gland transcription factor/signal transducer and activator of transcription
TEB	Terminal end bud
GH	Growth hormone
IGF	Insulin-like growth factor
ECM	Extracellular matrix
RSPO	R-spondin
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
BMP	Bone morphogenetic proteins
MaSC	Mammary stem cell
ASC	Adult stem cell
ESC	Embryonic stem cell
iPSC	Induced pluripotent stem cell
rhEGF	Recombinant human epidermal growth factor
PBS	Phosphate buffer solution
LD	Lactogenic differentiation
EM	Expansion media
LDM	Lactogenic differentiation media
BM	Basal media
qPCR	Quantitative real-time PCR
ELF5	E74-like Transcription Factor 5

Chapter 1 – Introduction

Dairy products are a dietary staple for humans due to their abundant content of essential nutrients. Populations in developed countries, especially in North America, have the highest consumption of dairy products and relatively low prevalence of lactose intolerance (Wang and Li 2008). In 2015, Canadian adults consumed an average of 1.36 servings of dairy products per day. On average, dairy products contributed 11% of daily energy intake, 39% of daily vitamin D, and 53% of daily calcium (Auclair et al 2019). Thus, dairy products are a rich source of essential nutrients for Canadians.

Despite the important contributions of dairy food to human nutrition, milk production has a detrimental impact on the environment. Reports from the Government of Canada estimate that animal production, mainly dairy and beef, represents the largest sector of agricultural emissions, accounting for 3.42% of total national greenhouse gas (GHG) emissions (Environment and Climate Change Canada 2022). GHG emissions from the agriculture sector increased by 33% between 1990 and 2020 (Environment and Climate Change Canada 2022). While GHG emissions per dollar of gross domestic product generated in the agriculture sector has decreased by 50% since 1997, increased demand for animal products has led to a steady increase in total GHG emissions (Environment and Climate Change Canada 2020). One potential strategy to mitigate GHG emissions by the dairy sector is to increase milk productivity, thereby decreasing the number of cows needed to meet the demand. Although advances in genetic selection and nutritional management of dairy cows have been instrumental in driving milk production to its current level, the development of cellular and molecular tools to understand the mechanisms of milk synthesis are needed to fully unleash the lactogenic potential of the bovine mammary gland.

Thus, the establishment of an organotypic model of bovine lactation is urgently needed to maximize the efficiency of dairy production.

In vitro cultures of bovine mammary cells are used to model the mechanisms of lactation. For decades, researchers have relied on 2-dimensional cultures of mammary epithelial cells. However, 2-dimensional models are limited by longevity, genetic drift, and their functional and structural proximity to the *in vivo* bovine mammary gland (Matitashvili et al 1997). In recent years, organoids have emerged as a 3-dimensional method of *in vitro* cell culture. Organoids have a longer lifespan than 2-dimensional cell cultures, maintain *in vivo* cell lineages, and resemble the structure and function of the organ. However, organoids have yet to be applied to the bovine mammary gland. The purpose of this study was to establish and characterize mammary organoids from lactating dairy cows.

Chapter 2 – Literature Review

Nutritional Components of Dairy Products

1. Milk Proteins

Dairy products are a good source of high-quality protein as they contain all nine essential amino acids in appropriate proportions for human nutrition. Milk proteins have a higher ileal digestibility than plant-derived proteins (Mathai et al 2017). Amino acids in milk stimulate muscle development and maintenance, making milk especially valuable in the diets of children and older adults (Arentson-Lantz et al 2015). Thus, research has focused on mechanisms of milk protein production.

Mammary-specific proteins comprise approximately 3.2% of bovine whole milk (*FoodData Central* 2019). These proteins are categorized into two groups: caseins and whey proteins. Caseins represent approximately 80% and whey proteins represent approximately 20% of mammary-specific proteins in bovine milk. Casein exists in four peptide conformations: α_{S1}, α_{S2}, β, and κ. Caseins are present in milk in the form of micelles comprised of an inner layer of α_{S1}, α_{S2}, and β caseins, an outer layer of κ casein, and a core of calcium phosphate that binds the casein molecules within its isoelectric point. Casein micelles are aggregations of thousands of casein molecules and can be up to 500 nm in diameter (Głab and Boratyński 2017). Whey proteins refer to a variety of mammary-specific proteins including β-lactoglobulin and α-lactalbumin. β-Lactoglobulin represents approximately 50% of whey proteins in bovine milk. It is present in milk as a compact globular structure with several ligand binding sites, which has led researchers to hypothesize that it has a role in nutrient transport. α-lactalbumin represents approximately 20% of whey proteins. α-Lactalbumin is a complex folded helical structure with

calcium binding sites. α-Lactalbumin is present in the Golgi apparatus where it interacts with a UDP-galactose to promote lactose synthesis. Minor whey proteins are present in milk in lower concentrations. Immunoglobulins are present primarily in the bovine colostrum, and their role is primarily to transfer the mother's immunity to its calf. Blood serum albumin has no known role in milk, and its presence is suspected to be due to leakage from blood (O'Mahony and Fox 2013). While bovine milk contains a diverse range of mammary-specific proteins, casein expression is commonly used as an indication of functional differentiation of mammary cells due to their responsiveness to lactogenic cues.

Casein is transcribed during lactation through hormonal stimulation of the JAK/STAT pathway. When lactation is initiated, the production of progesterone ceases, allowing prolactin (PRL) to bind to its receptor on the plasma membrane. The activation of the PRL receptor stimulates the homodimerization of Janus tyrosine kinase 2 (JAK2) in the cytoplasm (Akers 2006). This interaction activates the dimerization of the mammary gland transcription factor/signal transducer and activator of transcription (MGF/STAT) isoforms 5a and 5b, which bind to form either homodimers or heterodimers (Figure 1). The MGF/STAT5 complex is transported to the nucleus where it binds to specific binding sites in the casein promoter region to induce transcription. Two MGF/STAT5 binding sites have been identified: the low-affinity sequence ATTTCTTGGGA between -136 and -146, and the high-affinity sequence ACTTCTTGGAATT between -87 and -99 (Groner and Gouilleux 1995).

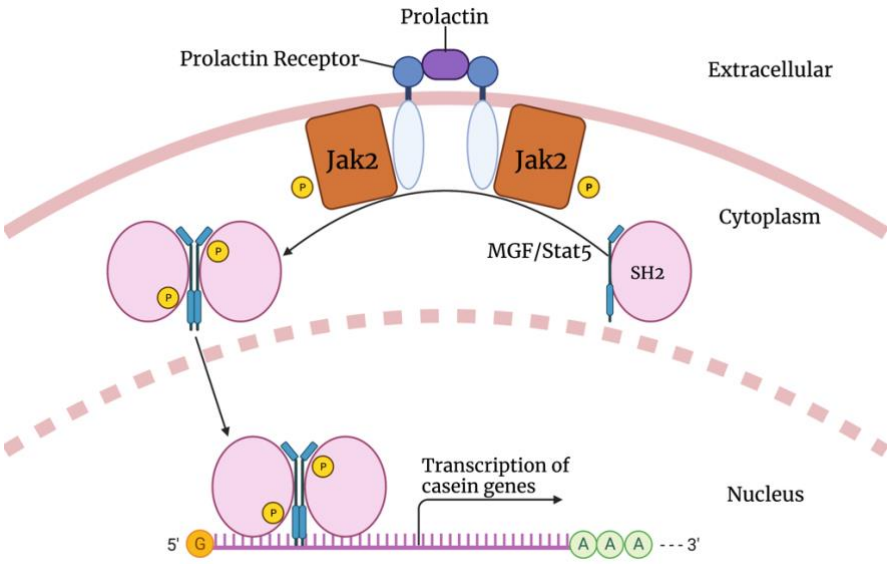

Figure 1. The JAK/STAT pathway induces the transcription of casein genes during lactation. PRL binds to its receptor on the cell surface, inducing homodimerization of JAK2 in the cytoplasm. JAK2 activates dimerization of the MGF/STAT5 complex. The MGF/STAT5 complex translocates to the nucleus where it binds to the casein promoter region to induce casein transcription.

Mammogenesis and Lactogenesis

1. Mammogenesis

It is important to consider the stages of mammary development *in vivo* when developing *in vitro* models of lactation. Mammogenesis begins in the embryo and the mammary gland grows isometrically between birth and the onset of puberty (Hovey et al 2002). Puberty initiates the allometric growth of the mammary ductal system that continues throughout the peripubertal period and pregnancy. The onset of pregnancy initiates the establishment of a more expansive ductal system and the development of alveolar buds (Macias and Hinck 2012). Prior to parturition, stage I lactogenesis initiates the completion of lobulo-alveolar structures, and stage II lactogenesis initiates the induction of pathways that transcribe milk components (Akers 2002).

Embryonic mammogenesis describes the development of the fetal mammary gland prior to parturition. Approximately 30 days into embryonic development, the ventral ectoderm of the bovine fetus thickens around the midline to develop a mammary band. Ectodermal cells around the mammary band proliferate to form the mammary streak. Mesenchymal cells proliferate and compact below the mammary streak, forming the mammary line. The mammary line shortens as the ectoderm grows into the mesenchyme to form the mammary crest. The bovine mammary crest develops into four protruding mammary buds that will develop into the udder. Mammary bud cells proliferate into the mesenchyme to form elongated networks referred to as primary sprouts. Primary sprouts develop into teats and the gland cistern, and secondary sprouts develop from primary sprouts to form a rudimentary ductal network. Fibroblasts and adipocytes proliferate in tandem with the mammary gland to form stromal tissue referred to as the mammary fat pad (Akers 2002). Between the calf's birth and the onset of puberty, the mammary gland undergoes isometric growth (Hovey et al 2002).

The onset of puberty initiates allometric growth of the mammary gland under the influence of reproductive hormones and growth factors. Terminal end buds (TEB) drive the expansion of the ducts. The cap cells of the TEB differentiate to form the outer layer of ductal myoepithelial/basal cells that encase the inner layer of luminal cells. Between puberty and pregnancy, the bovine mammary gland fills with an increasingly complex ductal structure, resulting in a mature mammary gland that can properly develop at the onset of pregnancy (Macias and Hinck 2012).

The stages of mammogenesis are distinguished by changes in the concentration of specific hormones that coordinate physiological adaptations. In prepubertal cattle, estrogen receptors have been detected in numerous lines of functional epithelial cells in the ducts of the

bovine mammary gland. As the mammary gland develops during puberty, mammary epithelial cells undergo conformational changes to permit estrogen to bind to its receptors. Estrogen stimulates the proliferation of mammary epithelial cells, allowing for allometric growth of the ductal system (Hovey et al 2002). This is evidenced by the formation of only a rudimentary ductal tree when the estrogen receptors of the mouse mammary gland are disrupted (Lubahn et al 1993).

Growth hormone (GH) plays an essential role in the proliferation and differentiation of mammary myoepithelial/basal and luminal progenitor cells. GH is first evident in the bovine mammary gland at the onset of puberty and increases in concentrations through adult mammogenesis and pregnancy (Akers 2006). Studies using immunofluorescent labelling show that GH receptors are most concentrated in ductal cells, less concentrated in alveolar cells and non-secretory cells, and absent in regressive mammary cells (Lincoln et al 1995). The increased concentration of GH receptors in the ductal system of the mammary gland suggests its role in mammogenesis is primarily the differentiation and proliferation of ductal cells (Akers 2006, Lincoln et al 1995).

Insulin-like growth factors (IGF) regulate the growth, proliferation, differentiation, and function of the bovine mammary gland (Akers 2002). IGF-I receptors are localized in the extracellular matrix (ECM) of mammary stromal cells, where IGF signaling initiates cell-cell communication. IGF-II receptors are localized on the luminal surface of mammary epithelial cells (Collier et al 1993). Starting at puberty, IGF regulates the development of parenchymal tissue through its induction of cell proliferation (Akers 2006). The increased concentration of GH in the mammary gland during puberty initiates transcription of IGF. The IGF produced in the mammary gland under the influence of GH, as well as the IGF produced in the liver, acts

synergistically with estrogen to enhance outgrowths of the mammary ductal system (Macias and Hinck 2012). As with estrogen, mammary glands with disrupted IGF production have a diminished capacity for ductal morphogenesis, forming only a rudimentary ductal tree with few outgrowths (Richards et al 2004). Although the mammary gland cannot be maintained with only IGF in absence of other mammogenic hormones, it has been determined that it plays an essential role in the maintenance of myoepithelial/basal cells by promoting cellular metabolism (Wood et al 1974). Under the guidance of these hormones, epithelial cells proliferate and differentiate so that the peripubertal mammary gland can grow allometrically until the onset of pregnancy.

Pregnancy initiates rapid development of the ductal and alveolar systems to prepare the mammary gland for lactation. In early pregnancy, secondary and tertiary ducts develop off pre-existing ducts to fill the mammary fat pad. Once the ductal system is established, mammary epithelial cells at the terminal ends of ducts proliferate into alveolar buds. The cells continue to proliferate and differentiate into increasingly distinct alveoli with hollow lumens (Macias and Hinck 2012). The development of the mammary gland during pregnancy are dependent on appropriate hormone exposure.

Pregnancy is regulated by the same hormones as puberty, in addition to progesterone and PRL. Progesterone initiates the development of secondary and tertiary branches in the ductal system. In a study performed in pregnant mice, both estrogen-receptor and progesterone-receptor knockout mice did not develop ductal side-branches or undergo lobuloalveolar development (Lydon et al 2000). The addition of estrogen or progesterone alone cannot induce normal mammary morphogenesis, thus estrogen and progesterone act synergistically to induce ductal elongation and alveolar development. The proposed mechanism of synergy suggests that

estrogen induces cellular proliferation and progesterone drives the differentiation of lobuloalveolar cells (Bocchinfuso et al 2000).

Progesterone and estrogen act synergistically with PRL to induce the development of alveoli at the terminal ends of ducts. If the mammary gland is lacking these hormones or their receptors, the ductal tree is not able to develop alveolar structures suitable for milk production (Schams et al 2003). PRL incorporates signals that lead to the establishment of essential pathways, such as JAK2/STAT5, that regulate the differentiation of alveolar cells and the eventual onset of lactation (Macias and Hinck 2012).

2. Lactogenesis

Lactogenesis is induced in late pregnancy in response to signaling prior to parturition and occurs in two stages. Stage I lactogenesis begins in the final third of pregnancy, before the epithelial cells of the mammary gland are fully developed for milk production. During stage I of lactogenesis, the production of milk is limited due to a high volume of undifferentiated mammary epithelial cells. Prior to differentiation, mammary cells can not produce or secrete milk. Stage I lactogenesis is regulated by progesterone and estrogen (Akers 2006). To initiate this stage, the concentrations of progesterone and estrogen must increase simultaneously. Progesterone and estrogen work synergistically to initiate lobulo-alveolar development necessary to produce copious milk (Tucker 1981). Although the alveoli are structurally well-developed during the first stage of lactogenesis, the functional capacity of the luminal cells are limited, as the cells have not been fully differentiated to their terminal-state (Akers 2002). The structure of epithelial cells and the presence of excess progesterone during early lactation leads to incomplete tight junctions, exhibited by the presence of milk components in the blood prior to cellular maturity (Kessler et al 2019). Additionally, excess progesterone prevents the onset of copious

milk secretion by inhibiting PRL and glucocorticoids from binding to their receptors (Tucker 1981, Akers 2002).

Stage II lactogenesis begins immediately prior to parturition. While the first stage of lactogenesis terminates in the maturity of lobulo-alveolar structures, stage II lactogenesis is marked by the functional completion of the mammary gland. Stage II lactogenesis is initiated by the synthesis and synergistic activity of positive lactogenic hormones PRL, glucocorticoids, estrogen, IGF, as well as the reduction of progesterone levels. The concentration of PRL in the mammary gland increases dramatically through late pregnancy. PRL regulates both the maturation of secretory cells and the synthesis of milk. PRL is associated with the maturation of the Golgi apparatus and the development of vesicles in secretory cells. PRL activates the JAK/STAT5 pathway as described previously. The pathway initiates the synthesis of mRNA encoding casein. The absence or inhibition of PRL in the mammary gland leads to a decrease in caseins and α-lactalbumin. A massive increase in the concentration of glucocorticoids coincides with the event of parturition. Glucocorticoids, specifically cortisol in ruminants, are essential in the maturation of the rough endoplasmic reticulum of mammary epithelial cells. The presence of glucocorticoids is associated with the synthesis of milk proteins casein and α-lactalbumin. Although the exact mechanism of glucocorticoid action is unknown, there is evidence that copious milk production is dependent on both glucocorticoids and PRL. Estrogen is present in the mammary gland throughout pregnancy and reaches its maximum concentration several days before parturition, then rapidly decreases. Estrogen priming of the mammary gland prior to the onset of PRL and glucocorticoids increases the production of milk proteins. However, the continuous presence of estrogen after parturition has been found to decrease milk production. One proposed mechanism of synergy between PRL, glucocorticoids, and estrogen suggests that

estrogen and glucocorticoids increase the number of PRL receptors on mammary epithelial cells (Akers 2002). Conversely, progesterone concentration decreases several days prior to parturition.

As mentioned previously, progesterone inhibits the binding of PRL and glucocorticoids to receptors. Immediately prior to parturition, a decrease in progesterone is necessary for the initiation of milk production by lactogenic hormones (Tucker 1981). In addition to lactogenic hormones, the role of insulin-like growth factors in the onset of lactogenesis has been extensively studied and is highly disputed. There is an increase in the concentration of IGF receptors on mammary epithelial cells in late gestation (Akers 2002), however there is no evidence that IGF has a direct role in the synthesis of milk or milk proteins. *In vivo* studies have elucidated that the increase in IGF receptor concentration is a function of mammary growth regulation via cellular maintenance and proliferation (Collier et al 1993). Cellular and structural integrity must be maintained for the continued production and secretion of milk.

Tissues of the Mature Bovine Mammary Gland

1. Parenchyma

The mature, lactating bovine mammary gland consists of a large stroma with a complex network of primary, secondary, and tertiary ducts (Hovey et al 1999). At the terminal ends of ducts, hollow, milk-producing alveoli cluster to form lobules. Each lobule has a separate lactiferous duct, all of which join in the gland cistern that drains into the teat canal (Figure 2). The teat canal opens upon stimulation by suckling or milking (Alhussien and Dang 2018). The functionality of the mature mammary gland relies on fully differentiated cell types in the appropriate locations.

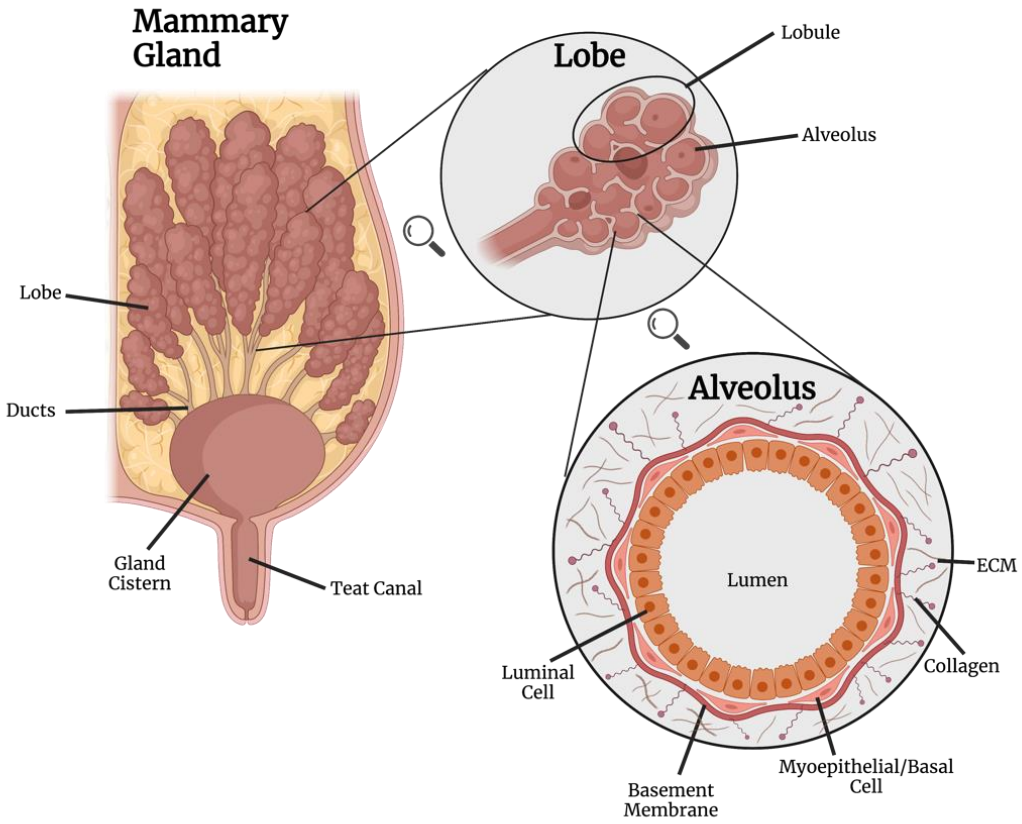

Figure 2. The mature bovine mammary gland consists of functional alveolar units clustered to form lobes. Each lobe leads to a separate lactiferous duct. The ducts culminate in the gland cistern that drains into the teat canal. Each alveolus consists of an outer layer of myoepithelial/basal cells and an inner layer of luminal cells surrounding a hollow lumen. Alveoli are surrounded by ECM components including the basement membrane and several collagens.

The lactating bovine mammary gland has two primary functional cell types: myoepithelial/basal cells and luminal cells. Mammary alveoli and ducts, collectively referred to as the mammary epithelium, are bi-layered structures composed of an outer layer of myoepithelial/basal cells and an inner layer of luminal cells surrounding a hollow lumen. The primary function of alveolar luminal cells is to produce milk (Macias and Hinck 2012). The

primary function of the outer layer of myoepithelial/basal cells is to contract upon stimulation during suckling. When the teat canal is stimulated, the pituitary gland releases oxytocin, which binds to receptors on the myoepithelial/basal cells causing them to contract around the inner layer of luminal cells. The contraction forces the milk produced within the alveoli through the lactiferous ducts, into the gland cistern, and finally into the teat canal (Sumbal et al 2020).

Both luminal and myoepithelial/basal cells are generated from mammary stem cells (MaSC). Studies in the mammary glands of mice have confirmed that MaSCs are concentrated in the TEB that drives morphogenic development. In the presence of regulatory hormone IGF, the number of cells expressing MaSC markers $CD24^{+}$ and $CD49^{hi}$ increased significantly in the TEB niche in both pre- and post-pubertal mice (Luo et al 2021). Cell lineage tracing using surface markers has elucidated the differentiative cascade of MaSCs (Figure 3). When MaSCs proliferate, they either self-renew or differentiate into progenitor cells. Bovine studies found that progenitor cells with surface markers $CD24^{+}$ and $CD49^{low}$, often referred to as "common progenitor cells", are multipotent and can differentiate into both luminal-committed progenitor cells and myoepithelial/basal-committed progenitor cells (Finot et al 2019). Multipotent luminal-committed progenitor cells with surface markers $CD24^{hi}$ and $CD49^{low}$ differentiate progressively until they reach a terminally differentiated state. Terminally differentiated luminal cells include ductal, alveolar, and secretory luminal cells with surface markers $CD24^{med}$ and $CD49^{low/neg}$. Myoepithelial/basal-committed progenitor cells expressing $CD24^{neg}$ and $CD49^{hi}$ are unipotent and differentiate into only terminally differentiated myoepithelial/basal cells (Rauner and Barash 2016). Although the stem cell differentiative hierarchy has been extensively researched, differences in progenitor cell differentiation among species and developmental stages remain disputed.

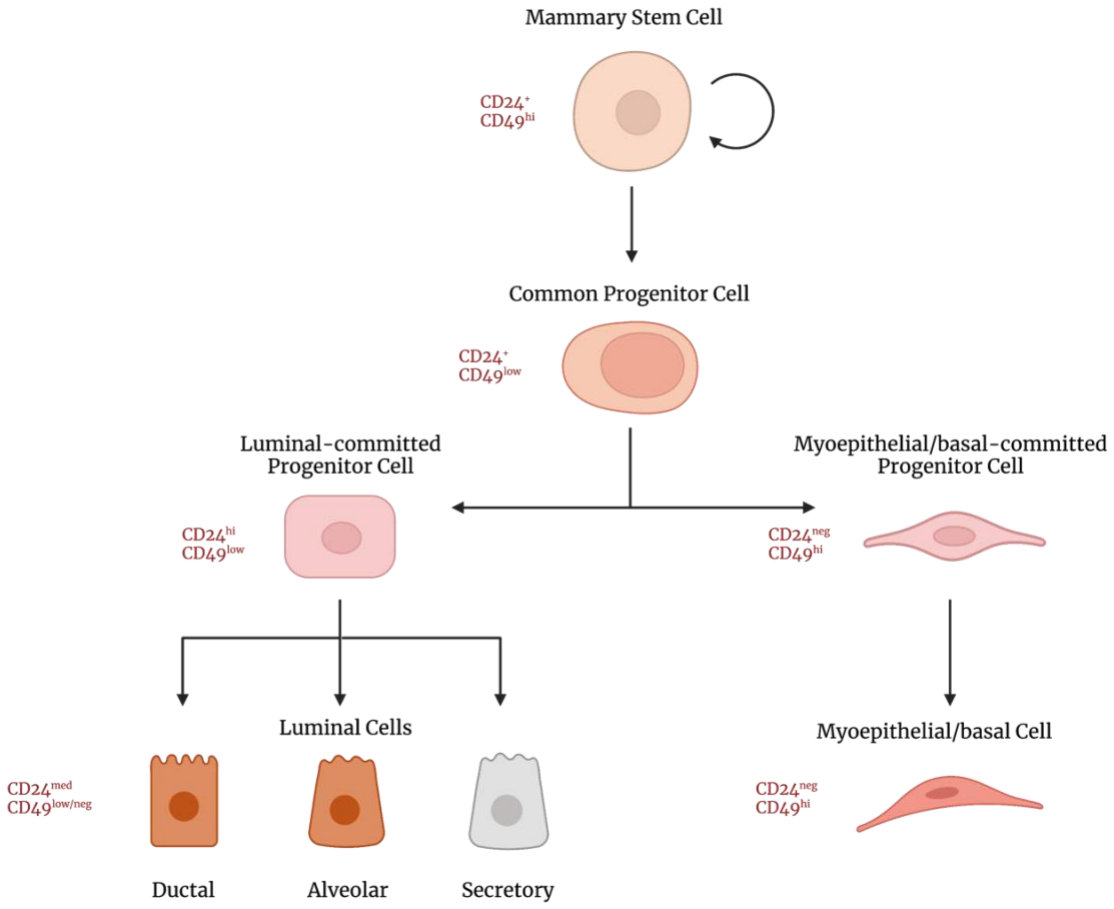

Figure 3. The proposed differentiative hierarchy of mammary stem cells and surface markers for cell types (left, in red). MaSCs can self-renew or differentiate into common progenitor cells. Common progenitor cells differentiate into either luminal-committed progenitor cells or myoepithelial/basal-committed progenitor cells. Luminal-committed progenitor cells are multipotent and differentiate into ductal, alveolar, and secretory luminal cells. Myoepithelial/basal-committed progenitor cells are unipotent and differentiate into myoepithelial/basal cells.

Research in bovine MaSCs suggests that progenitor cells are more highly concentrated in the pubertal mammary gland than MaSCs, and their proportion increases throughout mammogenesis until lactogenesis (Finot et al 2019). Researchers found that common progenitor cells have a greater proliferative capacity than MaSCs, thus their high concentration starting at puberty is essential in allometric growth of the mammary gland and ductal branching (Capuco et

al 2012). However, the presence of common progenitor cells in the bovine mammary gland is uncertain. Studies using prepubertal bovine MaSCs found no evidence of common progenitor cells, instead finding that MaSCs differentiate directly into either multipotent luminal-committed progenitor cells or unipotent myoepithelial/basal-committed progenitor cells (Rauner and Barash 2016). Further research is necessary to elucidate the bovine MaSC differentiative hierarchy through all stages of mammogenesis.

2. Stroma

The bovine parenchyma is surrounded by supportive stromal tissue composed of non-secretory and non-ductal tissue in the udder. Stromal tissue is derived from the mesenchyme and includes connective tissue, adipose tissue, nerve tissue, and blood vessels (Akers 2002). Two distinguishable layers of stroma surround the bovine mammary parenchyma. A fibrous layer consisting primarily of fibroblasts surrounds the parenchyma, and a fatty layer consisting primarily of adipocytes surrounds the fibrous layer. The fibrous layer supports ductal elongation into the stroma during mammary gland development. Fibroblasts in stromal tissue express proteins encoding the *Wnt* pathway, indicating that fibrous stromal tissue may have a role in the induction of ductal morphogenesis. Both fibrous and fatty stromal tissue in the bovine mammary gland express genes encoding ECM proteins, which has led some researchers to conclude that the stroma is integral to the development of the ECM (Kosenko et al 2022).

3. Extracellular Matrix

The expansion and structural formation of mammary epithelial cells is aided by ECM components. A thin layer of ECM referred to as the basement membrane surrounds mammary epithelial cells to form a barrier between the mammary gland and surrounding stromal tissue. The ECM adheres to specific regions of mammary epithelial cells to maintain cell structure and

normal cellular function. ECM proteins laminin, collagens I and IV, and heparan sulfate interact with mammary stem cells to assist in cellular differentiation. ECM proteins are produced by mammary epithelial cells and are regulated by exogenous matrix proteins (Matitashvili and Bauman 2001). Due to its role in the regulation of mammary cells *in vivo,* researchers have focused on incorporating ECM components in *in vitro* cultures to elucidate their role in mammary gland function.

Tissue Culture Models of the Ruminant Mammary Gland

1. Mammary Explants

Explant cultures are used for *in vitro* studies of whole tissue slices or biopsies. In this procedure, mammary tissue is minced (typically 1 mm^3) or sliced and connective tissue is manually removed to the highest degree possible. The explants are then placed on a floating paper or suspended in a dish filled with media (Topper et al 1975)

Using this approach, researchers found that mammary explants relied on the presence of mammogenic hormones found *in vivo* for the survival of lobulo-alveolar structures and cells developed in late pregnancy (Elias 1957). A comprehensive methodology for mammary explant culturing was published nearly two decades later (Topper et al 1975), however it was only in 1977 that bovine mammary explants were successfully cultured. When bovine mammary explants were cultured for 48 hours with media containing lactogenic hormones insulin, PRL, and cortisol, the lumen of the explant alveoli expanded with milk proteins and lipids synthesized within the alveoli (Collier et al 1977).

Explant studies have been integral in understanding the importance of hormones and growth factors in the synthesis of milk components. Lactogenic studies in bovine mammary

explants found that the presence of insulin was essential in the survival of explants due to its role in cellular maintenance and metabolism (Collier et al 1977). However, the study of whole tissue leads to several challenges. The presence of multiple cell types causes difficulty in determining the specific cellular target of hormones and growth factors. The variable cellular composition among explants is a source of biological variability. Additionally, the presence of endogenous growth factors and hormones can pose challenges due to carryover effects. Moreover, explants cannot be sustained in culture for long periods (Matitashvili et al 1997).

2. Acini

Acini are basic functional units of the mammary gland. To isolate bovine mammary acini, minced mammary tissue is enzymatically digested with collagenase and mechanically dissociated. The resulting sample is centrifuged at low speed to separate the heavier acini from acinar fragments and single cells. Acini are suspended in growth media and plated on a collagen-coated dish, where they take root in collagen. Within 1-2 days after the plating of acini, cells proliferate into outgrowths of 2-dimensional cells spread around the original acini. After 6-10 days in culture, the original acini reorganize to join the 2-dimensional outgrowth, forming a continuous sheet of 2-dimensional cells (Talhouk et al 1990).

Researchers found significant increases in the metabolic rate of lipid precursors in the mammary acini of rats, leading to greater lipid production in acini than in explants. The relative efficiency of metabolism in acini may be due to the lack of stromal cell types present in explants (Katz et al 1974). Later studies confirmed that mammary acini could be isolated from the bovine mammary gland and used to model the response to lactogenic hormones. The synthesis of caseins and β-lactoglobulin can be maintained in culture for 14 days (Talhouk et al 1990).

Bovine mammary acini are a more reliable method of *in vitro* studies than explants. The cell types present in acini are more consistent due to the more rigorous isolation procedure. This decreases the risk of population drift towards stromal cell types. Additionally, the increased metabolism and protein synthesis of acini exhibits an increased functional capacity and a closer proximity to the behaviour of mammary alveoli (Katz et al 1974, Park et al 1979). However, research in rats found that the method of acini isolation dissolves the basal membrane, greatly reducing the presence of myoepithelial/basal cells in acini cultures (Katz et al 1974). The lack of myoepithelial/basal cells limits the culture's functional proximity to *in vivo* mammary glands. As explants, acini cultures have limited long-term culture potential. Acini cultures only maintain the capacity to synthesize lactogenic proteins for 14 days, limiting the ability to research the long-term effects of mammogenic and lactogenic factors (Talhouk et al 1990).

3. Primary Cell Culture

Primary cell cultures are established from a suspension of isolated single cells seeded on a substratum. Mammary cell cultures consist of single mammary epithelial cells isolated from a pregnant or lactating cow. The culture technique has been used extensively in the research of mammogenic and lactogenic hormones, growth factors, cellular differentiation and proliferation, and the effects of different substratum. Both myoepithelial/basal and luminal progenitor cells are present in culture. Cells that enter the culture in their terminally differentiated state are non-proliferative, therefore progenitor cells quickly become the dominant cell-type. As progenitor cells are not committed to a lactogenic cell type, primary cell cultures have provided valuable information regarding the induction of lactogenic cells and milk protein synthesis (Matitashvili et al 1997). Milk proteins, especially caseins, are synthesized in primary cell cultures when supplemented with lactogenic hormones (Talhouk et al 1998, Matitashvili et

al 1997). However, synthesis of casein proteins was observed for a maximum of 14 days after seeding the cells, and α-S1 casein was only synthesized for four days (Talhouk et al 1998).

To combat the longevity limit of primary cultures, cells can be passaged. While a primary cell culture can be maintained for only 14 days (Talhouk et al 1998), repeated passaging of cells can increase the lifespan of the culture to at least eight weeks. It has been established that bovine mammary cells maintain the capacity to synthesize lactogenic proteins, including α-S1, α-S2, β, and κ caseins for up to three passages after primary culture. Interestingly, α-S1 and κ caseins were not expressed in passage 1, however the expression of all caseins was restored in passages 2 and 3 (Jedrzejczak and Skatkowska 2014). Although passaging increases the lifespan of the culture, a limited number of passages can be performed before cells undergo genetic drift and the cellular makeup of the culture is no longer representative of cell types *in vivo.* After more than 3 passages, cellular viability and proliferative potential decreases, preventing cultures from reaching confluency and expressing detectable quantities of milk proteins (Matitashvili et al 1997).

The substratum of a cell culture impacts cell morphology, differentiation, growth patterns and protein expression. Common substrata include tissue-grade plastic plates, collagen, and isolated ECM or ECM components. Matrigel, also referred to as EHS-matrix or Cultrex, is a commercially available alternative to isolated ECM. Matrigel contains ECM components such as laminins, collagen, and proteoglycans (Freshney 2016). In a comparison of primary cell cultures from a single cow grown on these substrata, notable differences were observed between the growth behaviours of cells. In cultures grown on plastic or a collagen-coated plate, large epithelial cells and fibroblasts grew in a monolayer. After 10 days, cells reached 4 layers of confluency. In cultures grown on Matrigel or with cells embedded in collagen, cells grew in 3-

dimensional, acini-like structures. The structure did not display any cellular organization or alveolar structures (Talhouk et al 1998). A study in bovine mammary epithelial cells showed that in the presence of ECM, bovine mammary gland progenitor cells differentiate into a greater diversity of cellular lineages compared to the same cells grown in cultures without ECM components (Holland et al 2007). In a study performed in the mammary epithelial cells of mice, it was discovered that the presence of isolated laminin promotes cellular differentiation into more varied cell types. Both Matrigel and purified laminin dramatically upregulate the expression of α, β, and κ caseins compared to cells cultured on plastic (Streuli et al 1995, Holland et al 2007). The variety of culture substrata allows for more diverse research directions than previously described methods.

Primary cell cultures are the most versatile and longest surviving of the established methodologies of *in vitro* mammary research. Cell cultures can be used to effectively study the effects of growth factors, hormones, and cellular components on cell morphology, differentiation, and protein synthesis. However, the impact of cell culture studies is limited by two-dimensionality, the presence of contaminating cells, and the limited passages possible before cells begin to lose their characteristics (Matitashvili et al 1997). The varied protein expression depending on the passage of the culture limits the consistency of long-term studies (Jedrzejczak and Skatkowska 2014). Another limitation of cell cultures is the lack of proximity to *in vivo* structures and cellular arrangement, which restricts the ability of cultures to accurately represent the behaviours of the mammary gland (Talhouk et al 1998).

4. Continuous Mammary Cell Lines

When freshly isolated bovine mammary cells are inaccessible, continuous cell lines are commercially available. The most common and best-established bovine mammary cell line is

MAC-T. MAC-T cells were first established and characterized in 1991 by Huynh and colleagues (Huynh et al 1991). To establish the MAC-T cell line, mammary tissue was collected from a lactating cow, and mammary epithelial cells were isolated and cultured on a collagen-coated plate as a primary cell culture. The cells were transfected with the SV40 antigen, which acts to immortalize the cells (Huynh et al 1991). After the establishment of the MAC-T cell line, other immortalized cell lines, such as HH2a, were established to offer greater variability in baseline gene expression (Huynh and Pollak 1995). Continuous bovine mammary cell lines are an important tool in long-term effect studies and characterization of the role of growth factors and hormones. Because continuous cell lines have already been established and characterized, researchers can save time on preliminary studies to establish baseline protein expression. However, immortalized cell lines are limited due to heterogeneity in the population (Matitashvili et al 1997). Researchers have identified three subtypes of the MAC-T cell line that differ in morphology, growth properties, and differentiative capacity. One subtype of the MAC-T cell line was terminally differentiated, thus proliferative and differentiative capacities of these cells were limited (Zavizion et al 1995). Additionally, MAC-T cells do not proliferate in response to EGF, despite its role in bovine mammary cell growth and function (Matitashvili et al 1997). Thus, cultures of immortalized cell lines are limited in their functional resemblance to the *in vivo* mammary gland.

Organoids

1. Overview of Organoids

Organoids are a relatively recent development in *in vitro* modeling systems. Organoids are established from a suspension of single stem cells cultured in a 3-dimensional gel containing ECM components. When supplied with a combination of growth factors, hormones, and

nutrients, the stem cells proliferate and differentiate into a variety of *in vivo* cell types that self-organize into a 3-dimensional structure resembling an organ. In addition to structural resemblance, the genotype of the original donor tissue is maintained, allowing for reliable experimentation that closely resembles donor-specific *in vivo* behaviours. Organoids have been established from stem cells derived from a wide variety of organs and species, however human and mouse organoids have been studied most thoroughly (Dutta et al 2017).

For over a century, researchers have been interested in developing a 3-dimensional culture model to recapitulate the mechanisms of organogenesis. In the early 20th century, researchers found that when tissue fragments were cultured in a hollow slide permitting 3-dimensional growth, the tissue continued to grow into the medium for extended periods of time (Harrison 1906). This method was applied to single cells, which regenerated tissue-like structures when suspended in a 3-dimensional substrate (Wilson 1907). Later studies found that single cells cultured on floating collagen gels had a greater capacity for differentiation and more sustained protein expression than cells grown in 2-dimensional culture (Emerman and Pitelka 1977). The development of commercially available ECM gel allowed researchers to explore the roles of ECM components in cellular proliferation, differentiation, and protein expression in a 3-dimensional environment (Li et al 1987). While the definition of what qualifies as an organoid has varied through time and literature, modern definitions have included the use of an ECM gel as a criterion of organoid culture as it recapitulates the *in vivo* microenvironment. Because of the structural and functional similarity of organoid culture models to *in vivo* conditions, organoid research has coincided with research on the mechanisms and differentiative capacity of stem cells (Simian and Bissell 2016).

Organoids can be established from either pluripotent stem cells or organ-specific adult stem cells (ASCs). Pluripotent stem cells are characterized by their capacity to self-renew and differentiate into nearly any cell type. They are divided into two categories: embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs are found in the blastocyst of the early embryo of all mammalian species. IPSCs are pluripotent stem cells generated from adult somatic cells that are reprogrammed to the state of an ESC. ESCs and IPSCs are not committed to a specific organ, thus they are most useful in research pertaining to embryonic organogenesis (Dutta et al 2017).

ASCs are derived from the tissue of a non-embryonic animal. ASCs are characterized by their capacity to self-renew and differentiate into organ-specific cell types (Dutta et al 2017). When seeded in a 3-dimensional gel and supplied with growth factors that mimic the *in vivo* environment, ASCs differentiate into all the functional cell types found in the organ of origin. These cells come together to form 3-dimensional organoids resembling the organ's *in vivo* structure. Lgr5 is a marker of ASCs derived from all known organs. Lgr5 is a receptor and a product of the *Wnt* pathway involved in tissue regeneration (Clevers et al 2014). The presence of Lgr5 in stem cells was modelled in organoids established from the intestinal ASCs of mice. A single $Lgr5^{+}$ cell was plated in each dome of ECM gel, and the cultures were supplied with intestinal growth factors. Within four days, the gels contained structures resembling crypt-villi, structures characteristic of the intestine, comprised of approximately 100 cells. The rate of cellular proliferation seen in the intestine was maintained in the organoid culture. Four epithelial cell types found in the intestine were identified in the organoid structures, and all stromal cell types were absent. This study confirms that $Lgr5^{+}$ cells have the capacity to proliferate, differentiate, and self-organize into 3-dimensional structures in the absence of all other cell

types. Organoid cultures established from intestinal ASCs maintain functionality for up 14 months when passaged every two weeks (Sato et al 2009). These methods have been applied to establish organoids modelling a variety of organs from several species, including the human and mouse mammary gland.

2. Mammary Organoids

Mammary organoids have been established from the mammary epithelial cells of humans and mice. When human mammary epithelial cells were seeded in floating collagen gels and provided with media containing appropriate growth factors, mature luminal, luminal progenitor, and myoepithelial/basal progenitor cells generated branched 3-dimensional mammary organoids (Linnemann et al 2015). Further studies found that freshly isolated human mammary epithelial cells separated by cell type and seeded in 3-dimensional ECM gel established organoids of different structures. Mature luminal cells formed spherical, acini-like structures with a hollow lumen. Luminal progenitor cells formed smaller spherical organoids with smaller lumens. Myoepithelial/basal progenitor cells formed complex, disorganized structures with spherical outgrowths, potentially mimicking luminal budding (Rosenbluth et al 2020). An experiment performed with freshly isolated mouse mammary epithelial cells seeded in ECM gel found that both luminal and myoepithelial/basal progenitor cells gave rise to organoid colonies and complex budding structures (Jamieson et al 2017). Similar structures were observed when freshly isolated mouse mammary acini were seeded in ECM gel (Sumbal et al 2020). Studies performed on mouse mammary organoids found a correlation between the presence of fibroblast growth factors (FGF) in the culture and the formation of budding or branching structures (Jamieson et al 2017, Sumbal et al 2020). The addition of the ROCK inhibitor Y27632 for the first three days of culture increased organoid budding formations (Jamieson et al 2017). The structural resemblance

of mammary organoids to the *in vivo* structure allows researchers to further explore mechanisms of mammary cellular function.

Mammary organoid research has elucidated the differentiative potential of mammary epithelial cells. Studies have examined the ability of different cell types to arise in cultures established from an isolated cell type. These studies revealed that both luminal and myoepithelial/basal progenitor cells can generate organoids with both types of progenitor cells and mature luminal cells. The presence of the three cell types was confirmed in both humans (Linnemann et al 2015, Rosenbluth et al 2020) and mice (Jamieson et al 2017, Sumbal et al 2018) via staining of established cell surface markers. Epithelial cell types were determined to be in the appropriate locations according to *in vivo* morphology of mammary alveoli. The mentioned studies found that myoepithelial/basal cell lineages were located on the basal side and luminal cell lineages lined the luminal side of the organoids. The cells maintained their original phenotypes, with elongated myoepithelial/basal cells surrounding spheres, buds, and branches of cuboidal luminal cells (Jamieson et al 2017).

Research suggests that the efficiency of organoid formation and maintenance depends on both the original cell type and the presence of certain growth factors. One study found that human mammary organoids are established more efficiently from progenitor cell types than mature cell types. Myoepithelial/basal and luminal progenitor cells established organoids 11× and 5× more efficiently, respectively, than mature luminal cells. This is consistent with the increased proliferative and differentiative potential of progenitor cells. However, these results seem to be dependent on the presence of both luminal and myoepithelial/basal cell types in the original culture (Dekkers et al 2019). When luminal and myoepithelial/basal cells were separated prior to plating, isolated luminal progenitor cells were 12× more efficient and mature luminal

cells were 7× more efficient at organoid formation than myoepithelial/basal progenitor cells (Rosenbluth et al 2020). A study performed with the mammary cells of mice found that both luminal and myoepithelial/basal progenitor cells generated organoids with equal efficiency. When the cell types were seeded together, 37% of cells of both lineages generated an organoid. The presence of FGF in the culture increased the formation efficiency of myoepithelial/basal-derived organoids and was essential for the formation of luminal-derived organoids (Jamieson et al 2017). Although organoids can be generated from the freshly isolated mammary cells of both humans and mice, methods have been established to increase the efficiency of organoid establishment.

Organoid formation efficiency was increased when cells were plated in 2-dimensional culture prior to seeding them in 3-dimensional culture. About 50% of freshly isolated mammary epithelial cells are viable in culture. Non-viable cells do not proliferate in culture and are eliminated due to single-cell apoptosis. By culturing cells in 2-dimensional culture first, all non-viable cells are filtered out. A study performed in human mammary epithelial cells found that 2-dimensional culturing increased the formation efficiency of branched structures by up to 12× and spherical structures by up to 4× (Linnemann et al 2015). Increasing the efficiency of organoid formation allows researchers to optimize productivity.

Mammary organoids address the longevity limitation of previous culture methods. Human mammary organoids can be passaged more than 20 times (Sachs et al 2018) and survive for up to 16 months (Rosenbluth et al 2020) without losing regeneration efficiency. Mouse mammary organoids can be passaged four times and survive for up to four weeks (Jamieson et al 2017). The increased longevity of organoid cultures permits more extensive long-term studies with closer proximity to *in vivo* cellular function.

One of the most pertinent functions of mammary organoids is the ability to express proteins more similarly to *in vivo* tissues than previous culture models. One study used β casein and κ casein as markers for milk synthesis in organoids established from pre- and post-pregnant mice. They found a 300-fold increase in β casein mRNA and a 48-fold increase in κ casein mRNA in pre-pregnancy organoids supplemented with pregnancy hormones, compared to organoids fed with only essential media. A further increase in β casein mRNA was detected in the organoids of post-pregnant mice. The expression of casein mRNA was detected within six hours of hormone induction (Ciccone et al 2020). Another study found that pretreatment of mouse mammary organoids with FGF2 prior to the addition of lactogenic hormones further increased β casein production. FGF2 stimulates mammary epithelial cell proliferation and increases the presence of branching structures conducive to milk synthesis. Immunohistochemistry confirmed that β-casein was produced in the luminal space of the organoids, consistent with *in vivo* lactogenesis. Additionally, they found that lipid droplets were produced in the intraluminal space and secreted with contraction. Contraction was induced by the addition of oxytocin to the media, and time-lapse photography confirmed that myoepithelial/basal cells contracted around the lumen, consistent with *in vivo* cell behaviour (Sumbal et al 2020). The capacity of human and mouse mammary organoids to establish organized structures that accurately model *in vivo* systems provides a versatile culture model for lactation studies.

Understanding the mechanisms of bovine mammogenesis and lactogenesis is critical in the development of new methods of lactation research. Researchers have used 2-dimensional cellular models to elucidate cellular interactions throughout mammary gland development and lactation, which has shed light on the factors that influence the synthesis of milk-specific

proteins. However, some of the mechanisms underlying milk synthesis remain unknown, possibly due to limitations in existing cellular models. The development of 3-dimensional models of organoids has addressed some of these limitations. Organoids have a longer lifespan, a greater potential for cellular differentiation, and more diverse structural organization than 2-dimensional cellular models. Research in organoids from the mammary cells of humans and mice has confirmed that cell lineages, structures resembling the *in vivo* mammary gland, and expression of milk-specific proteins can be maintained *in vitro* for extended periods. However, the absence of research in bovine mammary organoids limits the implications of organoid research in dairy sciences. Therefore, the objective of this study is to establish and characterize organoids from the mammary cells of lactating dairy cows.

Chapter 3 – Methodology and Results

Materials and Methods

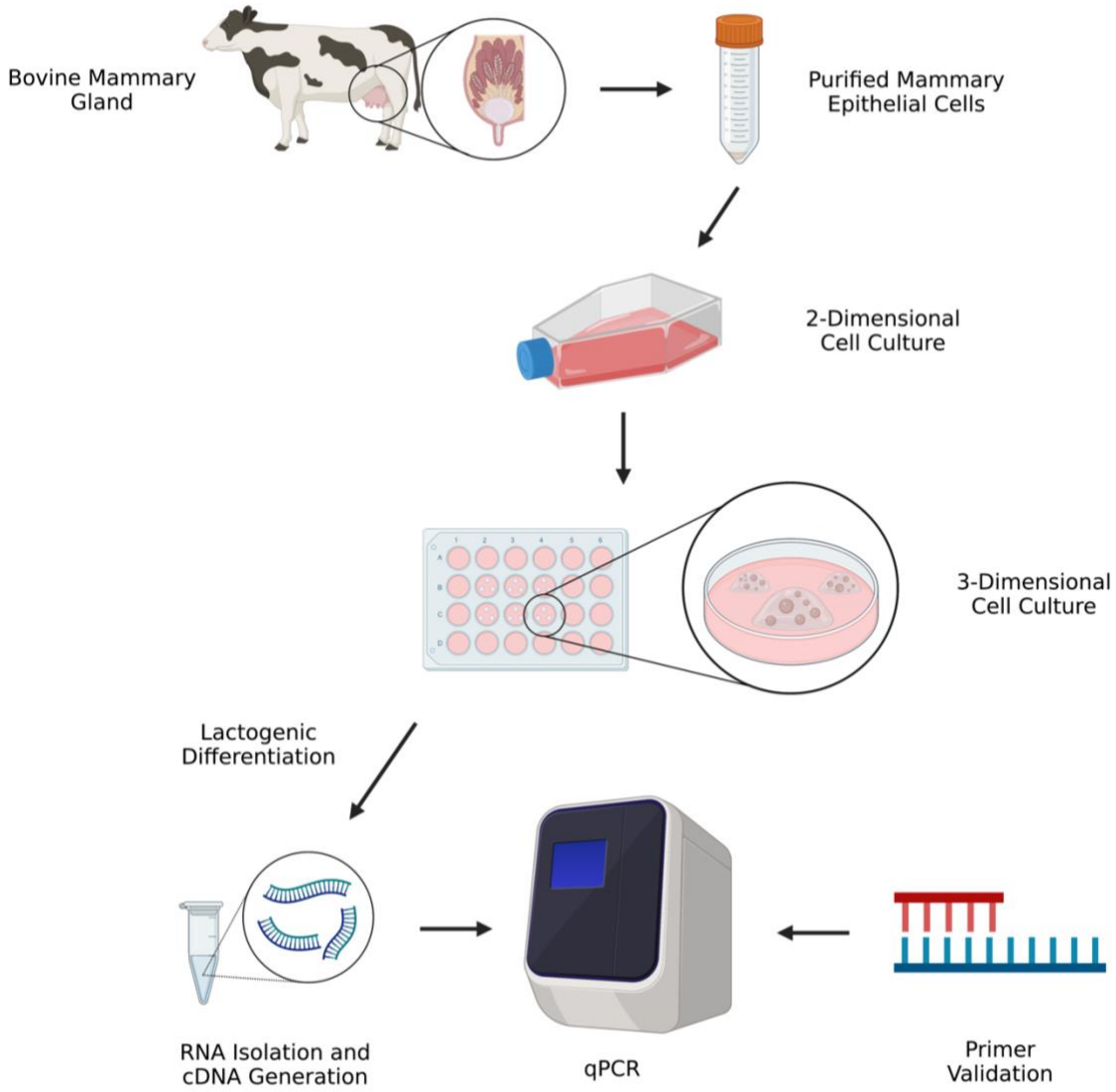

Figure 4. A graphical schematic of the research methods. Epithelial cells were isolated from the bovine mammary gland. The cells were grown in 2-dimensional culture and then plated in 3-dimensional culture as organoids. Lactogenic differentiation was induced with lactogenic hormones. RNA was isolated from organoids and cDNA was generated for qPCR analysis using validated primers.

1. Isolation and Culture of Primary Bovine Mammary Epithelial Cells

Mammary glands from three Holstein cows in late lactation (MCF2040, MCF5871, and MCF8500) culled for reproductive reasons from the Macdonald Campus Dairy Farm were collected upon slaughter at a local abattoir. Immediately after, mammary epithelial cells were harvested and plated according to the protocol outlined in Huang et al 2020. Briefly, mammary tissue was aseptically sliced from the mammary gland and placed in Hanks' balanced salt solution, then transported to the laboratory on ice. In the biosafety cabinet, visible adipose and connective tissue, as well as blood vessels, were excised. The remaining tissue was minced with scalpels to ~1 mm^3 pieces. Blood and milk were removed through repeated rinsing with Ham's F12 media supplemented with 1× antibiotics/antimycotics. The tissue was digested in Dulbecco's Modified Eagle Medium/F12 supplemented with 300 U/mL type-3 collagenase, 400 U/mL hyaluronidase, and 1 mg/mL DNase I supplemented with 1× antimycotic/antibiotic and enzymatically digested for 16 hours at 37°C with constant shaking at 80 rpm. The digested tissue was filtered through a 200-μm mesh sieve into a 50 mL centrifuge tube and centrifuged at 80×*g* for 30 seconds. The remaining pellet consisted of mammary acini composed of mammary epithelial cells. The mammary acini were suspended in M87A-X growth media modified from Garbe et al 2009. The media was composed of 1:1 (v/v) Mammary Epithelial Basal Medium MCDB170 (US Biological) and Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 supplemented with 0.25% fetal bovine serum, 0.1% Albumax II, 7.5 μg/mL insulin solution from bovine pancreas, 0.3 μg/mL hydrocortisone, 5 ng/mL EGF, 2.5 μg/mL apo-transferrin, 5 μM isroproterenol, 5 pM triiodothyronine, 0.5 pM β-estradiol, 0.1 nM oxytocin, 1× antimyotic-

antibiotic, and 50 μg/mL gentamicin. Acini were seeded on a collagen-coated T-175 flask. M87A-X was replaced every 3-4 days. Once cells reached 70-80% confluency, media was aspirated, and the flask was rinsed with 15 mL phosphate-buffered saline (PBS). 15 mL TrypLE was added, and the flask was incubated at 37°C until all cells had dissociated from the flask. The cell suspension was placed in a 50 mL centrifuge tube and the flask was rinsed with 5 mL PBS which was added to the tube. The cell suspension was centrifuged at 500×*g* for 5 minutes. The remaining pellet was resuspended in M87A-X and subcultured or cryopreserved. The cell culture reagents were from ThermoFisher Scientific or Sigma-Aldrich unless otherwise stated.

2. Bovine Mammary Organoid Culture

Bovine mammary organoids were established from cells harvested from 2-dimensional culture. Cells were seeded for organoid establishment as described by Dekkers et al 2021. Briefly, cells were lifted from flasks and pelleted as described for passaging. A portion of the cell suspension was used to count viable cells using a hematocytometer. Cells were centrifuged at 500×*g* for 5 minutes and the remaining cell pellet was resuspended in ice-cold Cultrex RGF Basement Membrane Extract Type 2 (R&D Systems) at a density of ~2,500 cells/μL. Three 40 μL drops were placed at equal distances in the middle wells of ultra-low attachment 24-well plate (Sigma-Aldrich). The plate was immediately inverted and incubated at 37°C for 30 minutes to prevent gel flattening. Upon gelation, 500 μL bovine mammary organoid expansion media (EM) was added to each well. The media was composed of 1× Advanced Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 supplemented with 10 mM HEPES solution pH 7.0-7.6, 10 mM nicotinamide, 1.25 mM N-acetyl-L-cysteine, 1 μM SB202190, 0.5 μM A83-01, 5 μM Y-27632, 5 ng/mL recombinant human EGF (rhEGF), 5 nM rhHeregulin β-1, 5 ng/mL rhFGF-7 (PeproTech), 20 mg/mL rhFGF-10 (PeproTech), 100 ng/mL Noggin (PeproTech), 10% rhR-

Spondin-1 Conditioned Media, 1× B27 supplement, 1× GlutaMAX, and 100 μg/mL Primocin (InvivoGen). The plate was incubated at 37°C and media was aspirated and replenished every 3-4 days. Organoids were passaged every 7 days. For passaging, 500 μL of ice-cold TrypLE was added to each well and forcefully pipetted at the gels 10× to dissociate the gel from the plate. TrypLE was pipetted up and down 10× to initiate organoid dissociation. The plate was incubated at 37°C and pipetting was repeated every 5-10 minutes until organoids had dissociated into a single-cell suspension. The cell suspension was pipetted into a 15 mL centrifuge tube and the plate was rinsed 2× with ice-cold PBS, which was added to the centrifuge tube. The cells were pelleted and reseeded as described. The organoid culture reagents were from ThermoFisher Scientific or Sigma-Aldrich unless otherwise stated.

3. Treatment and Harvesting of Organoids

To determine the capacity of bovine mammary organoids to produce milk proteins, organoids were incubated in lactogenic differentiation media (LDM). LDM was composed of 1× Advanced Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 medium supplemented with 10 mM HEPES solution pH 7.0-7.6, 1× GlutaMAX, 1 μg/mL insulin solution from bovine pancreas, 1 μg/mL bovine prolactin (a gift from A.F. Parlow, National Hormone & Peptide Program, UCLA), 1 μg/mL hydrocortisone, and 100 μg/mL primocin (InvivoGen). Basal media (BM) without lactogenic hormones was used as a control. Media was replenished every 2 days. Organoids were harvested on day 4 of treatment for gene expression analyses. The treatment media was composed of reagents from ThermoFisher Scientific or Sigma-Aldrich unless otherwise stated.

4. Image Acquisition

Organoids were imaged using phase-contrast microscopy on an EVOS® Digital

Microscope (ThermoFisher Scientific) on days 0, 2, 6 and 7 of each passage and on days 0 and 4 of BM and LDM treatment. Images were downloaded for ImageJ analysis.

5. ImageJ Analysis of Organoids

ImageJ software (Rasband 1997) was used to analyze phase-contrast images. Images were batch-processed to crop all images to the same size. Cropping was performed to limit interference of shadows at edges. The "Find Edges" function was used to convert organoid edges to white and the background to black. A calibrated scale was added to the image before the "Color Threshold" function was used to highlight the edges of organoids. "Analyze Particles" was set to a threshold particle size of 1,500 μm^2, holes were included, but particles on edges were excluded to eliminate partial organoids and shadow interference. Average organoid size and number of organoids was calculated and exported to Microsoft Excel (2022). Due to the analysis of a single 2-dimensional image, organoid size is reported as the 2-dimensional area covered by the organoid. The Pearson correlation coefficient between average organoid size and number of organoids throughout passages was calculated in Microsoft Excel (2022).

6. Isolation of RNA from Organoids, RNA Integrity, and cDNA Synthesis

Organoids were harvested in 1 mL of ice-cold TRIzol reagent (ThermoFisher Scientific) to each well and pipetted 10× to dissociate gels from the plate and incubated on ice to initiate dissociation of cells. The TRIzol suspension from each well was then pipetted into a separate, labelled RNAse-free microcentrifuge tube. RNA was isolated according to manufacturers' instructions (TRIzol Reagent User Guide 2020). Once RNA was isolated and solubilized in nuclease-free water, RNA concentration was quantified through absorbance at 260 and 280 nm using a Take3 micro-volume plate in an Epoch microplate spectrophotometer using the Gen5 software (BioTek, Winooski, VT). RNA was diluted with nuclease-free water to a final

concentration of 250 ng/μL. RNA integrity was assessed using a 0.7% agarose bleach gel electrophoresis (Aranda et al 2012). The gel was imaged using the Bio-Rad ChemiDoc Imaging System (Bio-Rad Laboratories). The image was annotated and analyzed using ImageLab software (Bio-Rad Laboratories) to verify the presence and relative quantities of the 28S and 18S bands. RNA integrity was defined by the presence of two clear bands and a ratio of 28S:18S quantity greater than 2:1. RNA (1 μg) was reverse transcribed using the iScript™ cDNA Synthesis kit (Bio-Rad Laboratories) in a T100 Thermal Cycler (Bio-Rad Laboratories).

7. Quantitative Real-Time PCR

Quantitative real-time PCR (qPCR) was performed in accordance with MIQE guidelines (Taylor et al 2019). Briefly, 10 μL of qPCR reaction mix was prepared in 12-tube PCR strips (Bio-Rad Laboratories) containing 1× SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad Laboratories), 0.5 μM gene-specific primers, and 10 ng of cDNA. qPCR was performed in the CFX96 Thermal Cycler (Bio-Rad Laboratories), and results were analyzed using CFX Maestro software (Bio-Rad Laboratories). The stability of five reference genes, UXT, PPIA, GAPDH, EIF3K, and ACTB, was assessed using pooled samples from each treatment group. Average M value and gene stability were generated using the gene study function of CFX Maestro software (Bio-Rad Laboratories). The results were used as input for the geNorm algorithm (https://genorm.cmgg.be/). Reference genes with an M value below 0.5 qualified as stable (Taylor et al 2010). The M value was 0.014 for UXT and PPIA, 0.069 for GAPDH, 0.135 for EIF3K, and 0.294 for ACTB. UXT and PPIA were selected as optimal reference genes. For each primer-pair of interest, a melting curve analysis was generated, and amplicon length was verified using 2% agarose gel electrophoresis. A 4-fold serial dilution was performed to generate the standard curve and efficiency of each primer-pair, and efficiencies were calculated by CFX-

Maestro (Bio-Rad Laboratories). Primers used for qPCR are described in Table 1. All replicates with a Cq standard deviation greater than 0.50 were excluded from analysis. ΔΔCq values were calculated in CFX-Maestro (Bio-Rad Laboratories) using the geometric mean of 2 reference genes (PPIA and UXT1) to determine relative gene expression.

8. Statistical Analysis

The experimental design is schematically represented in Figure 5. Experimental design. Wells within a plate were randomly assigned to BM and LDM using SAS. The experimenters treating the wells, isolating RNA, and plating the qPCR experiment were blind to the treatment allocation until qPCR analysis was completed. The experiments were performed in three independent wells per cow, with three cows per experiment. Treatments were randomly assigned to wells within cows (block) according to a randomized block design using PROC Plan SAS software (SAS Institute Inc.; Cary, NC). The outcome assessor was blind to treatment allocation. Data was analyzed using a two-way ANOVA. Fixed effects included in the model were cow, treatment, and cow-by-treatment interaction. The means were compared using Tukey's test to control the Type I experiment-wise error rate. A P-value < 0.05 was considered significant.

Table 1. Primers used in qPCR gene expression analysis.

Gene Symbol	**mRNA Accession No.**	**Forward Primer (3'→5')**	**Reverse Primer (5'→3')**	**Primer Start Position**	**Exon Junction**	**Product Size**	**Efficiency (%)**
UXT	NM_ 001037471.2	GTC CTG GAC CAT CGT GAC AA	CCG AGT GGT TAG CTT CCT GG	133	210/211	94	95.2

PPIA	NM_ 178320.2	CTG AGT GGT TGG ATG GCA AG	TGT CCA CAG TCA GCA ATG GT	367	373/374	134	97.9
CSN1S1	NM_ 181029	AGT GCT GAG GAA CGA CTT CA	CCA GGC ACC AGA TGG ATA GG	488	496/497	150	98.6
CSN2	NM_ 181008	CAG CAG CAA ACA GAG GAT GAA C	AGG GAT GTT TTG TGG GAG GC	252	N/A	114	84.4
CSN3	NM_ 174294	GCC AAC TGA ACC TAC TGC CA	CTC CTG GGC ACC CAA AAA TG	15	123/124	121	94.6
ELF5	XM_ 024975448.1	TGG AAG GCT GAA CAG AGG TG	GAG TCC AAC ATC ACC CAA GC	3	N/A	137	101.8

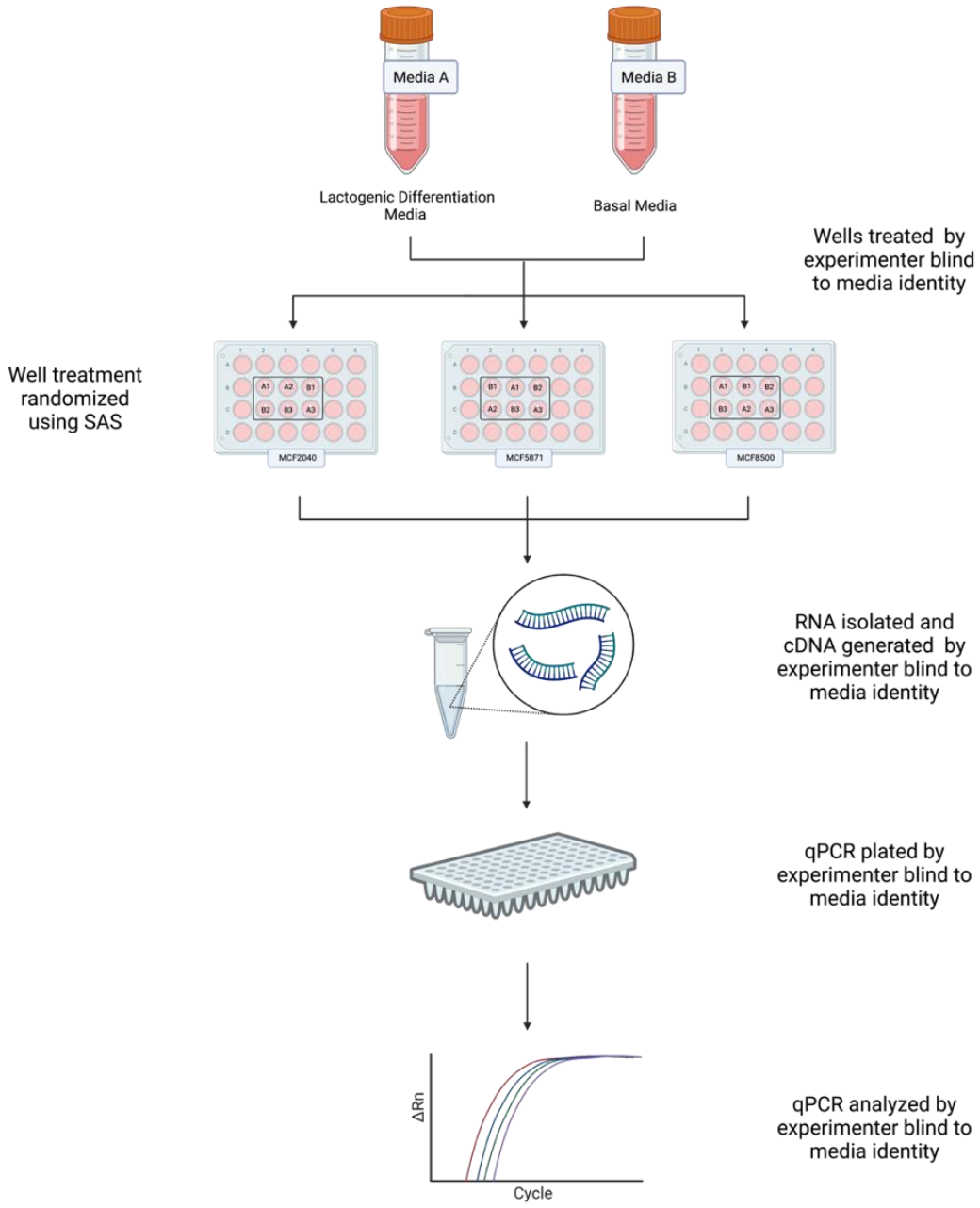

Figure 5. Experimental design. Wells within a plate were randomly assigned to BM and LDM using SAS. The experimenters treating the wells, isolating RNA, and plating the qPCR experiment were blind to the treatment allocation until qPCR analysis was completed.

Results

1. Generation of Mammary Organoids

Images were acquired using phase-contrast microscopy to observe organoid growth and morphology between passages and treatments. All organoids observed in our study had a spherical morphology. We observed the formation of organoids within 2 days of passaging, and growth continued with further treatment with EM (Figure 6). Large organoids sank in the gel and attached to the plate. Thus, organoids were passaged on day 7 of treatment with EM. Organoid density decreased progressively through subsequent passages (Figure 7A). Organoids increased in size after treatment with BM and LDM. No morphological differences were visible between organoids treated with BM and LDM (Figure 7B).

Figure 6. Representative images of organoids in passage 2 taken on days 0, 2, 6 and 7 of expansion. Scale bars = 200 μm.

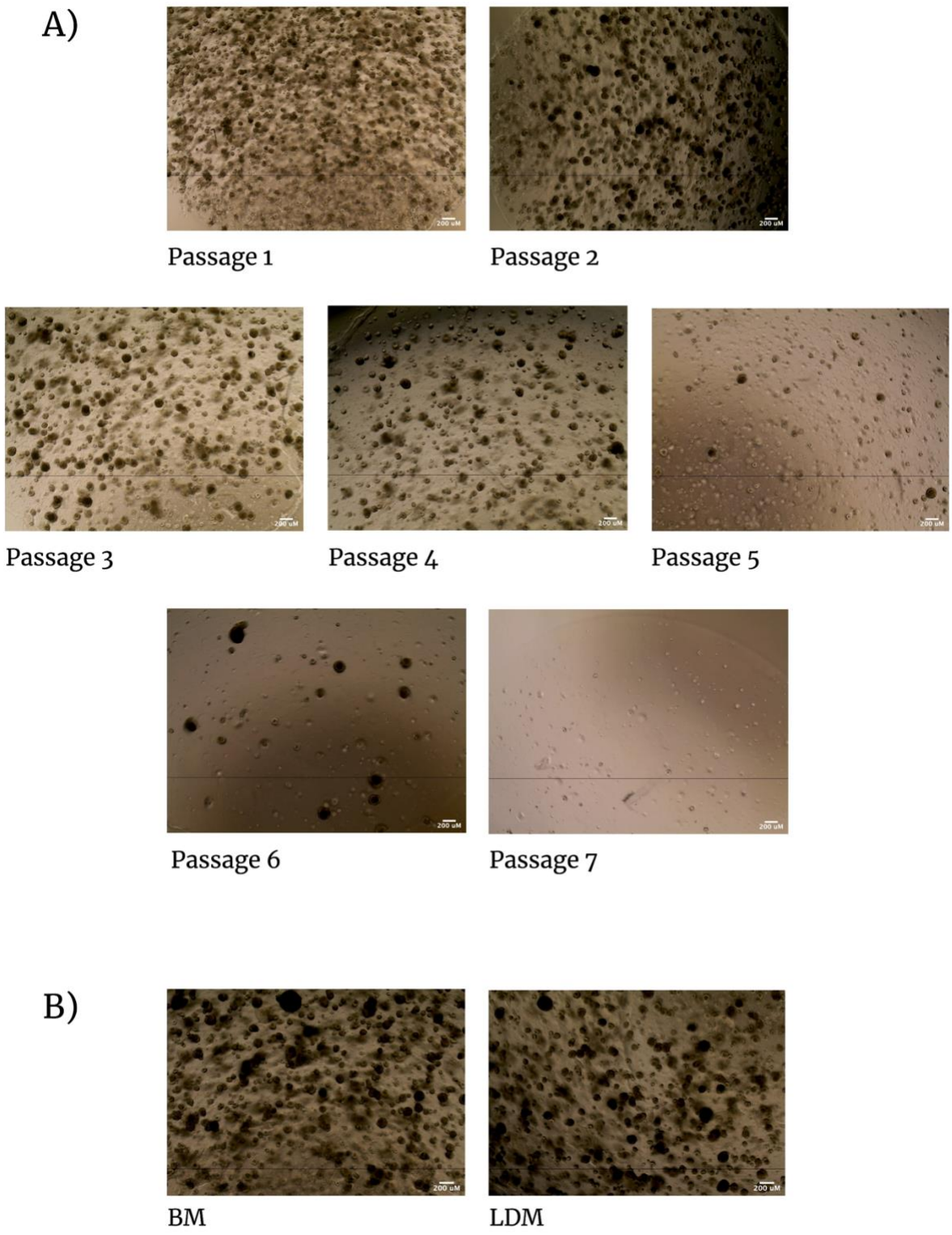

Figure 7. A) Images taken of organoids on the final day of passages 1 through 7. B) Images taken of organoids on the final day of treatment with BM and LDM. Scale bars = 200 μm.

2. Organoid Number and Size

To measure organoid number and size, images of organoids in passage 2 were acquired on day 7 of culture in expansion media and analyzed in ImageJ (Figure 8). Average 2-dimensional area was calculated to determine the size of organoids. Cultures from cow MCF2040 had an average of 88.5 organoids per drop and an average 2-dimensional area covering of 2954.6 μm^2. Cultures from cow MCF5871 had an average of 100 organoids per drop and an average 2-dimensional area covering 3628.2 μm^2. Cultures from cow MCF8500 had an average of 209 organoids per drop and an average 2-dimensional area covering 3130.7 μm^2.

To analyze morphological differences between organoids treated with BM and LDM, images were taken on day 4 of treatment and analyzed using ImageJ (Figure 8). In cultures from cow MCF2040, average organoid size increased 2.3-fold in wells treated with BM and 2.2-fold in wells treated with LDM. Organoid number increased 1.9-fold in cultures treated with BM and was approximately the same in cultures treated with LDM. In cultures from cow MCF5871, average organoid size increased 1.7-fold in wells treated with BM and 1.3-fold in wells treated with LDM. Organoid number increased 1.3-fold in cultures treated with both BM and LDM. In cultures from cow MCF8500, average organoid size increased 1.1-fold in wells treated with BM and 1.2-fold in wells treated with LDM. Organoid number decreased 0.7-fold in cultures treated with both BM and LDM.

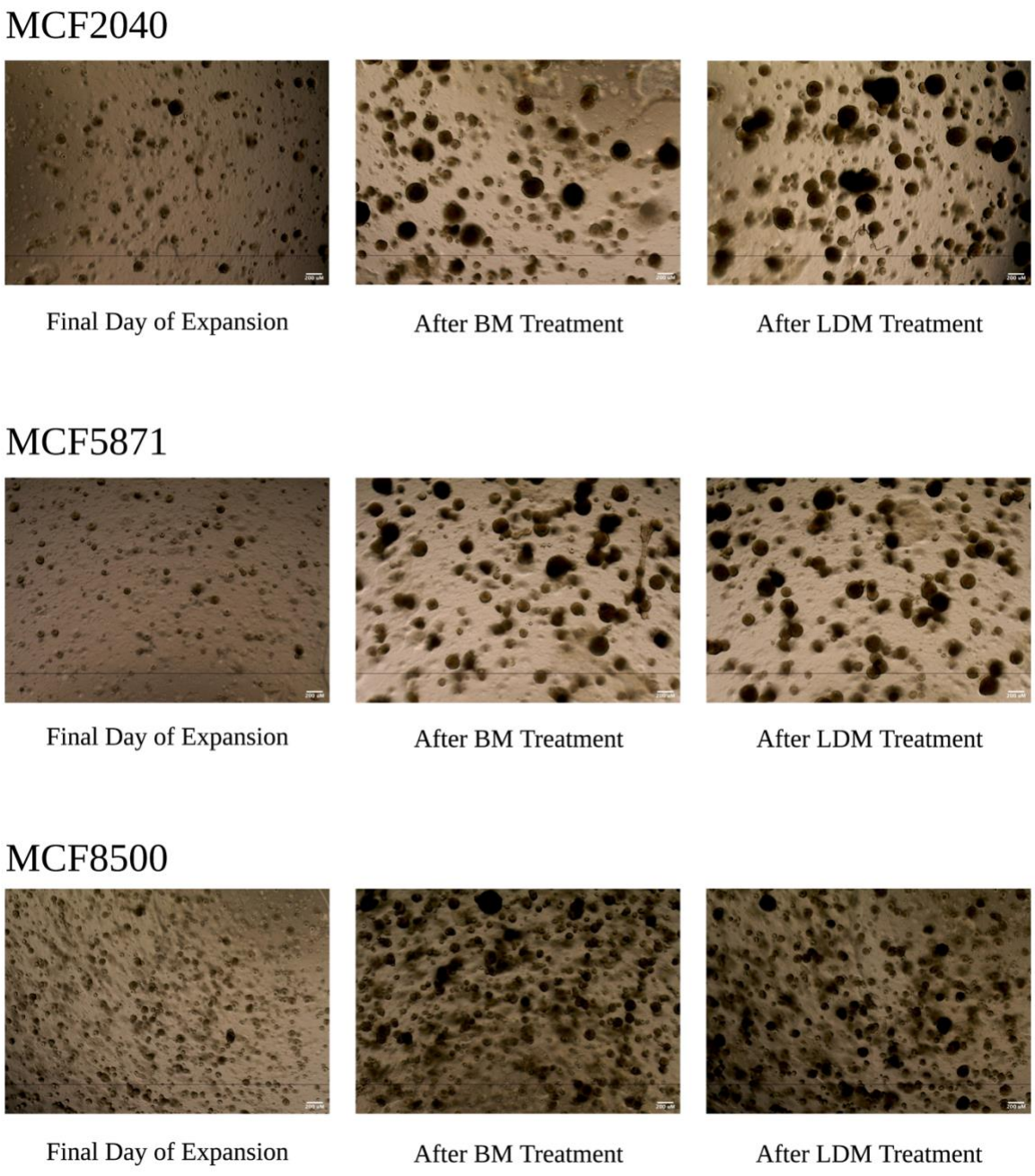

Figure 8. Representative images of organoids from each cow on the final day of expansion in passage 2 and on the final day of treatment with BM and LDM. Scale bars = 200 μm.

3. Passaging Efficiency

A characteristic feature of organoids is their long-term passage potential. Therefore, we serially passaged organoids to determine their long-term culture potential under our conditions. Organoids from cows MCF5871, MCF8500, and MCF2040 were maintained through passages 8, 7, and 6, respectively. The initial cell density was visibly low in passage 3 of organoids from cows MCF2040 and MCF5871, thus organoids were grown for longer to recover cells before further passaging.

To assess changes in organoid size and number at different passages, organoids were imaged on the last day of each passage and processed using ImageJ. There was no consistent trend in average organoid size with subsequent passages (Figure 9). However, the number of organoids decreased with each passage in all 3 cows (Figure 10). Pearson's correlation coefficient calculated between average organoid size and organoid number suggests a strong positive correlation in cow MCF2040 ($r=0.74$), a weak positive correlation in cow MCF8500 ($r=0.30$), and a weak negative correlation in cow MCF2040 ($r=-0.28$).

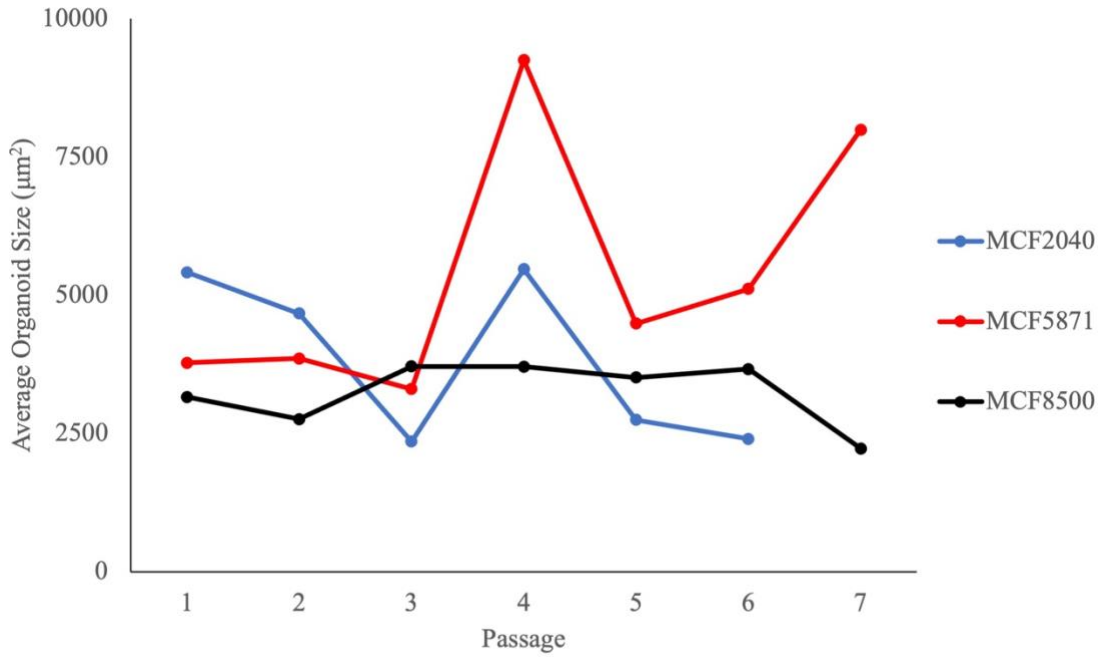

Figure 9. Average 2-dimensional organoid size from passages 1 through 6 (MCF2040) and 7 (MCF5871 and MCF8500). The dots represent the average organoid size on the last day of each passage.

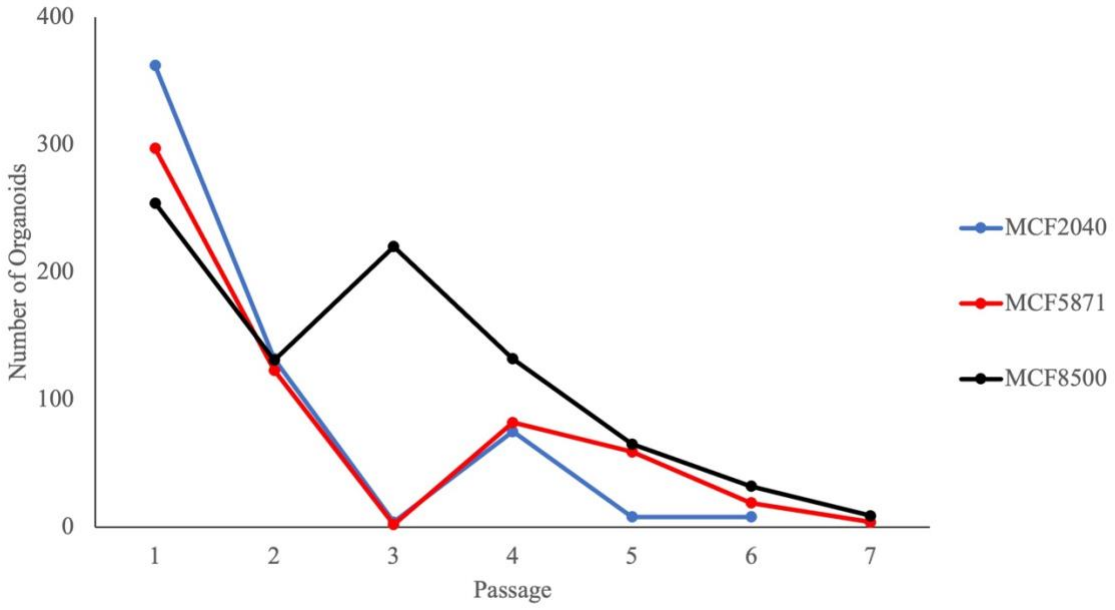

Figure 10. Number of organoids from passages 1 through 6 (MCF2040) and 7 (MCF5871 and MCF8500). The dots represent the number of organoids on the last day of each passage. Due to

the low initial cell density, drops from cows MCF2040 and MCF5871 contained 4 and 2 organoids, respectively, on the final day of passage 3.

4. Primer Validation

Primer efficiency was measured for all primers of interest. We identified efficient primers for markers of α-S1 casein (CSN1S1), β casein (CSN2), κ casein (CSN3), and E74-like Factor 5 (ELF5) (Table 1). Notably, no efficient primers were identified for markers of α-S2 casein, α-lactalbumin, or β-lactoglobulin. Melt curve analysis of all efficient primers generated a single peak, thus the amplification of a single amplicon was confirmed. Base-pair analysis of agarose gel electrophoresis confirmed the amplification of the target gene.

5. Expression of Milk Protein Genes

Expression of target gene CSN1S1 increased 6.9-fold in cow MCF2040, 7.4-fold in cow MCF5871, and 2.3-fold in cow MCF8500 in organoids treated with LDM compared to organoids treated with BM (Figure 11). The effect of LDM treatment on CSN1S1 gene expression compared to BM treatment was significantly different between cows (P-value=0.0002). Treatment with LDM stimulated CSN1S1 mRNA upregulation in organoids from all cows, but upregulation was only significant in cow MCF5871 (P-value<0.0001). The overall effect of LDM on CSN1S1 gene expression was significantly greater than the effect of BM (P-value<0.0001). The combined effect of treatment on CSN1S1 gene expression was significantly different between cows (P-value<0.0001).

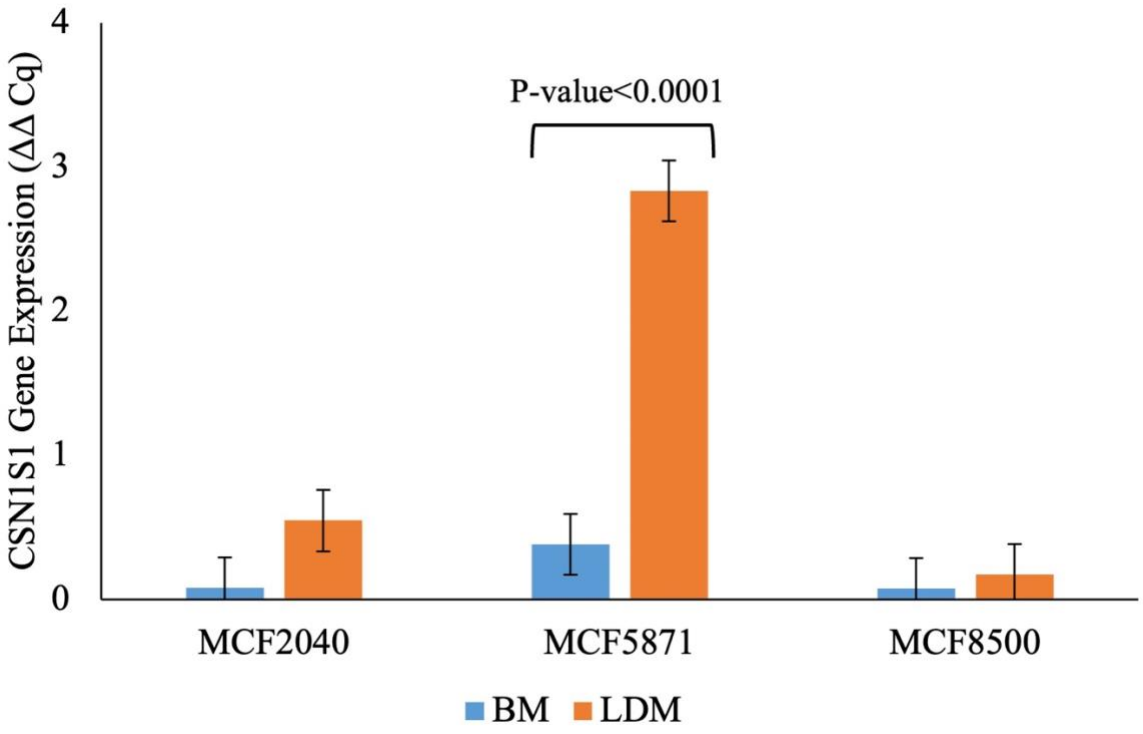

Figure 11. Least square mean CSN1S1 gene expression in organoids treated with BM and LDM in cows MCF2040, MCF5871, and MCF8500. Error bars represent standard error of the mean. P-value indicates a significant difference in the effect of BM and LDM treatments in cow MCF5871.

Expression of target gene CSN2 increased 1.4-fold in MCF2040 and 4-fold in MCF5871 in organoids treated with LDM compared to organoids treated with BM, and increased 1.6-fold in MCF8500 in organoids treated with BM compared to organoids treated with LDM (Figure 12). The effect of LDM treatment on CSN2 gene expression compared to BM treatment was significantly different between cows (P-value<0.0001). Treatment with LDM stimulated CSN2 mRNA upregulation in organoids from cows MCF2040 and MCF5871, but upregulation was only significant in cow MCF5871 (P-value<0.0001). CSN2 mRNA expression was higher in organoids treated with BM in cow MCF8500, but the difference in CSN2 expression between treatments was not significant. The overall effect of LDM on CSN2 gene expression was

significantly greater than the effect of BM (P-value=0.0010). The combined effect of treatment on CSN2 gene expression was significantly different between cows (P-value=0.0059).

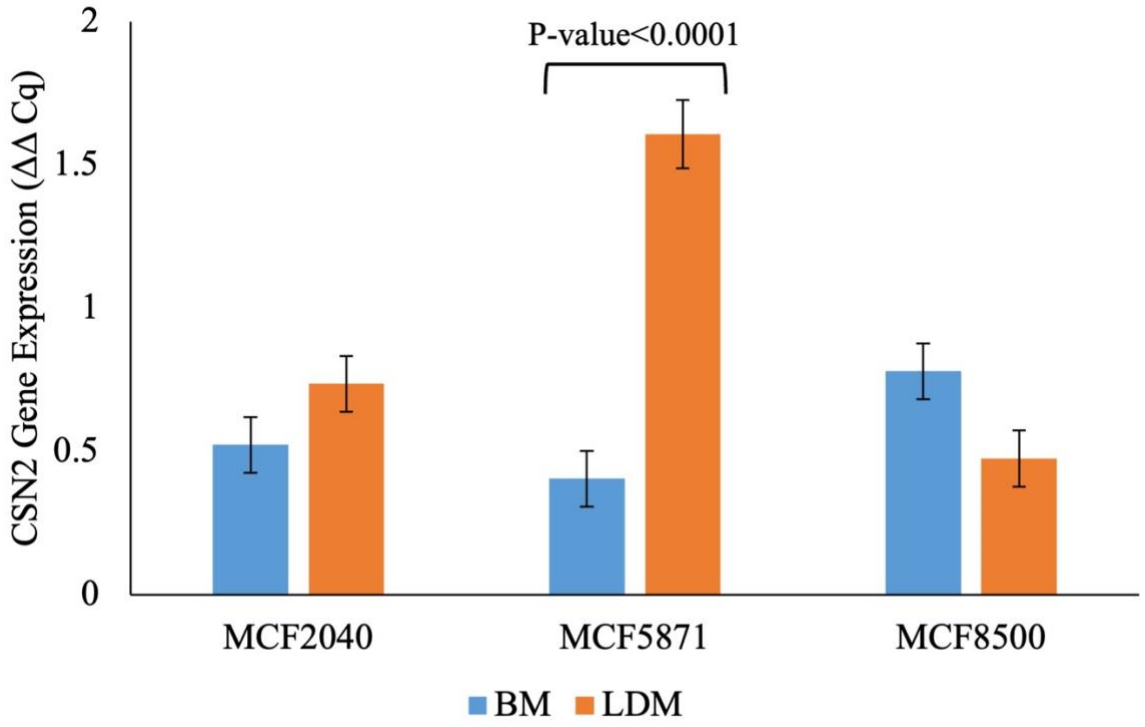

Figure 12. Least square mean CSN2 gene expression in organoids treated with BM and LDM in cows MCF2040, MCF5871, and MCF8500. Error bars represent standard error of the mean. P-value indicates a significant difference in the effect of BM and LDM treatments in cow MCF5871.

Expression of target gene CSN3 increased 2-fold in cow MCF2040, 1.5-fold in cow MCF5871, and 1.8-fold in cow MCF8500 in organoids treated with LDM compared to organoids treated with BM (Figure 13). The overall effect of LDM on CSN3 gene expression was significantly greater than the effect of BM (P-value=0.0002). The effect of treatment did not differ between cows.

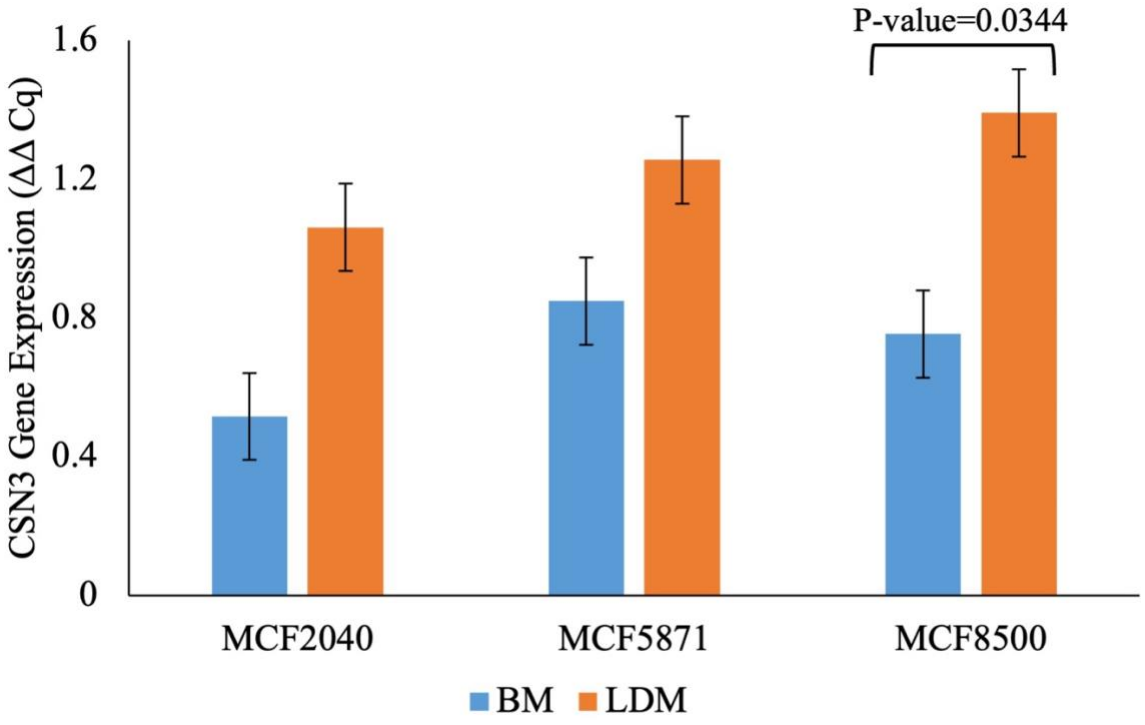

Figure 13. Least square mean CSN3 gene expression in organoids treated with BM and LDM in cows MCF2040, MCF5871, and MCF8500. Error bars represent standard error of the mean. P-value indicates a significant difference in the effect of BM and LDM treatments in cow MCF8500.

Expression of target gene ELF5 increased 1.4-fold in cows MCF2040 and MCF5871 in organoids treated with LDM compared to organoids treated with BM, and increased 3.5-fold in cow MCF8500 in organoids treated with BM compared to organoids treated with LDM (Figure 14). The effect of LDM treatment on ELF5 gene expression compared to BM treatment was significantly different between cows (P-value=0.0238). Treatment with LDM stimulated ELF5 mRNA upregulation in organoids from cows MCF2040 and MCF5871, but upregulation was not significant. EFL5 mRNA expression was significantly higher in organoids treated with BM in cow MCF8500 (P-value=0.0465). The combined effect of treatment on ELF5 gene expression

was significantly different between cows (P-value=0.0441). There was no significant difference between the effects of BM and LDM.

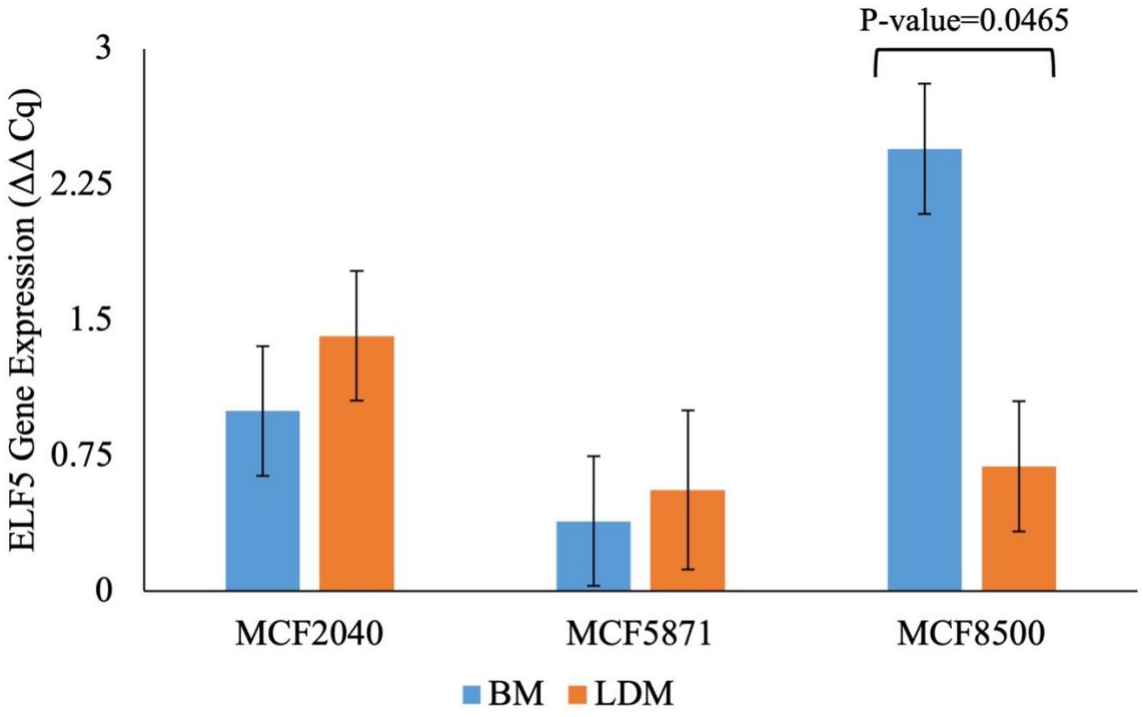

Figure 14. Least square mean ELF5 gene expression in organoids treated with BM and LDM in cows MCF2040, MCF5871, and MCF8500. Error bars represent standard error of the mean. P-value indicates a significant difference in the effect of BM and LDM treatments in cow MCF8500.

Chapter 4 – General Discussion

We adapted a methodology for the generation of bovine mammary organoids from established methods used in humans and mice. Phase-contrast images confirm that bovine mammary organoids supplemented with EM were established within 2 days of initial seeding. Previous studies observed organoid growth from human mammary cells within 10-12 days of seeding in floating collagen gels (Linnemann et al 2015) and from mouse mammary cells within 7 days of seeding in ECM gel (Sumbal et al 2020). Linnemann et al (2015) used a commercially available media for 2-dimensional cell cultures supplemented with antibiotics and chemical inhibitors, but did not contain growth factors. Sumbal et al (2020) used DMEM/F12 media supplemented with antibiotics and growth factors FGF2, FGF7, FGF10, and a combination of Wnt pathway stimulators. The results of our research in combination with the research of Linnemann et al (2015) and Sumbal et al (2020) indicate that the species of origin and media components may affect the establishment rate of mammary organoids. As we did not have access to samples from the mammary glands of humans and mice, we were unable to compare the establishment rates of mammary organoids from different species under our culture conditions. Therefore, future studies should perform comparative studies to determine the roles of inter-special variation and media components in the establishment rate of mammary organoids.

All organoids observed in our study had a spherical morphology throughout all treatments and passages. Previous studies in human (Linnemann et al 2015, Rosenbluth et al 2020) and mouse (Jamieson et al 2017) mammary organoids saw more varied structures. A study by Linnemann et al (2015) in human mammary organoids found a correlation between the formation of spherical structures and the size of the luminal progenitor population in the culture. Thus, it was concluded that spherical structures arise primarily from luminal progenitor cells.

However, there was significant variation in organoid structure between donors and passages, and luminal progenitor derived organoids from some donors could generate branched structures. Interestingly, Linnemann et al (2015) found that the addition of chemical inhibitor Y27632 for the first 3 days of culture increased the formation of branched structures 5-fold. However, continuous treatment with Y27632 initiated cell-cell dissociation and inhibited morphogenesis (Linnemann et al 2015). Another study in human mammary organoids by Rosenbluth et al (2020) found that separated mature luminal and luminal progenitor cells both formed spherical structures, while myoepithelial/basal cells formed spheres with budding and branching outgrowths. Myoepithelial/basal derived organoids were larger than luminal derived organoids, however no numerical results were reported (Rosenbluth et al 2020). Conversely, a study in mouse mammary organoids by Jamieson et al (2017) found that 15% of luminal cells and 20% of myoepithelial/basal cells formed structures with branched or budding morphologies. They found that treatment with Y27632 for the first 3 days of culture increased budding formation in all organoids, and the addition of FGF2 and heparin was essential for the formation of budding structures in organoids derived from luminal cells (Jamieson et al 2017). Results from these studies suggest several hypotheses for the absence of complex budding or branching structures established in our experiments. In our study, we isolated cells from lactating cows. Previous studies that have reported more complex mammary organoid morphologies isolated cells from non-lactating humans (Linnemann et al 2015, Rosenbluth et al 2020) and mice (Jamieson et al 2017). Therefore, it is possible that our initial cell populations contained more luminal and luminal progenitor cells than previous studies. This could be addressed by cell sorting or staining prior to initial seeding. Additionally, the absence of FGF2 and heparin in EM could have prevented luminal cell populations from forming branched structures. We included Y27632 in

EM through all 7 days of expansion. It is possible that the presence of Y27632 after day 3 of expansion initiated cell-cell dissociation and inhibited the formation of more complex morphologies. Future studies should observe morphological changes that occur in bovine mammary organoids with the addition of FGF2 and heparin to media and withdrawal of Y27632 after 3 days.

Organoids from cows MCF2040 and MCF5871 increased in number and average size after treatment with both BM and LDM. Organoids from cow MCF8500 increased in average size and decreased in number after treatment with both BM and LDM. We interpret the different morphological responses to treatment with LDM in organoids from different cows to mean that organoids maintain individual characteristics of the cows. No notable differences in growth or morphology were observed between organoids treated with BM or LDM. A previous study by Sumbal et al (2020) used time-lapse technology to observe growth of mouse mammary organoids after lactation was induced with lactogenic hormones. After 6 days of growth in media supplemented with growth factors, organoids supplemented with PRL and hydrocortisone increased ~1.4-fold in size and ~2-fold in relative density after 4 days (Sumbal et al 2020). These observations are comparable to increased size and number in LDM organoids from cows MCF2040 and MCF5871. In the experiment by Sumbal et al (2020), PRL and hydrocortisone were withdrawn from some organoids after lactation was induced. Organoids switched to media without lactogenic hormones decreased ~2-fold in size and relative density after 5 days. They concluded that the decrease in size after lactogenic hormones were withdrawn was due to an involution-like process. They also observed lipid droplets accumulating in lumens during treatment with lactogenic hormones which disappeared after the hormones were withdrawn. This observation was visual confirmation of milk synthesis and an involution-like process (Sumbal et

al 2020). No previous research has reported on growth and morphology of mammary organoids treated with BM and LDM simultaneously. Due to the similar morphologies of organoids treated with BM and LDM simultaneously, it appears that lactogenic hormones did not have a key role in organoid growth. Due to the absence of morphological differences, including lumen darkening suggesting the accumulation of milk components, we did not obtain visual confirmation that lactation was induced in LDM organoids. Future research should use immunofluorescent staining of milk components and fluorescent microscopy to determine the presence of milk components in the lumen. Additionally, whether the withdrawal of lactogenic hormones induces an involution-like process in bovine mammary organoids is not known. These experiments could elucidate the functional proximity of organoids to the development of the mammary gland during pregnancy, lactation, and involution.

Organoids were maintained in continuous culture for up to 2 months (8 passages) before losing regeneration efficiency. 2-dimensional culture models of bovine mammary cells have been limited to a maximum of 3 passages before genetic drift prevents cells from reaching confluency (Matitashvili et al 1997). Thus, this method of bovine mammary organoid culture provides a longer lasting model than previous 2-dimensional models. The decrease in number of organoids through serial passaging suggests a progressive loss in regeneration capacity. No pattern was detected in change in average organoid size between passages, and the spherical structure of organoids was maintained through all passages. Thus, organoid morphology was maintained throughout passages. There was a strong positive correlation between average organoid size and organoid number in cow MCF2040, a weak positive correlation in cow MCF8500, and a weak negative correlation in cow MCF5871. Taken together, no correlation between average organoid size and organoid number can be detected. Future research using a

larger sample size could further elucidate whether there is a correlation between average organoid size and organoid number throughout passages. Previous research in mammary organoids suggests significant variability in passaging efficiency and longevity between species. A study by Jamieson et al (2017) in organoids derived from mammary myoepithelial/basal cells from mice reported a maximum of 4 weekly passages before organoids underwent morphological changes. Initial seeding density was 2,500 cells per 1 mL gel (Jamieson et al 2017). Another study in mouse mammary organoids by Wrenn et al (2020) compared organoid outgrowth in cultures with varying initial seeding densities. They found that organoid outgrowth was maximized in cultures seeded at 150,000 cells per 1 mL gel suspension. At this seeding density, organoids were passaged biweekly 10 times and no growth arrest or morphological changes were detected (Wrenn et al 2020). Thus, it can be hypothesized that there is a correlation between initial seeding density and passaging efficiency. While we aimed for an initial seeding density of 250,000 cells per 1 mL gel, limitations in cell counting technology prevented us from accurately determining initial seeding density between passages. Therefore, the effect of initial seeding density on passaging efficiency and growth is unknown. Organoids established from human mammary epithelial cells were maintained for more than 20 passages performed every 1-4 weeks (Sachs et al 2018). Another study reported that human mammary organoid cultures were maintained for up to 16 months with passages performed every 2-4 weeks (Rosenbluth et al 2020). No initial seeding density was reported for the studies by Sachs et al (2018) and Rosenbluth et al (2020), thus it is uncertain whether it was consistent between passages. The inconsistent time interval between passages may suggest that organoids were passaged based on growth observations. Future studies in bovine mammary organoids should passage organoids at a consistent cell density rather than a consistent time interval to determine if culture longevity can

be increased. This could be accomplished by determining that a fixed confluency is reached prior to passaging.

We measured CSN1S1 gene expression to determine the role of lactogenic hormones in α-S1 casein expression in bovine mammary organoids. As expected, CSN1S1 gene expression was significantly upregulated in organoids treated with LDM compared to organoids treated with BM in all cows. Additionally, we observed a significant difference in the response to LDM between cows, suggesting that individual characteristics of the *in vivo* mammary gland are maintained in organoids. A previous study by Riley et al (2009) cultured mammospheres composed of mammary stem cells from nonlactating cows in Matrigel. They observed that CSN1S1 gene expression increased 100-fold in mammospheres supplemented with 3 μg/mL PRL compared to mammospheres supplemented with media lacking PRL (Riley et al 2009). While we found that supplementation with lactogenic hormones significantly upregulated CSN1S1 gene expression in bovine mammary organoids, we observed that CSN1S1 was also expressed in organoids supplemented with BM. One explanation for the basal expression of CSN1S1 is the use of cells from lactating cows, suggesting that the JAK/STAT pathway may have been induced *in vivo* and upregulated with *in vitro* supplementation of lactogenic hormones. Future studies should characterize basal gene expression prior to inducing lactation to determine upregulation relative to the basal gene expression level. Measuring basal gene expression would also be useful in characterizing individual characteristics of cows, thus providing further insight into the responsiveness of each cow to lactogenic hormones. Additionally, the mammospheres established by Riley et al (2009) did not undergo a 3-dimensional growth phase prior to the addition of PRL. Therefore, it is likely that the organoids we established contained more varied cell types that contributed to basal CSN1S1 expression. Future studies should use

immunofluorescent surface marker staining to characterize the role of different cell types in CSN1S1 gene expression. No previous studies have reported CSN1S1 gene expression in mammary organoids from mice or humans.

CSN2 gene expression was measured to determine the role of lactogenic hormones in the expression of β casein in bovine mammary organoids. We observed significant upregulation of CSN2 gene expression in organoids treated with LDM compared to organoids treated with BM. However, CSN2 gene expression was downregulated in LDM organoids from cow MCF8500. The significant difference in the effect of LDM on CSN2 gene expression between cows suggests that individual characteristics of the cows *in vivo* are maintained in culture. The upregulation of CSN2 gene expression after treatment with LDM is consistent with previous studies in mammary organoids established from mice. A study by Ciccone et al (2020) established mammary organoids from nonlactating mice. They observed an 11-fold upregulation of CSN2 gene expression in organoids treated with media supplemented with PRL and progesterone compared to organoids treated with essential media. Additionally, they found that the addition of FGF2 to media supplemented with lactogenic hormones inhibited CSN2 gene expression (Ciccone et al 2020). A study by Sumbal et al (2020) observed a 4 to 5-fold increase in CSN2 gene expression in freshly isolated mouse mammary organoids treated with LDM supplemented with PRL and hydrocortisone compared to organoids in which lactogenic hormones were withdrawn after four days. Further upregulation of CSN2 gene expression was observed in organoids supplemented with FGF2 for six days prior to the addition of lactogenic hormones. Notably, organoids were established from nonlactating mice, thus the supplementation and subsequent withdrawal of lactogenic hormones modeled lactation induction and involution (Sumbal et al 2020). The results from these studies suggest that lactogenic

hormones stimulate CSN2 gene expression in mouse mammary organoids, and FGF2 can either limit or increase CSN2 gene expression depending on when it is supplemented. Thus, it is possible that growth factors supplemented to bovine mammary organoids during the expansion phase affected CSN2 gene expression. One explanation for the different response to LDM treatment between cows is that organoids from cow MCF8500 had a basal expression of inhibiting growth factors during the expansion phase and expression continued during the treatment phase. Differences in basal expression of growth factors between cows could lead organoids from different cows to respond differently to treatment with LDM. Future studies should characterize the production of growth factors by organoids from all cows through the growth and treatment phases to determine the impact on gene expression.

CSN3 gene expression was measured to determine the role of lactogenic hormones in expression of κ casein in bovine mammary organoids. We observed significant upregulation of CSN3 gene expression in organoids supplemented with LDM compared to organoids supplemented with BM. A previous study by Ciccone et al (2020) observed a 48-fold increase in CSN3 gene expression in mouse mammary organoids supplemented with LDM compared to organoids supplemented with BM. Similarly, our results suggest that the presence of lactogenic hormones in bovine mammary organoid cultures upregulates CSN3 gene expression. No significant difference in the effect of LDM on CSN3 gene expression was found between cows. This may suggest that the concentration of κ casein in milk is less variable between cows than the concentration of α-S1 casein and β casein. κ casein is found in a lower concentration in cow's milk than α-S1 casein and β casein (Głąb and Boratyński 2017). Therefore, inter-cow variability in CSN3 gene expression in response to LDM treatment may be less detectable than that of proteins found in higher concentrations. Future research should measure milk protein

concentrations in fresh milk prior to establishing organoids, thus providing insight into whether donor-specific milk protein concentrations are maintained in organoid culture.

ELF5 is a milk protein transcription factor located downstream of PRL receptors in the JAK/STAT pathway. The activation of ELF5 receptors regulates the expression of β casein and several other milk-specific proteins (Zhou et al 2005). Thus, we measured ELF5 gene expression to determine the role of lactogenic hormones in the expression of a PRL-mediated β casein regulator. We observed a significant difference in the combined effect of treatments on ELF5 gene expression between cows. Additionally, we observed a significant difference in the effect of LDM treatment on ELF5 gene expression between cows. We observed that ELF5 gene expression was upregulated in organoids treated with LDM in cows MCF2040 and MCF5871, but downregulated in organoids treated with LDM in cow MCF8500. As expected, these results correspond to the effects of LDM treatment on CSN2 gene expression in each cow. However, we did not observe a significant difference in the overall effects of BM and LDM treatments on ELF5 gene expression. A previous study by Finot et al (2018) measured the expression of ELF5 surface markers on several bovine mammary cell types. They observed high concentrations of ELF5 surface markers on stem cells and common progenitor cells, low concentrations on luminal-committed progenitor cells, and no ELF5 surface markers on myoepithelial/basal-committed progenitor cells. Additionally, they observed the highest concentration of PRL receptor surface markers on luminal-committed progenitor cells, lower concentrations on stem cells and common progenitor cells, and no surface markers on myoepithelial/basal-committed progenitor cells (Finot et al 2018). One explanation for the significant difference in ELF5 gene expression and responsiveness to LDM between cows is the presence of different cell types in cultures from different cows. The mean of ELF5 gene expression between treatments was

highest in organoids from cow MCF8500. This may suggest that organoids from cow MCF8500 were primarily composed of stem cells and common progenitor cells, suggesting a high basal concentration of ELF5 surface markers but a low responsiveness to PRL. Contrarily, it is possible that organoids from cows MCF2040 and MCF5871 were composed primarily of luminal-committed progenitor cells, suggesting a low basal concentration of ELF5 surface markers but a high responsiveness to PRL. Future studies should use surface-marker staining to characterize the cell types present in organoid cultures and elucidate the roles of different cell types in the expression of milk protein transcription factors.

There were several limitations to our study. One limitation was the lack of a reliable method to determine initial cell density. The same method of cell counting was applied at initial seeding of cells from the three cows in passage 1. However, initial seeding density could not be maintained between passages. Thus, a consistent passaging ratio could not be calculated. Another limitation was the lack of validated primers to measure gene expression of milk-specific proteins α-S2 casein, α-lactalbumin, and β-lactoglobulin. Despite attempting several primer designs, we were unable to confidently validate primer sets for these genes due to poor annotation of the bovine genome. Standard curves for these primers did not generate an efficiency between the threshold values. Thus, gene expression of these key milk components could not be confirmed.

This model of bovine mammary organoids offers a novel direction for the future of lactation research. Our cultures had a longer lifespan than 2-dimensional cultures of bovine mammary epithelial cells, suggesting that organoid models may be a more reliable option for long-term effect studies. Future research should focus on optimizing the culture methods to determine whether longevity and passaging efficiency could be improved. We observed

significant differences in the response to lactogenic hormones between cows, suggesting that individual *in vivo* characteristics of the mammary gland may be maintained in organoids. Future research should characterize gene expression levels *in vivo* to determine whether organoids accurately model individual behaviours. Additionally, researchers should explore the potential of organoids for modeling and treating bovine mammary diseases. Because organoids maintain the genotype and cell lineages of the *in vivo* organ, they have been used to elucidate the cellular mechanisms of bacterial infections (Dutta et al 2017). This suggests that bovine mammary organoids could be used to understand the mechanisms of bovine mastitis and to develop diagnostic methods, vaccines, pharmacological treatments, and preventative measures. Establishing a reliable *in vitro* method for the prevention, diagnosis, and treatment of bovine mastitis would decrease financial strain on dairy farmers and increase the efficiency of dairy production. Finally, bovine mammary organoids should be used to develop technologies that improve the environmental sustainability of dairy production. The upregulation of milk-specific proteins after LDM treatment suggests that organoids can be used to model lactation. Future research should utilize this model to determine the impact of hormonal and nutritional supplements on the production rate of milk and its nutritional components. Understanding the interactions between mammary epithelial cells and supplements could aid in the development of technologies that improve the efficiency of dairy production and decrease environmental impact.

Conclusion

This study has developed a method for establishing bovine mammary organoids and characterizing the synthesis of milk-specific proteins. We found that spherical 3-dimensional organoids were established from bovine mammary epithelial cells within 2 days of initial seeding throughout all passages. Organoid cultures were maintained for up to 2 months or 8 weekly passages. Furthermore, we have detected a significant upregulation in gene expression of milk-specific proteins α-S1 casein, β casein and κ casein after lactogenic differentiation was induced. This suggests that bovine mammary organoids are responsive to lactogenic hormones, thus necessitating further research into the reliability of organoids as a method for in vitro lactation studies. Additionally, we observed a significant difference in the effect of lactogenic differentiation in α-S1 casein, β casein, and ELF5 gene expression between cows. This suggests that individual characteristics of the cow *in vivo* are maintained in organoids. In conclusion, this work provides a novel method for the growth, passaging, and gene expression characterization of bovine mammary organoids.

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