TRIENNIAL LACTATION SYMPOSIUM/BOLFA: Dietary regulation of allometric ductal growth in the mammary glands[1,2]

G. E. Berryhill, J. F. Trott, A. L. Derpinghaus, and R. C. Hovey[3]

Department of Animal Science, University of California, Davis, One Shields Avenue, Davis 95616

ABSTRACT: Although mammary gland growth and development in females is a lifelong process, it builds on isometric and allometric phases of mammary growth to establish a complex ductal network before and during puberty. Only then can other phases of branching and alveologenesis, differentiation, lactation, and involution proceed. Although the ductal network of various species differs in its histomorphology, all glands undergo a common phase of allometric growth when the mammary ducts penetrate into the supporting stromal microenvironment. Perhaps not surprisingly, different aspects of diet and nutrition can influence this allometric growth, either directly or indirectly. In this review, we outline some of the fundamental aspects of how allometric ductal growth in the mammary glands of various species is influenced by diet and nutrition and identify opportunities and questions for future investigation.

Key words: allometric, conjugated linoleic acid, terminal end bud

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IN THE BEGINNING – GROWTH AND ELONGATION OF THE MAMMARY DUCTS

The mammary ducts first arise during embryogenesis after inductive signaling from the mammary mesenchyme that leads to formation of the epithelial anlagen from the subtending epidermis. In mice, the ducts first appear around d 15.5 of gestation (Propper et al., 2013), whereas in heifers, the primary sprout first appears when the fetus is around 12 cm long (Turner, 1952). This primary sprout then canalizes followed by further secondary and tertiary branching, which in heifers occurs when the fetus is approximately 20 cm long (Turner, 1952). The mammary ducts in male rats and some strains of male mice are subject to testosterone-induced ablation by the adjacent mesenchymal cells during fetal development; in contrast, the mammary glands in males for the majority of livestock species (except stallions) undergo relatively typical ductal and nipple/teat development in utero (Turner, 1952).

By birth, the ducts are present as a simply branched anlagen that is embedded within a distinct embryonic mesenchyme and are positioned adjacent to a mature depot of differentiated white adipose tissue into which it subsequently extends (Turner, 1952; Propper et al., 2013). The primary goal for the expanding ducts is to establish a network of tubular structures to drain milk to a collecting cistern/sinus and then to the teat/nipple. A parallel consideration is that the terminal regions of the ductal network in humans (the terminal ducts) are considered to be the site of origin for many human breast cancers, which distinguishes them from the site of origin for many mammary cancers that arise in mice (Tsubura et al., 2007).

Our understanding of ductal elongation in rodents is centered around the formation and actions of the

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[3]Corresponding author: rchovey@ucdavis.edu

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terminal end bud (**TEB**), a bulbous epithelial structure that forms at the tips of ducts in response to mitogenic stimulation (Paine and Lewis, 2017). The TEB has been a focus in numerous studies of factors regulating ductal development, not only because they are the site of extensive mitosis alongside accompanying apoptosis that directs lumen formation but also because they represent candidate sites for cancer initiation (Russo and Russo, 1996). These TEB respond to a range of endocrine and local signals. A primary mechanism underlying TEB development involves the IGF1/GH/estrogen axis whereby endocrine estrogenand GH induce the local paracrine synthesis of IGF1 that acts on epithelial cells via IGF1 receptors (Kleinberg et al., 2000). Other signals that promote TEB formation include progestins (Ruan et al., 2005), fibroblast growth factors (Parsa et al., 2008), and members of the epidermal growth factor family (Sternlicht et al., 2005; Ciarloni et al., 2007).

When considering development of the ductal network, it is important to recognize that its architecture significantly differs across species and often does not reflect that portrayed for the widely studied mouse. For example, the human breast has ductal structures that present as a range of terminal ductal lobular units (**TDLU**) manifest as increasingly branched structures prior to lactation that can be classified as types 1through 3 depending on the number of branched ductules arising from the terminal duct (Russo et al., 1992). That said, the site of active ductal elongation in the human breast appears to be a TEB-like structure (Russo et al., 2001), although a complete morphological description of this structure is lacking in the literature. Interestingly, pigs appear to have a similar range of morphological development; their ductal terminations can be defined as TDLU using the aforementioned human classification system, whereas initial elongation of the ducts is directed by TEB-like structures that also undergo estrogen-induced mitosis (Horigan et al., 2009). In contrast, heifers and sheep apparently expand their ductal network en bloc, whereby the epithelial parenchyma advances with a TDLU-like morphology but does not elongate the ducts at sites of focused proliferation such as in TEB (Capuco and Ellis, 2005). These differences in ductal morphology across species raise questions relevant to their potential nutritional regulation. For example, do ruminants lack TEB as a function of a different digestive and metabolic environment, such as a low glucose environment and the ruminal biosynthesis of VFA, CLA, and SFA? Given the demonstrated effects of all these metabolites on the growth and survival of various types of epithelial cells in vitro (Wicha et al., 1979; Wilson and Gibson, 1997; Keating et al., 2008), it could be speculated that these metabolic differences between nonrumiants and ruminants could account for some of the histomorphological differences seen during ductal development

DUCTAL HISTOMORPHOGENESIS

Consideration of the gland's ductal architecture would be incomplete without a parallel assessment of the accompanying histomorphogenesis. The TEB in the mammary glands of rodents are composed of multilayered luminal epithelial cells in the "body" of the TEB that subtend a leading layer of "cap cells" considered to be multipotent by virtue of their being able to give rise to luminal and myoepithelial (also often referred to as "basal") cell populations that can be distinguished by their expression of markers such as different keratins (*KRT8* and *KRT18* in luminal cells and *KRT5* and *KRT14* in myoepithelial cells), smooth muscle actin and *TP63* (myoepithelial markers), or *EPCAM* (epithelial; Paine and Lewis, 2017). Subtending the body cells of the TEB is an inner zone of apoptotic cells that fulfill a crucial role in canalizing the duct; the TEB is also flanked by an outer layer of longitudinally aligned myoepithelial cells (Williams and Daniel, 1983). Notably, TEB in mice are intimately associated with adipocytes (typically white and unilocular), particularly around the leading edge of their invasion (Hovey et al., 1999), whereas the trailing flank of the TEB and the subtending ducts are enveloped by a single layer to several layers of stromal fibroblasts. The basement membrane is also approximately 10 times thicker in the neck region of the TEB compared with its leading edge (Williams and Daniel, 1983). In contrast, the epithelium of the TDLU in nulliparous female humans, pigs, and ruminants is surrounded by an "intralobular" stroma of connective tissue (Rowson et al., 2012), which is then positioned within a more collagenous "interlobular" connective tissue that is also an extension of the connective tissue veins that run throughout the mammary adipose tissue. Some authors have suggested that these veins are responsible for directing formation of TDLU and the lobules they generate (Mayer and Klein, 1961; Anbazhagan et al., 1991; Hovey et al., 1999).

STAGES OF DUCTAL GROWTH

The rate of parenchymal progression into the mammary fat pad is by no means linear but rather advances through key developmental stages in concert with the female's acquisition of reproductive competence. Generally speaking, growth of the ductal network in fetal mice, humans, and heifers is isometric during late fetal development (Hovey et al., 2002). At the same time, the mammary epithelium remains sensitive to the changing

maternal endocrine environment around parturition, as illustrated by altered ductal morphology in human infants during this period (Anbazhagan et al., 1991).

The ductal structures remain relatively dormant after birth until the subsequent onset of allometric advancement. In rats and mice, the mammary ducts begin to grow allometrically (3.5–5 times that of the rest of the body) after around 4 wk of age, which continues to 40 and 56 d of age, respectively (Flux, 1954; Sinha and Tucker, 1966). In humans, the onset of allometric breast development (thelarche) occurs with the onset of puberty, which can be defined by outward appearance of the breasts according to the Tanner classification (De Sanctis et al., 2016). Notably, thelarche in girls occurs earlier in the present day, coincident with earlier onset of puberty as a function of factors including improved nutrition (Villamor and Jansen, 2016). In heifers, allometric growth (3.5 times faster than metabolic live weight gain) starts around 2 to 3 mo of age and continues until around 9 mo of age, concomitant with the onset of puberty (Sinha and Tucker, 1969; Meyer et al., 2006). In ewe lambs, allometric growth commences between approximately 8 and 16 wk of age (Anderson, 1975). Although these are generalized descriptions for each species, it is likely there is also variation within species, which requires further resolution. It has been proposed that age at the onset of puberty in heifers can be genetically linked, likely as a function of BW differences (Macdonald et al., 2007), whereas others suggest that a greater predisposition to accrete lean mass (and lesser deposition of fat) may explain differences among genotypes (Lohakare et al., 2012). Similarly, differences exist in the regulation of allometric growth across different strains of mice (Berryhill et al., 2012; Hadsell et al., 2015), which is at least partly independent from any contribution from BW (Hadsell et al., 2015).

The onset of allometric growth is widely held to begin in response to the onset of ovarian cyclicity, as deduced from ovariectomy experiments across a range of species (Hovey et al., 2002). An exception appears to be sheep, which undergo allometric mammary growth even after ovariectomy (Ellis et al., 1998). The primary signal from the ovaries is likely elevated concentrations of circulating estrogen, consistent with the demonstration that genetic deletion of the estrogen receptor (Mallepell et al., 2006), or its pharmacological inhibition (Tucker et al., 2016) blunts ductal progression. Certainly the converse is true, where exogenous estrogen can restore ductal elongation and TEB development in a wide range of species (Hovey et al., 2002). A noteworthy side note is that many of these studies have used supraphysiological doses of 17β-estradiol to establish this response, whereas many studies in reproductive biology use lower doses that more closely mimic those seen in the cyclic female.

Although the rate of ductal elongation is most pronounced during allometric growth, it is worth noting that the epithelium within the ductal network also proliferates and branches during and after this phase. Depending on the species, branching is manifest either as more sympodial branching of the TDLU structures (as in ruminants, humans, and pigs) or as secondary and tertiary lateral branching from ducts (as is frequently recorded in mice and rats; Hovey et al., 1999; Rowson et al., 2012). Waves of branching morphogenesis and mitosis continue to occur during each estrous cycle; in humans and rats, the greatest amount of epithelial proliferation occurs during the luteal phase (Söderqvist, 1998), whereas in immature rats, there was also clear evidence for increased proliferation of epithelium within TEB during estrus (Dulbecco et al., 1982). In heifers, the DNA content of the mammary gland was recorded to be greatest at estrus (Sinha and Tucker, 1969).

GENETIC FACTORS REGULATING DUCTAL GROWTH

Although development of the mammary ductal network is influenced by a variety of factors, there is clearly a genetic component that contributes to the extent of ductal growth. This consideration is first evident in a range of species during embryogenesis that leads to inappropriate positioning of teats/nipples along the mammary lines (Veltmaat et al., 2013). Furthermore, strains of male mice differ in the presentation and penetrance of ductal development, where some males lack development of the ductal apparatus and others have variable presence of the ducts at different teats when examined during adulthood, whereas others have a considerable number of ductal branches (Nagasawa et al., 1988). Similarly, recent work by Hadsell and colleagues demonstrated that numerous strains of female mice also differ in the extent of their ductal development (Hadsell et al., 2015). A similar situation exists in beef and dairy cattle; although the extent of ductal development in various breeds has not been fully characterized, there is widespread appreciation that dairy breeds develop a mammary parenchyma that is larger and penetrates farther into the mammary fat pad than that of their beef breed counterparts (Turner, 1952). Although the basis for these genetic differences are likely a function of altered endocrine or local signals, their changes warrant consideration for understanding the nutritional regulation of mammary growth and interactions that might be at play across different regulatory mechanisms.

OBESITY, EXCESS DIETARY ENERGY, AND PLANE OF NUTRITION

The impact of a female's plane of nutrition and her metabolic state on mammary growth has been studied in a variety of models and applied to a variety of translational endpoints. From a human health perspective, the obesity epidemic has led to questions about lifetime breast cancer risk. From an agricultural perspective, decades of discussion have centered around the question of how plane of nutrition and excess fatness impacts mammary development in heifers during allometric growth. From a basic science perspective, numerous studies have investigated the effects of caloric intake on mammary growth in rodents and implications for tumorigenesis.

In rodents, an elevated plane of nutrition reduces the extent of ductal development, likely as a function of reduced metabolic size of the female (Engelman et al., 1994; Zhu et al., 1999). Conversely, in obese mice, the volume of the mammary fat pad was increased, yet the extent of ductal branching was reduced (Kamikawa et al., 2009). When assessed as future breast cancer risk, the link between caloric intake and breast development in humans is somewhat surprising, given that obesity is typically implied to confer increased risk for various diseases. For breast cancer risk, the impact of obesity depends on age and the stage of breast development. In postmenopausal women, there is clear evidence that obesity increases the chance of developing breast cancer (Matthews and Thompson, 2016). The opposite is true for women who were obese during childhood (Matthews and Thompson, 2016). Such an outcome is counterintuitive given that obese girls typically enter menses earlier and would therefore be exposed to ovarian secretions at a younger age, potentially increasing the extent of hormone-induced parenchymal development. The most likely hypothesis seems to be that obesity suppresses the rate of epithelial mitosis in the prepubescent/pubescent female, thereby reducing the opportunity for mutagenesis to occur. This paradox may be explained by our understanding of mammary development in ruminants, including heifers.

For decades, the question of how excess nutrition fed to heifers impacts mammary development built on the observation by several groups that a high plane of nutrition/gain led to reduced development of the mammary parenchyma in combination with a "fatty udder" (Sejrsen et al., 2000). A similar outcome has been recorded in ewe lambs (Johnsson and Hart, 1985) and beef heifers (Johnsson and Obst, 1984). The phenomenon attracted considerable attention because of the finding that the excess fat accumulation and associated reduced parenchymal development also translated to reduced milk yield during lactation (Sejrsen et al., 2000). More recent data suggest that excess rate of gain does not impair the rate of parenchymal development per se but rather induces puberty at an earlier age, thereby shortening the window of time available for allometric growth to proceed (Meyer et al., 2006). However, it is fair to say that further studies are needed to fully complete this picture, particularly given that a primary goal of dairy producers is to have heifers bred at as early an age as possible, leading to the continued potential for mammary development to be suppressed in these females.

Combined, these studies appear to paint a picture that excess rate of gain/obesity suppresses one or more aspects of mammary development during the peripubertal period across a range of species. Whether there is a common, conserved mechanism, such as a shortened period for allometric growth resulting from the early physiological attainment of puberty remains to be confirmed. Certainly, endocrine changes likely also underlie and contribute to the impairment of mammary growth, where heifers fed a high plane of nutrition had reduced serum GH concentrations (Sejrsen et al., 1983); ironically, IGF1 concentrations are increased by this same treatment. These same authors also identified that prolactin concentrations were inversely correlated with the extent of mammary development but dismissed these changes by apparently accounting for glucocorticoid and insulin concentrations (Sejrsen et al., 1983). Further insight into the endocrine basis for this effect may come from studies of humans, for whom the rate of prepubertal obesity is on the rise. For example, prepubertal obesity leads to earlier adrenarche, reduced concentrations of circulating sex hormone-binding globulin and increased free sex steroids, increased insulin resistance, and altered circulating concentrations of adipokines such as leptin that modulate hypothalamic–pituitary activity and ovarian function (Dunger et al., 2006).

Alternatively, a myriad of local negative changes may also be at play, including those associated with increased fat deposition in the mammary fat pad, which could physically impede parenchymal development by modifying components of the extracellular matrix (Kamikawa et al., 2009) or change the chemical microenvironment by altering the stromal synthesis of various growth factors, enzymes, or inflammatory cytokines (Ford et al., 2013). Further discussion of some of these candidate mechanisms and pathways follows below.

THE ROLE OF THE MICROENVIRONMENT IN MEDIATING THE EFFECTS OF DIET ON DUCTAL GROWTH

One recurring finding over the last 50 yr has been the highlighted importance of the stromal microenvironment in support of ductal growth and development. Perhaps the most convincing demonstration of a crucial function for the adipose stroma comes from transplantation studies in mice showing that mammary epithelial cells have a unique requirement for adipose tissue in which to grow and elongate (Hoshino, 1978). Such a finding continues to be relevant given the recent demonstration that an extract from the mammary fat pad and its associated glandular elements, but not from other adipose depots, was able to support the establishment of transplanted testicular stem cells (Bruno et al., 2017). There also seems to be important species-specific requirements for the type of mammary stroma, as evidenced by the fact that the mouse mammary fat pad must be "humanized" with human stromal fibroblasts to support the growth of human breast epithelium (Proia and Kuperwasser, 2006), where similar relationships have also been described for bovine mammary epithelium (Rauner and Barash, 2016).

Much remains to be appreciated about how the adipose microenvironment regulates ductal growth and, in turn, how diet affects both of these aspects. The mammary fat pad is clearly a source of growth-regulatory molecules able to influence cell growth and ductal morphogenesis, including unsaturated fatty acids, various paracrine growth factors such as IGF1, hepatocyte growth factor, and fibroblast growth factors and extracellular matrix components and enzymes able to alter the physical environment (including matrix metalloproteases and cathepsins; Hovey et al., 1999; Hovey and Aimo, 2010). Furthermore, the amount and metabolic activity of adipose tissue can impact the form and extent of mammary ductal development, as illustrated in mice with a targeted disruption of adipocyte formation (Couldrey et al., 2002). The metabolic state of the mammary adipose tissue also contributes to the function and morphology of the ductal network. For example, mice with stage-specific depletion of adipocytes had either impaired TEB formation or precocious formation of side branching and milk protein production, depending on when adipocytes were ablated (Landskroner-Eiger et al., 2010). Given the dynamic nature and metabolic fluctuation within the adipose microenvironment, it is not surprising that it remains difficult to directly implicate these cells and their metabolism in any response or responses by the mammary epithelium.

More recently, attention has been directed to how partnering cell types can co-occupy the stroma and modulate epithelial proliferation. Among the various immune cells that infiltrate the mammary stroma and the ductal network, eosinophils and macrophages play a key role in the direction of ductal development (Sun and Ingman, 2014). In the absence of epithelium-derived colony stimulating factor, macrophages were not recruited to the vicinity of TEB and failed to activate an *EGFR*-dependent crosstalk between the cell types (Coussens and Pollard, 2011). Moreover, we now understand that the adipose tissue itself functions as a metabolically active organ that is susceptible to dysregulation during states such as obesity, leading to local alterations in inflammation, lipid metabolism and metabolic flux, angiogenesis, and recruitment and activation of cells such as macrophages (Cleary, 2013; Ford et al., 2013).

The physical environment of the stroma and the basement membrane also likely provides important cues for ductal development and allometric growth. The amount of fibrous connective tissue in the adipose stroma of the mammary glands varies across species, as does the different forms of stroma within the TDLU (Rowson et al., 2012). The extracellular matrix from different species can differentially regulate the morphogenic behavior of epithelial cells in culture (Dhimolea et al., 2012), as can the ratio of various extracellular matrix components (Campbell et al., 2011). Differential abundance of these components alters the physical "stiffness" of the matrix, which, in turn, modifies cellular behavior (Tung et al., 2015). Although relatively little is known about how these matrix variations affect ductal elongation in vivo, the abundance of connective tissue elements is a key determinant of the fibroglandular elements that can be visualized by mammographic imaging of the human breast (Ironside and Jones, 2016), where parenchymal development and breast cancer risk are proportional to the relative abundance of this compartment (Ironside and Jones, 2016). One might expect that similar relationships exist in the mammary glands of domestic livestock.

STIMULATION OF DUCTAL GROWTH BY DIETARY FATTY ACIDS AND *TRANS*-10, *CIS*-12 CLA

Although total caloric intake certainly can impact ductal growth, the type of dietary fat can also affect the extent and nature of ductal development, as evidenced in vivo and in vitro. Generally speaking, diets enriched with unsaturated fatty acids tend to promote ductal development in mice (Welsch and O'Connor, 1989), whereas those containing elevated concentrations of saturated fats are typically inhibitory (Welsch and O'Connor, 1989). These findings align well with the effects of various fatty acids on mammary epithe-

lial cell proliferation in vitro (Wicha et al., 1979). In an interesting parallel study, ewe lambs fed rumen-protected PUFA had similarly increased mammary growth (McFadden et al., 1990).

Conjugated linoleic acids in the isomeric forms *cis*-9, *trans*-11 (*c*9,*t*11-CLA) and *trans*-10, *cis*-12 (*t*10,*c*12-CLA) originally attracted attention due to their ruminal biosynthesis and appearance in ruminant meats and milk followed by the subsequent demonstration of their anticancer properties in chemically induced and xenografted models of mammary cancer (Ip et al., 2001; Kelley et al., 2007). At the same time, *t*10,*c*12-CLA has become a widely used weight loss supplement due to its ability to promote lipolysis (Lehnen et al., 2015); in rodents, *t*10,*c*12-CLA also stimulates hepatic steatosis, hyperlipidemia, and hyperinsulinemia similar to certain aspects of metabolic syndrome (den Hartigh et al., 2017).

Given these effects of CLA on mammary tumorigenesis and the potential human health impacts of CLA health supplements, we investigated whether CLA could modulate aspects of normal ductal development. Pubescent female mice fed *t*10,*c*12-CLA at a rate of 1% had a small increase in ductal elongation over that induced during normal allometric growth (Berryhill et al., 2012), whereas there was a lesser effect of *c*9,*t*11-CLA. We subsequently fed these CLA to females that were ovariectomized prior to puberty. Interestingly, the mammary ducts in mice fed *t*10,*c*12-CLA initiated allometric growth evidenced by the formation of TEB at the ductal termini. Also, *c*9,*t*11-CLA and other *trans*-fats such as those found in partially hydrogenated vegetable oils did not elicit a similar effect (Berryhill et al., 2017b), coincident with their inability to evoke pronounced metabolic dysregulation. We subsequently showed that the mammogenic effect of *t*10,*c*12-CLA reflected IGF1R-dependent signaling alongside diet-induced hyperinsulinemia and that the effect of *t*10,*c*12-CLA could be ameliorated by rosiglitazone, a PPAR-γ agonist (Berryhill et al., 2012).

Potentially, the most important outcome from these experiments demonstrating diet-induced ductal growth was the finding that the response was entirely independent of estrogenic stimulation, given that allometric growth occurred not only in mice that were ovariectomized prior to puberty but also in mice that were co-treated with the ER antagonist ICI 182,780 or the aromatase enzyme inhibitor letrozole (Berryhill et al., 2012). Notably, *t*10,*c*12-CLA never induced uterine hypertrophy, further emphasizing that ductal elongation occurred in the absence of estrogenic stimulation.

More recently, we sought to resolve the mechanism or mechanisms by which *t*10,*c*12-CLA evokes estrogen-independent ductal growth by directly comparing the acute spatiotemporal response to *t*10,*c*12-CLA with that induced by estrogen (Berryhill et al., 2017a). In these mouse studies, we compared oral delivery of dietary *t*10,*c*12-CLA with orally administered low-dose 17β-estradiol at various intervals during the first 180 h of exposure. Our goal was to establish exactly when the ductal epithelium first initiated a histomorphogenic response to the 2 treatments in the form of TEB development. To answer this question, we detected cellular proliferation within the entire ductal network by labeling dividing cells with 5-ethynyl-2-deoxyuridine and then visualized and quantified the fluorescent signal using widefield epifluorescence (Berryhill et al., 2016). Using this approach, we were able to obtain tissues enriched for the proliferating epithelium from estrogen- and *t*10,*c*12-CLA–stimulated mammary glands at the precise points when epithelial proliferation was initiated, with the goal of comparing gene expression profiles in each of these treatments using RNA sequencing.

From the outset, we anticipated that these treatments would use common gene expression networks to direct cellular proliferation. Indeed, this was the case, whereby estrogen- and *t*10,*c*12-CLA induced signaling pathways downstream of key mitotic regulators such as Tp53. At the same time, RNA sequencing allowed us to identify gene expression networks that were unique to either estrogen- or *t*10,*c*12-CLA–induced proliferation. Not surprisingly, estrogen induced gene networks previously reported in the literature as being upstream of target genes including amphiregulin and the progesterone receptor, but these were not affected by *t*10,*c*12-CLA. Conversely, *t*10,*c*12-CLA induced significant gene expression changes that aligned with pathways associated with immune responses, including genes downstream of colony stimulating factor 2.

Combined, these unique experiments unexpectedly emphasized that certain dietary components may be able to directly impact ductal development in the mammary glands. Although *t*10,*c*12-CLA is not abundant in the food chain, its widespread use as a supplement raises a host of questions regarding its impact on human and even animal health. Any role for *t*10,*c*12-CLA during mammary growth in domestic livestock is unknown.

EFFECTS OF A DIETARY ALCOHOL ON MAMMARY GROWTH IN A PIG MODEL OF HUMAN BREAST DEVELOPMENT

An often-overlooked dietary component that increases breast cancer risk and appears to affect the normal developing mammary glands is alcohol. Although this lifestyle choice is unique to breast development in humans, the opportunity to study its effects on the

mammary glands of pigs represents a unique window into the physiological factors regulating mammary growth. Epidemiological studies have highlighted that a woman's risk for developing breast cancer is directly proportional to the amount of alcohol she regularly consumes, such that her relative risk for the disease increases by approximately 10% for each drink regularly consumed (Mostofsky et al., 2016). Yet the impact of alcohol on the normal developing mammary glands prior to pregnancy or before a round of lactation-associated development has remained far from clear. Surprisingly, this uncertainty exists despite the fact that there is now a much higher frequency of young women who regularly drink, raising the likelihood that this behavior may have a long-term impact on breast cancer risk across populations (Colditz et al., 2014).

A challenge in addressing this question is the fact that most animal species do not voluntarily consume alcohol per os. Studies in rodents have typically administered lower concentrations of alcohol in the drinking water to address this limitation, which has provided insights to the consequences for glandular development that include increased ductal proliferation in TEB (Singletary and McNary, 1992; Polanco et al., 2011). An alternative approach for ascertaining the effects of alcohol on mammary development is to examine its effects in a species that is more amenable to intake, such as pigs. To this end, we fed female pigs a standard diet with supplemental ethanol for a period of 4 wk and then assessed epithelial proliferation in the mammary glands alongside histomorphological changes (Schennink et al., 2015). The TDLU from alcohol-fed females showed increased epithelial proliferation that was associated with the accumulation of eosinophilic secretion in the ductular luminae, alongside progression of less developed TDLU type 1 to more advanced TDLU type 2. Although the basis for these changes is still being explored, candidate mechanisms for how alcohol increases parenchymal progression include increased circulating estrogen and/or prolactin. The established effects of these hormones on TDLU development in the mammary glands of pigs (Horigan et al., 2009) would be consistent with the alcohol-stimulated phenotype. Alternatively, as outlined above for excessive fattening in ruminants and for the effects of *t*10,*c*12-CLA in rodents, the inflammatory microenvironment of the mammary adipose tissue also appears to be altered in response to alcohol (Steiner and Lang, 2017), potentially increasing the provision of local growth stimulatory signals to the ductal epithelium. Collectively, these data highlight that various dietary elements, whether they be various fats or alcohol, can stimulate ductal development, possibly through modification of the local inflammasome.

DIET AS A MODIFIER OF THE EPITHELIAL RESPONSE TO ENDOCRINE AND LOCAL CUES

Although dietary components can impact ductal development, many of these so-called effects, whether they be pronounced or subtle, may well arise through the modification of other critical signals to which the epithelium responds. For example, dietary energy intake modifies the circulating concentration of numerous hormones implicated in the regulation of ductal growth including IGF1, GH, leptin, thyroid hormone, and the ovarian steroids (Sejrsen et al., 1983; Cleary, 2013). Similarly, the number of receptors for many of these hormones is altered by nutritional state. The effects of diet can also be realized intracellularly; for example, signaling involving intermediates such as various tyrosine kinase receptors, IRS1, IRS2, MTOR, STAT3, AKT1, and MAPK1 are all activated, amplified, or repressed under various nutritional states (Chen, 2011). The observation that the mammary fat pad serves as a potential reservoir of fatty acids also represents a unique situation among all tissues whereby lipids or their derivatives might impact the signaling and responsiveness of adjacent epithelial cells to external cues such as hormones and growth factors (Hovey and Aimo, 2010). At the same time, we still have only a cursory understanding of how specific nutrients, particularly various micronutrients or those that may be deficient in different diets, might affect ductal development. For example, zinc deficiency negatively impacts ductal development in mice in association with an altered microenvironment (Bostanci et al., 2015). Along similar lines, cadmium stimulates development of the ductal network in female mice by acting as an estrogen mimic (Johnson et al., 2003). These 2 examples highlight the great deal of uncharted territory that exists for our understanding of how specific nutrients impact mammary growth. Considering the range of micro- and macronutrients and contaminants in groundwater and feedstuffs across the world, one might raise the question of how this variation affects the mammary glands of humans, livestock, and wildlife alike.

AREAS OF OPPORTUNITY, AND QUESTIONS,FORUNDERSTANDINGDUCTAL GROWTH AND ITS REGULATION

Although our appreciation for how ductal growth is regulated has advanced tremendously, particularly on the heels of its role in the origins of breast cancer, there are still considerable gaps in our understanding, particularly when it comes to nutritional regulation, species differences, and their interactions. One key limitation

to progress has been the insufficiency of models, particularly in vitro, for studying the processes of ductal development. Although alveolar development in mice can be studied using the whole organ culture system that retains all aspects of the epithelial–stromal relationship (Atwood et al., 1995), extensive unpublished efforts by ourselves and others (using supplements up to, but short of, the veritable "kitchen sink") have all failed to develop conditions whereby the mammary ducts and TEB develop ex vivo in the mammary fat pad in situ. The key factor or factors limiting ductal growth in this system remain to be established, but such an approach, if realized, would be powerful for separating the genetic, nutritive, and systemic contributions. Most efforts to model TEB formation and ductal elongation in vitro continue to rely on 3-dimensional matrices based on either collagen or Matrigel (Becton Dickinson, Franklin Lakes, NJ; Nguyen-Ngoc and Ewald, 2013). These environments certainly mimic some aspects of ductal formation in vitro, including structures that partly resemble TEB or TDLU (Nguyen-Ngoc and Ewald, 2013). However, one must be cautious about interpreting such data, because "resemblance" does not guarantee "authentic mimicry," and hence, conclusions must be carefully drawn. In particular, the collagen/Matrigel 3-dimensional environment lacks many of the components and cell types found in vivo including, but not limited to, the stromal vasculature and lymphatics, adipocytes, and immune cells. Efforts to reconstitute these components, including in bioengineered scaffolds, continue to present new opportunities for recreating and studying the ductal network and stand to provide the means needed to ascertain how diet directly affects its growth.

At the same time, mammary gland biologists are extremely fortunate to have access to a pliable system for studying ductal development in vivo by transplanting cells into the supporting mammary stroma including that which has been cleared of the native epithelium, or the so-called "cleared" mammary fat pad (Hovey et al., 1999). This method continues to provide great utility for mouse biologists for testing the consequence of genetic modifications of not only the epithelium but also the stroma, particularly when combined with methods to sort epithelial cells into various types and/or lineages prior to transplantation (Buric and Brisken, 2017). Similarly, the ability to introduce additional genetic modifications into the epithelium using a lentivirus delivery system provides a powerful tool to study the genetic basis of mammary growth and, furthermore, how diet affects the associated processes. Until recently, these approaches, outside of xenografting experiments for transplanting nonmouse epithelium into immunodeficient mice, have been limited to mice. However, the principles of mammary gland transplantation (outside of the luxury of syngeneic animals) remain the same for other species, including livestock. To this end, we have been able to successfully adapt the cleared mammary fat pad approach to both sheep (Hovey et al., 2000) and pigs (Rowson-Hodel et al., 2015) and have recently reported on our ability to use lentivirus to genetically modify the mammary epithelium of pigs ex vivo prior to its reconstitution in the mammary glands in vivo (Rowson-Hodel et al., 2015). Although these strategies in large animals are expensive and demanding, they should help resolve the many unanswered questions faced by animal scientists regarding species-specific aspects of mammary growth and its dietary regulation.

The cellular origins of the ductal network and the types of epithelial cells populating it and directing its expansion (so-called stem cells/progenitors) remain contentious, oftentimes more so as a function of the methodologies and nomenclature in use. Outside of this confusion, considerable room still exists to define how diet and the associated mechanisms might affect the cellular hierarchy within the developing mammary gland (Ford et al., 2013). At the same time, these cells likely carry different genetic signatures and, hence, would be expected to differ in their sensitivity to a changing nutritional state or microenvironment, possibly as "nutrient sensing" precursors. Alternatively, cells with different genetic signatures might also be sensitive to different nutritionally directed epigenetic modifications, which would then influence the state and function of the mature gland, through into lactation. From a management standpoint, these considerations raise some interesting questions for livestock industries. For example, do strategies such as nutritional "flushing" to improve fertility modify ductal growth and future lactation potential? For that matter, to what degree does the extent of allometric ductal growth dictate future milk yield? Ironically, this question still remains unanswered; although there is no doubt formation of the ductal network is critical for future lactation, the degree of plasticity that exists to counter adverse events such as impaired nutrition or to capitalize on increased growth during this same phase is uncertain.

SUMMARY

From their very beginning, the mammary glands are invested in the successful establishment of a ductal framework that forms the basis for the future milk-secreting organ. This development is directed by a wide array of systemic, local, physical, and chemical cues acting on the target epithelium. Central to many of these cues is diet, which has the potential not only to directly affect the growth and function of the epithelium but also to modulate the effects of the many other

signals acting on these target cells. Ultimately, these effects are manifest in many ways but perhaps most importantly intersect with the translational endpoints of animal management and lactation as well as for human health in the form of breast cancer risk.

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