Mammary gland development—It's not just about estrogen¹

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ABSTRACT

The mammary gland (MG) is one of a few organs that undergoes most of its growth after birth. Much of this development occurs concurrently with specific reproductive states, such that the ultimate goal of milk synthesis and secretion is coordinated with the nutritional requirements of the neonate. Central to the reproductive–MG axis is its endocrine regulation, and pivotal to this regulation is the ovarian secretion of estrogen (E). Indeed, it is widely accepted that estrogens are essential for growth of the MG to occur, both for ductal elongation during puberty and for alveolar development during gestation. As the factors regulating MG development continually come to light from the fields of developmental biology, lactation physiology, and breast cancer research, a growing body of evidence serves as a reminder that the MG are not as exclusively dependent on estrogens as might have been thought. The objective of this review is to summarize the state of information regarding our understanding of how estrogen (E) has been implicated as the key regulator of MG development, and to highlight some of the alternative E-independent mechanisms that have been discovered. In particular, we review our findings that dietary *trans*-10,*cis*-12 conjugated linoleic acid promotes ductal elongation and that the combination of progesterone (P) and prolactin (PRL) can stimulate branching morphogenesis in the absence of E. Ultimately, these examples stand as a healthy challenge to the question of just how important estrogens are for MG development. Answers to this question, in turn, increase our understanding of MG development across all mammals and the ways in which it can affect milk production.

Key words: prolactin, progesterone, mammary epithelial, conjugated linoleic acid

INTRODUCTION

The mammary glands (MG) are unique among organs with respect to the large amount of postnatal development they undergo. The extent of epithelial proliferation within the gland is massive and often occurs during a very short interval; this topic has been reviewed extensively elsewhere (Hovey et al., 2002; Yart et al., 2014). These periods of allometric growth coincide with critical periods of reproductive development. The first is the beginning of allometric growth around the onset of puberty. Depending on species, growth in this period accomplishes expansion of the parenchymal ductal network via elongation and arborization of the ductal system. The second phase initiates specifically during pregnancy when the parenchyma expands allometrically in association with expansion of the alveolar population in anticipation of lactogenesis.

THE CASE FOR ESTROGENS AS PRIMARY REGULATORS OF MG DEVELOPMENT

There are many clear demonstrations that the ovaries are essential for the normal MG to develop, not least the fact that ovariectomy (OVX) of females from a variety of species, including rodents (Hovey et al., 2002), ruminants (Berry et al., 2003; Yart et al., 2014), and nonhuman primates (Cline et al., 1996), halts MG growth. An interesting exception appears to be sheep, which continue to undergo MG growth during the prepubertal period following OVX (Ellis et al., 1998).

Estrogens (E) are key contributors to the 2 aforementioned phases of allometric MG development. During elongation of the ductal network around the onset of puberty, activation of the hypothalamic–pituitary–gonadal axis leads to increased epithelial proliferation at the termini of ducts, either as terminal end bud (TEB) structures characteristically present at the leading edge of the mammary ducts in rodents, pigs, and humans (Hovey et al., 2002; Rowson et al., 2012) or as a peripheral zone of mitosis within the more complex terminal ductal lobular unit structures found within the MG of female ruminants such as heifers and ewe lambs (Ellis and Capuco, 2002). The ability of E to promote TEB formation in rodents is particularly pronounced: de-

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pending on the dose and route of exposure, the mitotic rate in epithelial cells following E exposure increases from <1 to >50% in a matter of days (Daniel et al., 1987). In heifers, the effect of OVX or exogenous E on mammary epithelial cell (MEC) proliferation is much less pronounced; OVX reduced the rate of mitosis from ~3 to ~1% (Meyer et al., 2006a), whereas exogenous E only increased the rate of mitosis from ~8 to ~14% (Capuco et al., 2002) or from ~3 to ~8% (Meyer et al., 2006a). During gestation, there is an equal requirement for estrogens to realize full lobulo-alveolar development, where hormone replacement experiments in rats demonstrated that the combination of E, progesterone (P), prolactin (PRL), and growth hormone (GH) was required for full development to occur (Lyons et al., 1958).

THE CASE FOR ESTROGEN-INDEPENDENT PATHWAYS DURING MG DEVELOPMENT

As summarized above, estrogens have undeniable effects on the MG epithelium. However, examples also exist that raise the question of whether MEC proliferation and development of the MG can occur independently of estrogens or at least with only partial dependence on them. The following review seeks to highlight these examples.

The In Vitro Paradox

Growth of the MG ultimately reflects an increase in the number of MEC within the parenchyma. In turn, the ability to grow and culture these cells provides an important tool for studying the regulation for normal MEC growth. However, one discrepant issue has been the longstanding inability to replicate several aspects of E-induced mammary growth in vitro.

An analysis of E receptor (ER) expression in vivo indicates that most ER within the MG are expressed by MEC, primarily in a luminal population (Anderson et al., 1998) and are also expressed by cells within the supporting adipose stroma (Hovey et al., 1999). The presence of epithelial ER raises the question of their function, where one might surmise that treatment of isolated MEC with E would promote their proliferation. However, this expectation is not the case for most studies of normal MEC in vitro. When normal MEC isolated from different species, including mice and humans, were treated with E, they failed to demonstrate a robust proliferative response over a range of E concentrations (Haslam and Levely, 1985; Richards et al., 1988; Xie and Haslam, 1997). One consistent observation during these studies is that normal MEC frequently lose most of their ER expression upon their

isolation and during culture (Xie and Haslam, 1997), possibly explaining the loss of E-responsiveness in vitro. An alternative hypothesis is that ER within the surrounding stromal environment, or these stromal cells in some other capacity, are required to mediate a proliferative effect of E, where exogenous E promotes a parallel proliferative burst in stromal cells surrounding the epithelium of mice (Woodward et al., 1998). Interestingly, in heifers, a round of E-induced stromal proliferation occurs after the phase of epithelial proliferation (Woodward et al., 1993). Several lines of evidence suggest that stromal cells (Haslam and Levely, 1985) or extracellular matrix proteins (Novaro et al., 2003) are required to confer a proliferative response to E by MEC in vitro. In many other ways, however, the magnitude of the E-induced proliferative response in the mouse MG in vivo is yet to be recapitulated in vitro. As a case in point, even when entire intact MG from mice are cultured in vitro using a whole-organ culture system, they fail to undergo ductal elongation or formation of TEB in response to supplemental E, in the presence or absence of GH or IGF-1, despite having a full complement of epithelial and stromal cell types (Vonderhaar, 1984). The suggestion that the effects of E on the MG may not be direct is further emphasized by responses to hormone priming of donor mice before organ culture; priming nulliparous mice with either E or P fails to facilitate hormone-induced responsiveness, which can only be achieved by priming donor animals with both E and P. Thus, the paradox exists that E, either alone or with its mediators IGF-1 and GH, cannot fully recapitulate the growth-promoting effects in vitro that it promotes in vivo.

A Paradox for the Role of E During Ductal Elongation In Vivo

Despite a clear role for E during allometric growth of the MG, a series of classic experiments highlighted that in vivo, E alone is ultimately unable to promote growth of the MG. Specifically, whereas abolition of MG development by OVX could be rescued by exogenous E, this response could not be achieved in females that were hypophysectomized (Lyons et al., 1958; Nandi, 1958). Subsequent experiments confirmed the combined requirement for the ovarian hormone E and pituitary GH during ductal development in rodents and that these hormones synergistically induce local synthesis of IGF-1 in the stromal microenvironment (Kleinberg and Ruan, 2008). In turn, exogenous IGF-1 can stimulate the proliferation of MEC in vivo to recapitulate the formation of TEB (Kleinberg and Ruan, 2008). The important conclusion from these experiments was that IGF-1 is the primary effector of mammary ductal growth during

puberty, downstream of the effects of E and GH, at least in rodents.

The Promotion of Ductal Growth by P Independent of E

Whereas the effects of E have garnered widespread attention for their ability to recapitulate ovariectomy-ablated ductal development, limited attention has been given to the ability of P to achieve a similar outcome. In fact, the actions of P alone were often assumed to be exclusively dependent on E, given that P administered to OVX mice failed to stimulate MEC proliferation as measured by ^3H -thymidine uptake (Haslam, 1988). However, this finding contrasts with results from other laboratories. For example, Skarda et al. (1989) found that exogenous P administered to C3H mice stimulated ductal growth in both males and females, and we showed that P induced formation of TEB in prepubertal BALB/c mice (Atwood et al., 2000). Furthermore, when mice lacking IGF-1 were administered exogenous P and IGF-1, their MG underwent extensive ductal elongation (Ruan et al., 2005). The effects of P on the developing mouse MG were most recently described in detail by Aupperlee et al. (2013). Those authors showed that P (0.1 mg, representing one-tenth of the amount used in most other studies) administered to OVX prepubertal female mice stimulated increased epithelial proliferation and TEB formation in the absence of E.

Taken together, these findings lend support to the notion that, independent of E, P can promote ductal elongation in the MG, at least in mice. Whether this effect of P is mediated exclusively by the local production of IGF-1 remains unclear (Ruan et al., 2005). Interestingly, a recent report indicated that the effects of P on mammary tumorigenesis is due to the P metabolite 5- α dihydroprogesterone as a result of the action of 5- α reductase (Wiebe et al., 2015); whether this metabolite serves to regulate normal MG development remains to be determined. Furthermore, the effects of P on ductal elongation may well be mediated by local synthesis of various growth regulatory molecules. For example, progestins induce the local synthesis of GH within the MG of dogs (Mol et al., 2000), whereas the co-localization of elevated P receptor (**PR**), GH, and IGF-1 in feline mammary tumors (Queiroga et al., 2008) suggests the presence of a local paracrine loop involving these molecules. Others showed that P-induced ductal elongation is mediated by the local synthesis of amphiregulin in the ductal epithelium that, in turn, recruits leukocytes that have been implicated in the regulation of ductal elongation (Aupperlee et al., 2013). A potential relationship between P-induced ductal elongation and a role for the innate immune system was also recently

borne out in our studies of mice lacking STAT1 (signal transducer and activator of transcription 1) that have a reduced capacity to undergo ductal elongation and an increased susceptibility to evolving mammary neoplasias that are uniquely ER-positive (Chen et al., 2015). Specifically, we found that their impaired ductal elongation, that was partly a function of a modified stromal environment, could be rescued by supplemental P + PRL (Chen et al., 2015). These relationships further highlight the concept that cross talk between epithelial and stromal cells, as well as interactions between the effects of individual hormones, may be just as important during ductal elongation as the singular effect of a hormone such as E.

A CASE FOR DIET-INDUCED, E-INDEPENDENT DUCTAL ELONGATION

The above discussion provides evidence that elongation of the mammary ducts may not ultimately be dependent on E per se, and could be promoted by other factors such as P or local mediators of hormone action, particularly IGF-1. An understanding of this hierarchy then raises the question of whether other influences relevant to reproductive development, physiology, or disease can elicit similar outcomes in the MG.

The relationship between diet and MG development has been widely studied and debated, including in livestock (Daniels et al., 2009), and it bears a broad relevance to an understanding of lifestyle effects on breast cancer risk in humans. The complexity of this situation partly reflects the wide range of consequences that diet and its composition has on females, ranging from its effects on reproductive development and pubertal onset (Meyer et al., 2006b) through to long-term effects on metabolic health and disease (Park et al., 2014). Although these consequences have been reviewed elsewhere, their effect on normal MG development remains understudied. Here we provide one such example wherein an isomeric form of linoleic acid leads to the surprising promotion of E-independent MG development.

Dietary CLA and MG Development

Conjugated linoleic acids are derivatives of linoleic acid (18:2n-6) with conjugated double bonds in the *cis* or *trans* configurations (Ha et al., 1987). Although many positional and geometric isomers of CLA exist, *cis*-9,*trans*-11 CLA (**9,11 CLA**) and *trans*-10,*cis*-12 CLA (**10,12 CLA**) are the most biologically active forms (Pariza et al., 2001). Moreover, 9,11 CLA is the most common CLA isomer in the diet of humans (Pariza et al., 2001), in which isomers of CLA (primarily as 9,11 CLA) constitute a small, but significant, component of

fats derived from the meat and milk of ruminant animals (Chin et al., 1992). The combined levels of CLA in most dairy products range from 2.5 to 7.0 mg/g of fat (Lin et al., 1995), whereas meat from ruminants contains 2.7 to 5.6 mg of CLA/g of fat (Chin et al., 1992). Plant oils also contain small amounts of CLA (0.1–0.7 mg/g of fat) (Chin et al., 1992). The profile of CLA isomers also differs between plant oils and fats derived from ruminants: 9,11 CLA comprises ~70 to 90% of all CLA in meat and dairy products, whereas 9,11 CLA and 10,12 CLA are present in roughly equal amounts in plant oils (Chin et al., 1992). Partially hydrogenated vegetated oils (PHVO) and margarines contain variable amounts of CLA depending upon the hydrogenation conditions used (Mossoba et al., 1991; Jung and Ha, 1999); a commercially prepared PHVO contained 4.24 mg of CLA/g (Banni et al., 1995). Mixtures of CLA have also become widely available as a weight loss supplement (Whigham et al., 2007), in which the 9,11 and 10,12 isomers are predominant and present in approximately equal amounts (Kramer et al., 2004).

Dietary CLA garnered interest as a nutraceutical through its ability to suppress chemical carcinogen-induced mammary tumor burden in rats (Ip et al., 1991, 1994). Feeding butterfat enriched with 9,11 CLA to peripubertal rats also reduced epithelial area and TEB density in the MG (Ip et al., 1999). However, these data contrast strikingly to the subsequent finding that dietary 10,12 CLA enhanced mammary tumor burden and metastasis in mice overexpressing the ErbB2 oncogene (Ip et al., 2007). Moreover, dietary 10,12 CLA increased TEB number and epithelial cell proliferation (Ip et al., 2007) and induced precocious lobuloalveologenesis (Foote et al., 2010). Similarly, we discovered that feeding peripubertal BALB/cJ mice 1% 10,12 CLA stimulated elongation of the mammary ducts (Berryhill et al., 2012).

Dietary 10,12 CLA and E-Independent MG Growth

Our investigation into the mechanism underlying 10,12 CLA-induced ductal development led us to examine effects on the MG in OVX peripubertal mice (Berryhill et al., 2012). Surprisingly, this same diet promoted significant ductal elongation and TEB formation relative to the quiescent and primitive ductal structures induced by OVX (Figure 1), representing the first demonstration that a dietary component could incite ovary-independent MG development. We next investigated whether this effect of 10,12 CLA was a response to E or was mediated by the ER by co-administering the aromatase inhibitor letrozole or the ER-antagonist ICI 182,780. In keeping with an E-independent mechanism, neither compound altered 10,12 CLA-induced mam-

mary growth. In the same way, 10,12 CLA stimulated ductal elongation in the MG of peripubertal male mice (Berryhill et al., 2012).

Mechanisms of CLA-Regulated MG Growth

Dietary intake of 10,12 CLA in growing mice dramatically reduces adiposity (Tsuboyama-Kasaoka et al., 2000; LaRosa et al., 2006), consistent with our demonstration that the mass of the MG fat pad is reduced by ~50% in mice fed 10,12 CLA (Berryhill et al., 2012). This lipolytic response is also accompanied by appreciable inflammation within white adipose tissue (Poirier et al., 2006) and concurrent hepatic steatosis (Clément et al., 2002). Coincident with these systemic metabolic changes is the onset of hyperinsulinemia (Clément et al., 2002; Berryhill et al., 2012) and insulin resistance (Chung et al., 2005). At the level of the MG, such changes are manifest as decreased expression of insulin receptor (IR)-B mRNA, and IR-A and IR-B protein (Berryhill et al., 2012). These findings closely align with epidemiological and experimental evidence pointing to a role for hyperinsulinemia in breast cancer progression and poor disease outcome (Ferguson et al., 2013). Indeed, hyperinsulinemia promoted the growth of normal and hyperplastic MG in a non-obese model of type-2 diabetes (Novosyadlyy et al., 2010). Given the crucial role for peroxisome proliferator-activated receptor gamma (PPAR- γ) as a ligand-activated transcription factor during adipogenesis (Rosen et al., 1999) and its role in insulin sensitivity (Lehmann et al., 1995), we examined the effect of the PPAR- γ agonist rosiglitazone on 10,12 CLA-stimulated MG growth. Rosiglitazone treatment completely abrogated ductal development while also ameliorating the associated hyperinsulinemia and lipoatrophy (Berryhill et al., 2012). Taken together, these findings strongly support a role for PPAR- γ signaling within adipose tissue, potentially at the level of the MG stroma, during E-independent MG growth induced by 10,12 CLA.

Mechanisms of Diet-Induced E-Independent MG Growth

Dietary 10,12 CLA elicits numerous changes in the stromal microenvironment concurrent with the reduction in MG fat pad mass. These changes include the increased local deposition of collagen (Russell et al., 2007), the recruitment of immune cells including macrophages and mast cells (Poirier et al., 2006; Russell et al., 2007; Shen et al., 2013), and an inflammatory state at both the systemic and tissue levels that manifests as decreased serum adiponectin (Purushotham et al., 2007) and elevated IL-6 and tumor necrosis factor- α

mRNA in adipose tissue (Poirier et al., 2006; Shen et al., 2013). At the same time, the volume of the supramammary lymph nodes within the MG is increased by dietary 10,12 CLA, along with significant splenomegaly (Meng et al., 2008) and splenocyte proliferation (Hayek et al., 1999). The local inflammatory phenotype induced by dietary 10,12 CLA is also dose-dependent (Foote et al., 2010; Li et al., 2012; Shen et al., 2013).

These changes within the MG microenvironment in response to a diet supplemented with 10,12 CLA support the growing body of evidence implicating inflammation and leukocyte recruitment in the stimulation of MG development (Gouon-Evans et al., 2002) and tumorigenesis (Bhatelia et al., 2014). Homing of macrophages to the MG is crucial for normal ductal development, as demonstrated by its failure to occur in the

Figure 1. Dietary *trans*-10,*cis*-12 conjugated linoleic acid (10,12 CLA) stimulates estrogen-independent allometric mammary gland growth. (A) Representative mammary gland whole mounts prepared from the 4th inguinal mammary gland from mice ovariectomized at weaning, and then fed either the control diet or a diet with 1% fat as 10,12 CLA for 14 d. Scale bar is 2 mm. (B) Linear regression of log₁₀ (ductal area) and log₁₀ (metabolic BW) from ovariectomized mice fed either the control or the 10,12 CLA diet from for 7, 14, or 21 d. A subset of mice was euthanized at 21 d of age (baseline). The 95% confidence limits for control and 10,12 CLA groups were -0.85 to 2.04 and 2.62 to 5.28 , respectively. Parts of this figure were originally published by Berryhill et al. (2012) and reused here with permission. Color version available online.

MG of mice lacking the macrophage chemoattractant colony-stimulating factor-1 (CSF^{op/op}; Gouon-Evans et al., 2000). However, the finding that supplemental E only partially rescued ductal development in OVX CSF^{op/op} mice (Gouon-Evans et al., 2000) suggests that E-induced and macrophage-dependent ductal growth may occur via different mechanisms.

These findings raise the possibility that the infiltration of macrophages that is required for normal MG growth may also be at play during 10,12 CLA-stimulated ductal development. Whereas 10,12 CLA reduces adipose mass in rodents, the inflammatory signature it evokes is similar to that which occurs in response to diet-induced obesity. Indeed, diet-induced obesity and its resultant inflammatory phenotype accelerated tumor growth in BALB/cJ mice administered a chemical carcinogen (Zhao et al., 2013), whereas obesity-stimulated macrophage infiltration also stimulated stromal changes, leading to increased angiogenesis and vascularization in support of tumor growth (Arendt et al., 2013). These data highlight the role for the innate immune microenvironment during normal MG development and point to a stimulatory role for inflammation during aberrant MG growth and tumorigenesis.

The potential also exists for a host of candidate mitogens to promote MG growth in response to 10,12 CLA, including activation of signaling downstream of the IGF-1 receptor (**IGF-1R**)/insulin receptor by 10,12 CLA-stimulated hyperinsulinemia (Rostoker et al., 2015). Total IGF-1R protein was increased in the MG of OVX mice fed 10,12 CLA for a period of 2 wk, whereas IGF-1R mRNA and total IGF-1R protein increased after 3 wk (Berryhill et al., 2012). When we co-administered the IGF-1R inhibitor picropodophyllotoxin to OVX mice fed 10,12 CLA, ductal elongation was abrogated, indicating that activation of the IGF-1R was indeed necessary during this induction of MG growth. In support of these findings, phosphorylation of the IGF-1R/insulin receptor was increased in MG tumors from 10,12 CLA-fed mice overexpressing ErbB2 (Meng et al., 2008).

E-INDEPENDENT ENDOCRINE COOPERATIVITY DURING DUCTAL ELONGATION, BRANCHING MORPHOGENESIS, AND ALVEOLOGENESIS

A key function of E is the role it serves in potentiating the effects of other hormones on the mammary epithelium. The classic example of this interrelationship is the cooperative effect of E and P, where ER and PR are co-expressed within a subset of luminal MEC within the MG, and PR are E-inducible (Anderson et al., 1998). This hormone combination ultimately increases proliferation and branching morphogenesis in the MG

in various species (Hovey et al., 2002). What come to the fore are scenarios where E collaborates with other regulatory factors. For example, the combined effects of E and PRL appear to represent a significant contribution during MG growth in both rodent models and breast tumorigenesis (O'Leary et al., 2013). Schams et al. (1984) similarly found that the mammary effect of E+P in ewe lambs was dependent on PRL. We made similar observations in the MG of gilts, where the combination of E and PRL stimulated increased proliferation more than either hormone alone and promoted the progression of the epithelial structures toward a more advanced histomorphological state (Horgan et al., 2009).

The endocrine regulation of MG growth can also occur independent of E, an example being the combined effect of P and PRL. The fact that both hormones are essential for aspects of branching morphogenesis and alveologenes (Hovey et al., 2002; Lee and Ormandy, 2012), reminiscent of the development that ensues following the onset of pregnancy, suggests that these hormones collaboratively participate during the regulation of MG growth. A first indication of this cooperation in vivo came from the demonstration that receptors for both hormones show similar localization patterns in the ductal epithelium of nulliparous female mice (Hovey et al., 2001). Moreover, the finding that MEC in mice lacking the transcription factor CEBP/β overexpressed PR led to our subsequent demonstration that the PRL receptor (**PRLR**) was also overexpressed in the same cells (Seagroves et al., 2000), implying some level of coordination between the receptors for these hormones. This suggestion aligns with the earlier demonstrations that P induces expression of the PRLR and vice versa (Goldhar et al., 2005; Lee and Ormandy, 2012). Recently, the co-expression of these receptors has been associated with a population of epithelial cells proposed as alveolar progenitors (Lee and Ormandy, 2012; Tarulli et al., 2013).

Further evidence for convergence between the effects of P and PRL comes from various readouts of the response by MEC to this combination of hormones. In one approach, we administered all possible combinations of E, P, and PRL to sexually mature OVX mice and measured cellular proliferation 5 d later (Hovey et al., 2001). As expected, E alone increased MEC proliferation, whereas P or PRL alone had no effect. Consistent with their cooperative effect, treatment with P+PRL initiated a synergistic response such that cell division was 400-fold higher following treatment with this combination. Interestingly, when E was co-administered, it suppressed the effect of P+PRL, perhaps further highlighting an ability of P and PRL to function independent of E. At the same time, there was prolifera-

tion in the adjacent MG stroma, although it remains unclear whether this response preceded or followed that in the epithelium. The synergistic response to P and PRL was also evident at the intracellular level through increased phosphorylation of the IR substrates 1 and 2 (Lee et al., 2003). Others have implicated additional local consequences that likely mediate the combined effects of P and PRL (Lee and Ormandy, 2012). For example, the osteoclast differentiating factor receptor activator of nuclear factor kappa-B ligand (RANKL) is expressed by MEC and is induced by both P and PRL; moreover, loss of RANKL impairs alveologenesis in the MG during pregnancy (Lee and Ormandy, 2012). In a similar way, expression of the transcription factor Elf5 was reduced in both PR- and PRLR-deficient MEC, consistent with the finding that deletion of Elf5 impaired alveolar development (Lee and Ormandy, 2012). Meanwhile, restoration of Elf5 expression in the MG of PRLR-null mice rescued the associated impairment of alveologenesis. (Lee and Ormandy, 2012)

We also used an in vitro strategy to determine signaling pathways that might mediate the combined effects of P and PRL. Given that normal MEC do not maintain high expression levels of PR and PRLR in vitro, we used the T47D human breast cancer cell line and examined the ability of P and PRL signaling to converge through activation of the mouse mammary tumor virus (MMTV) promoter, a unique virus that is expressed almost exclusively in MEC (Morabito et al., 2008). From this analysis, we identified that P and PRL synergistically activate the recruitment of a unique and mammary-specific transcription factor (mammary gland specific factor, MGSF) to the MMTV promoter, suggesting that MEC may have a specialized sensitivity to this hormone combination. This induction involved PRL-induced signaling that was dependent on c-Src/Fyn downstream of the PRLR, but not STAT5a/b, which are often implicated during other PRL-induced processes in the MG (Morabito et al., 2008).

Combined, these results indicate that P and PRL can cooperatively stimulate the proliferation of MEC, independent of E. Regardless, questions remain about the extent and biological significance of this relationship. One particular question is whether such relationships hold in the MG across species, particularly in livestock and humans that have a more complex epithelial histomorphogenesis within their MG (Rowson et al., 2012). Our experiments in gilts suggest this may not be the case. Using a hormone ablation and replacement protocol similar to those used above for mice, we analyzed the histomorphological and proliferative changes in their MG (Horigan et al., 2009). As mentioned above, we observed clear responses to E, E+PRL, and E+P, whereas the combination of P+PRL did not elicit the

same synergistic proliferative response as was recorded in mice. The reason for this species difference is unclear, although possibilities include differences in the stromal composition of the MG between mice and pigs (Rowson et al., 2012) and differences in the relative length of the luteal phase in these species.

SUMMARY AND CONCLUSIONS

It is clear that combinations of growth regulatory molecules and biological mechanisms can facilitate aspects of MG growth independent of the effects of estrogens. As is widely appreciated by animal scientists, broad variation exists among species with respect to the course of MG development and the factors regulating it. The question remains as to what extent these alternative mechanisms are at play in species other than widely studied rodents. Given the importance of MG development for dairy and animal production, more attention for understanding these differences is warranted, particularly given that mice are unlikely to become a major source of meat and milk for the world's ever-expanding population!

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