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Central Precocious Puberty Alters the Mammary Gland Epithelial Landscape in Female Rats

Alina M. Hamilton
The University of Texas Rio Grande Valley

CENTRAL PRECOCIOUS PUBERTY ALTERS THE MAMMARY GLAND EPITHELIAL LANDSCAPE IN FEMALE RATS

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ALINA M. HAMILTON

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ALINA M. HAMILTON

COMMITTEE MEMBERS

Dr. Robert K. Dearth
Chair of Committee

Dr. Megan Keniry
Committee Member

Dr. Robert Gilkerson
Committee Member

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ABSTRACT

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Precocious puberty (PP) is a serious endocrine disorder associated with an increased risk for development of breast cancer (BC) later in life. However, the lack of a strong *in vivo* model has made it difficult to study this relationship. Our group has established manganese (Mn) as a non-toxic activator of the pubertal process. Recently we demonstrated that Mn-induced precocious puberty (MnPP) accelerates E_2-regulated mammary gland (MG) development in prepubertal female rats, resulting in persistent proliferation and adult hyperplasia. Using this model, I demonstrate that precocious puberty alters steroidal regulation of proliferation in both the prepubertal and adult virgin MG, altering the MG epithelial landscape and consequently sensitizing the gland to proliferative stimulus and malignant transformation. I further highlight puberty as a critical developmental window, demonstrating that the timing of E_2 exposure is of greater importance in precocious puberty-related BC risk than the exposure itself.

DEDICATION

To my ever-supportive husband, Tim, and two wonderful daughters, Kaylee and Avery.

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CHAPTER I

INTRODUCTION

Epidemiological evidence has shown that earlier age at menarche confers an increased risk for the development of multiple diseases in adulthood, including breast cancer (BC) (Bodicoat *et al.*, 2014; and Day *et al.*, 2017). It has been hypothesized that any factor that increases the total number of menstrual cycles in a woman's lifetime, such as early puberty, late menopause, or shorter menstrual cycles, increases BC risk by extending lifetime exposure of the mammary gland (MG) to the mitogenic ovarian hormones, estrodial (E_2) and progesterone (P_4) (Brisken *et al.*, 2015; Chavez-MacGregor *et al.*, 2005; Bernstein *et al.*, 2002; and Pike *et al.*, 1983). However, a recent epidemiological study has suggested that the timing or age at hormonal exposure, rather than just the total number of menstrual cycles, may be of greater importance in BC risk than previously thought (Hamajima, *et al*., 2012). Supporting this theory, both human epidemiological studies and rodent studies have long established that an early pregnancy is protective against BC (MacMahon *et al.*, 1970; Russo *et al.*, 1982; and Ewertz *et al.*, 1990), even though this major reproductive event exposes a woman to the highest levels of circulating estradiol in her lifetime. This protective effect can be mimicked with timely E_2 and P_4 treatment in virgin rodents (Sivaraman *et al.*, 2001), adding to evidence suggesting that the age of hormonal exposure is an important contributing factor in BC risk. In this regard, puberty has been established as a critical window of development and susceptibility in the MG (Fenton *et al.*, 2006; Reilly *et al.*, 2014; and Dearth *et al.*, 2014). Inappropriate or untimely

exposure to either endogenous or exogenous hormones, endocrine disrupting chemicals (EDCs), or carcinogens during this developmental window can have lasting effects on health outcomes and BC risk (Fenton, 2006; Brisken, 2010). Thus, early stimulation of the MG by E_2 during this window, such as in precocious puberty (PP), could alter cell fate and have a lasting impact on MG development and susceptibility to BC.

The MG is unique in that its development is primarily postnatal, with the most dramatic development occurring during puberty (Russo & Russo, 1997) as it undergoes a series of dynamic changes and robust proliferation in direct relation to pre- and post-ovulatory cues from ovarian hormones, E_2 and P_4 (Brisken *et al.*, 2010; Macias & Hinck, 2012). During normal pubertal development, E_2, via estrogen receptor (ER) and its co-regulators (Brisken *et al.*, 2015; Hurtado *et al.*, 2011; and Liu et al., 2016), is the primary stimulus of proliferation and ductal elongation, while P_4 induces alveologenesis and side branching (Macias & Hinck, 2012). After puberty, ER expression is largely down regulated, and P_4 becomes the major steroidal activator of proliferation in the adult breast (Graham & Clarke, 1997). Supporting this, studies have shown that the highest levels of proliferation occur during the luteal phase of the estrous cycle, when P_4 is highest (Russo *et al.*, 1999; Navarette *et al.*, 2005; Graham & Clarke, 1997; and Brisken *et al.*, 2013). In the normal mammary gland, both E_2 and P_4 regulate proliferation primarily by a paracrine mechanism in that hormone receptor expressing (HR+) cells very rarely proliferate themselves (Russo *et al.*, 1999; Brisken *et al.*, 1998; and Clarke *et al.*, 1997). Rather, steroidal stimulus induces the production and secretion of paracrine factors from HR+ cells, including amphiregulin (AREG) and epiregulin (EREG), to stimulate proliferation in neighboring hormone receptor-negative (HR-) cells (Brisken *et al.*, 1998; Mallepell *et al.*, 2006; and Kariagina *et al.*, 2010). It has been hypothesized that the small population of cells that are HR+ and can

proliferate may be luminal progenitor cells in the normal MG, and could be a potential source of ER+ BC (Tarulli *et al.*, 2015; Russo *et al.*, 1999). Supporting this, in both hyperplasias of the breast and in BC, a paracrine to autocrine shift has been observed where a greater percentage of proliferative cells also express either or both ER or PR (Shoker *et al.*, 1999; Russo *et al.*, 1999; Obr *et al.*, 2012; Kariagina *et al.*, 2010). Interestingly, a decrease in the percentage of proliferating HR+ cells has been observed in a rodent model of pregnancy (Sivaraman *et al.*, 2001), suggesting that the protective effect of pregnancy against BC could be directly related to the proportion of this cell population. Thus, the level of autocrine-mediated proliferation in mammary epithelial cells could be proportional to BC risk.

Until recently, investigating the effects of central precocious puberty on MG development and BC risk has been difficult due to the lack of a strong experimental model to study this relationship. Our group has established manganese (Mn) as a natural activator of the pubertal process, acting centrally to stimulate the release of the natural hormonal milieu that accompanies pubertal development (Pine *et al.*, 2005; Srivastava *et al.*, 2013, Srivastava *et al.*, 2016). Using this model, we recently demonstrated that Mn-induced PP (MnPP) accelerates E_2-regulated mammary gland (MG) development, resulting in sustained proliferation of mammary luminal epithelial cells, aberrant ER expression, and hyperplasia in the adult gland of female rats (Dearth *et al.*, 2014). However, we have yet to establish the molecular mechanism of this PP-induced hyperplastic phenotype. While several investigators have assessed the baseline level of autocrine-mediated proliferation at different phases of MG development, no studies to date have assessed their presence at pubertal onset or with respect to precocious puberty and BC risk. Therefore, in the current study, we characterized the normal distribution of HR+ proliferative

cells in the MG of prepubertal female rats and the immediate and lasting impact central precocious puberty has on steroidal regulation of this cell population with respect to BC risk.

CHAPTER II

REVIEW OF THE LITERATURE

Overview of Puberty

The pubertal process is a critical developmental period in a woman's life encompassing the attainment of sexual maturation and the ability to reproduce. Several physically discernable markers present as a result of underlying hormonal and biochemical cues during pubertal development (Marshall & Tanner, 1969). Thelarche, or breast development, is the earliest identifiable sign of pubertal development in young girls, followed by the development of pubic hair (adrenarche). Menarche, the initiation of menstruation and onset of puberty, follows after adrenarche and eventually results in the attainment of regular menstrual cycles (Bodicoat *et al*., 2014). These physical markers of pubertal development are predominantly regulated by different cues. For example, thelarche is driven by rising levels of circulating E_2, while adrenarche is caused by androgen secretion from both the ovaries and adrenal glands. While these seperate pubertal events are stimulated by different hormones, they are all connected by positive and negative feedback loops which are central in origin and under intricate neuroendocrine control.

The onset of puberty, identified at first ovulation in rats, is initiated by a complicated cascade of biochemical reactions and hormonal interactions within the hypothalamic-pituitary-ovarian (HPO) axis, which ultimately results in the pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, and concomitant release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary gland. Several key

regulators have been identified in this process, including mTOR, kisspeptin (Kp), neurokinin B (NKB), and dynorphin (Dyn), in addition to estradiol (E_2), and insulin-like growth factor 1 (IGF-1), among others (Roa, *et al.,* 2009; Thompson, et al., 2004; Hiney *et al*., 1996; Navarro, *et al*., 2004; Navarro, *et al*., 2009). It has been established that kisspeptin neurons from the arcuate nucleus (Arc) and anteroventral periventricular (AVPV) nucleus release kisspeptin, which bind to their receptor, GPR54, on GnRH neurons within the Preoptic Area (POA) and median basal hypothalamus (MBH) to induce secretion of GnRH (Navarro *et al*., 2009). The classic pulsatile release of GnRH is a result of feedback from Kiss neurons, which in addition to kisspeptin, release NKB and Dyn (Feng *et al*., 2012; Navarro *et al*., 2009). This is thought to occur due to a combination of the stimulatory effect of NKB together with the inhibitory action of Dyn on kiss neurons (Li *et al*., 2012). GnRH pulse frequency determines the ratio of FSH to LH that is released, with a lower frequency stimulating the release of FSH, and faster frequency stimulating the release of LH (Savoy-Moore & Swartz, 1987; Wildt *et al*., 1981). During the follicular phase of the ovarian cycle, secreted FSH from the pituitary stimulates the production of E_2 from granulosa cells of the ovary (Dorrington *et al*., 1975), which is then released into circulation. The frequency of GnRH pulsatile release has been shown to increase with rising levels of E_2 during the preovulatory period, concomitantly stimulating the release of LH, and ultimately resulting in ovulation, or pubertal onset (Evans *et al*., 1996; Herbison, 2008). IGF-1 plays a supportive role in this process, in that it is required for sufficient intra-ovarian production of E_2 (Dees *et al*., 2001) and has been shown to centrally stimulate LH release (Hiney et al, 1996). Thus, while Kp, NKB, and Dyn all stimulate GnRH pulsatile release centrally, E_2, supported by IGF-1, plays a key role in setting GnRH pulse frequency, and therefore play a major role in the onset of puberty. Following LH surge, ovulation signifies the start of the luteal phase of the ovarian cycle.

During this phase, P_4 levels predominate, which is important in proliferation of the uterus, but declines in the absence of a pregnancy. The decline of P_4 and other hormones during the luteal phase reduces inhibitory feedback on the hypothalamus, and a new cycle subsequently begins. While much is known about the pubertal process in females, the trigger that initiates this process is yet to be elucidated.

Precocious Puberty

The age at pubertal onset in females is an important factor in predicting the health and overall well-being of a female later in life. Precocious puberty is a serious endocrine disorder in that it is associated with an increased risk for development of several diseases in adulthood including breast cancer, asthma, diabetes, cardiovascular disease, obesity, PCOS and multiple sclerosis as well as psychological and social disorders (Day *et* al., 2017; Fenton *et al*., 2012; Golub, *et al*., 2008; Chitnis, 2013; Garn *et al*., 1986; Hulanicka *et* al., 2007; Macsali *et al*., 2011; Franceschi *et al*., 2010). The age at pubertal onset in girls has been decreasing in recent decades, with the majority of presenting cases being of unknown origin (Parent *et al*., 2003; Euling, *et al*., 2008; Toppari & Juul, 2010; Aksglaede *et al*., 2009). Given the serious risk for development of disease in adulthood, much importance must be put on determining what is causing the higher incidence of precocious puberty in girls. Early activation of the hypothalamic–pituitary–gonadal (HPG) axis results in central precocious puberty and is gonadotropin-dependent. Currently, the clinical definition of precocious puberty is the development of secondary sexual characteristics, such as thelarche, before the age of 8 years in girls (Abreu *et al*., 2013), although disparities in clinical age have been defined (Fenton *et al,*. 2012).

There are several known factors that affect the timing of puberty including genetics (Day *et al*., 2017; Lomniczi *et al*., 2013), metabolic status (including BMI) (Burt Solorzano &

McCartney, 2010), race and ethnicity (Herman-Giddens, *et al*., 1997), intrauterine health and birth weight (Ibanez & de Zegher, 2006), and environmental factors including nutritional factors and exposure to endocrine disrupting chemicals (EDCs) (Fenton, 2006; Fenton *et al*., 2012; Reilly *et al*., 2013; Dearth *et* al., 2004; Dearth *et al*., 2014).

Genetics are an important factor in determining age at puberty, as is supported by the similarity in age of first menses between mothers and daughters (Ersoy *et al*., 2005; Towne *et al*., 2005). Furthermore, the incidence of precocious puberty in girls is highest in African Americans, followed by Hispanics, and finally whites (Herman-Giddens *et al*., 1997). Undeniably, while there is speculation about which genetic factors play the heaviest roles in deciphering what the trigger is for puberty, the racial disparities seen in the incidence of precocious puberty together with the tendency of mothers and daughters to undergo puberty at similar ages strongly suggests a genetic predisposition to altered pubertal timing. Interestingly, however, there is a negative correlation between Body Mass Index (BMI) and age of pubertal onset in girls (Burt Solorzano & McCartney, 2010). The racial groups that have the highest incidence of precocious puberty also have the highest BMIs (Herman-Giddens *et al*., 1997; Wang & Beydoun, 2007). Moreover, these respective groups also have the highest risk for aggressive breast cancer and obesity-related diseases (Chlebowski, *et al*., 2005) --diseases that are suggested to occur at higher rates in individuals that had precocious puberty. Given that BMI is largely influenced by environmental factors including cultural diet, it safely follows then that environment and nutritional or metabolic status can play a major role in the regulation of pubertal timing. Supporting this, undernourished or underweight individuals in early adolescence often experience a delayed pubertal onset (Soliman *et* al., 2014). However, the timing of malnourishment is important to

note given that poor prenatal nutrition and consequentially low birth weight is associated with earlier age of menarche (Ibanez & de Zegher, 2006).

EDCs have a well-accepted role in advancing or delaying the onset of puberty in females, and thus increase risk for adult disease (Mouritsen, et al., 2010). Early life exposure to EDCs such as lead and arsenic have been shown to delay the onset of pubertal onset by interfering with and suppressing circulating levels of IGF-1 (Reilly *et al*., 2014; Dearth *et al*., 2004), while exposure to elevated dietary manganese and phytoestrogens have been shown to advance the onset of puberty (Pine *et al*., 2005; Parent *et al*., 2015). Collectively, there is not a single factor that affects the timing of pubertal development but rather a collection of factors that act on and alter the intricate network of neuroendocrine pathways controlling the initiation of puberty (Lomniczi *et al*., 2013).

While there are many theories that attempt to explain how early puberty may result in the development of disease later in life, the exact mechanisms that result in this fate are still unknown. New diseases have emerged that have a positive correlation with precocious puberty, suggesting that other diseases with known endocrine influences may be affected by the aberrant timing of hormonal fluctuations in early pubertal development. Puberty in general results in classic and necessary sexual dimorphisms. Given the multitude of diseases with known sexual dimorphisms, more research is needed exploring the pubertal process, what is triggering its onset, and how altered timing at onset may play a role in the etiology of disease later in life, particularly those of which have endocrine influences.

Precocious Puberty and Breast Cancer

Breast cancer is the most commonly diagnosed cancer in women besides skin cancer, and is the leading cause of cancer deaths among women. While there is strong evidence that shows a

role of genetic mutation in this disease, such as aberrant expression of BRCA1 and BRCA2 genes, only 5-10% of total breast cancer cases are due to genetic mutation. Thus, the vast majority of breast cancer cases can be attributed to the aging process, lifestyle, reproductive history, and environmental factors, particularly during critical developmental windows. In this regard, epidemiological evidence has shown that precocious puberty significantly increases the risk of developing BC later in life (Kvale, 1992; Berkey *et al.,* 1999; Bodicoat *et al*., 2014; Titus-Ernstoff *et al*., 1998; Day *et al*., 2017). However, a mechanistic explanation of this association has yet to be elucidated.

The mammary gland (MG) is unique in that the majority of its development occurs after birth, primarily during pubertal development, as it undergoes a dynamic series of developmental changes in direct relation to pre- and post-pubertal hormonal cues (Russo *et* al., 2004; Brisken & O'Malley, 2010). A rudimentary ductal structure of the MG is present at birth, but until puberty, grows only isometrically to the rest of the body under the control of local growth factors in a hormone-independent manner (Macias *et al*., 2012; Kleinberg, 1998). It is important to note that although the gland is not actively growing until just before puberty, functional hormone receptors are still expressed in the MG before this time (Grimm *et al*., 2002; Saji *et al.* , 2000). Activation of these receptors at inopportune times can result in abnormal growth and development (Bern *et al*., 1987; Dearth *et al*., 2014). This distinctive feature highlights the window of susceptibility to environmental factors during pubertal development, not only suggesting that the underdeveloped gland is more sensitive to EDCs and carcinogens, but that activation at inappropriate times, such as in precocious puberty, can result in aberrant growth and disease (Dearth *et al.,* 2014; Prété *et al*., 2008).

Following ovulation, MG development is highly dependent on fluctuating ovarian hormones, primarily E_2 and P_4, which are responsible for ductal elongation and extensive side branching, respectively (Macias & Hinck, 2012; Brisken and O'Malley, 2010). During pubertal development, estrogen is the primary proliferative stimulus in the MG, priming the gland for progesterone by inducing PR (Haslam & Shyamala, 1979), and acting through ERα to induce ductal elongation via proliferation of terminal end buds (TEBs). Importantly, this E_2-induced proliferation occurs in a paracrine fashion, in that cells expressing ERα secrete paracrine factors to induce proliferation of neighboring ER- cells (Mallepell *et* al., 2006; LaMarca & Rosen, 2007). Following puberty, ER expression is largely down-regulated throughout the gland, and the gland is minimally responsive to circulating hormones (Reid *et al*., 2003). Thus, any factor that could alter the expression of ER in the normal MG could alter the way it responds to its normal environment, possibly increasing susceptibility to BC.

Epidemiological studies have shown an increased risk for the development of both ER- and ER+ breast cancers following precocious puberty (Kvale, 1992), an association that is now widely accepted. Specifically, puberty that occurs before 12 years of age increases the risk of breast cancer development by 50% when compared to girls 16 years of age at first menarche (Perrson, 2000), with risk decreasing by 4-9% for each year that puberty is delayed (Clavel-Chapelon & Gerber, 2002). Determining how early puberty relays this increased risk is complicated, given the lack of information in this regard. One hypothesis is that given the rise of E_2 at each menstrual cycle, early pubertal onset would result in a longer lifetime exposure to E_2 (Bernstein, 2002), which would consequently increase risk of breast cancer due to the mitogenic properties of estradiol within the mammary gland. Supporting this, epidemiological studies show a decreased risk of breast cancer in women who go through early menopause, whether it is

naturally or surgically induced (Titus-Ernstoff *et al*., 1998). Furthermore, the removal of both ovaries, and consequently circulating hormones, has been shown to significantly reduce BC risk (Rebbeck *et al*., 1999). In addition, a high BMI is associated with elevated BC risk (Furberg *et al*., 2004) as well as precocious puberty, which has been suggested to be due to the estrogenicity of adipose tissue. This supports a role for estrogen in BC risk. With regard to this, two-thirds of breast cancer cases are ER+ (Hernyk, 2004). However, this does not explain the protective role of pregnancy in BC risk, as pregnancy is associated with the highest naturally occurring levels of E_2 in a woman's life. Thus, it may be possible that the protective vs. risk-promoting effects of estradiol may be due to the timing of exposure, in direct relation to the type of cells present in the MG during that respective stage of development.

Another theory that is relatively new is the association between timing of different stages of pubertal development as outlined by Tanner (Marshall & Tanner, 1969), i.e. the age at thelarche compared to age at menarche, or the time spent between thelarche and menarche. Bodicoat and associates have elucidated a relationship between earlier thelarche and BC risk (Bodicoat, 2014), arguing that the plurality of studies investigating the relationship between human pubertal development and BC has used menarche, time of first menses, as an indicator of risk. Thelarche is defined as the start of pubertal breast growth prior to the onset of puberty, or first menstruation, and is associated with rising levels of ovarian E_2 secretion (Klein *et* al., 1999; Fenton *et al*., 2012). This period of development is marked by primarily undifferentiated cells within the gland and a high number of terminal end bud structures (TEBs), which are more susceptible to environmental insult (Russo *et al*., 2004; Russo & Russo, 1996; Russo *et al*., 1982; Reilly *et al*., 2014). Thus, earlier thelarche, or a longer period between thelarche and menarche could increase the risk of BC later in life due to an extended critical window of susceptibility to

endogenous or exogenous insult, which may cause an aberrant advancement of MG development (Klein *et* al., 1999; Bodicoat, 2014; Fenton *et al*., 2012; Reilly *et al*., 2014). Importantly, these data suggest that a precocious elevation in serum E_2 levels, although vital in normal MG development, results in precocious MG development resulting in increased BC risk (Dearth *et al*., 2014. Thus, any endogenous or environmental exposure to a substance that can directly or indirectly alter E_2 levels too early in life, such as precocious puberty (Prété *et al*., 2008), could result in aberrant MG development (Rasier *et* al., 2006), and subsequently increase breast cancer risk.

A common theme surrounding breast cancer risk and precocious puberty has been the susceptibility of the mammary gland to endogenous or exogenous insult, whether it be due to untimely activation of receptors within the gland in precocious puberty, or by exogenous insult on vulnerable undifferentiated mammary epithelial cells during thelarche. However, the molecular mechanism explaining vulnerability of the MG is not known. This vulnerability could be explained by the types of cells present in the gland at any given time. The luminal compartment of the MG is primarily composed of four classic luminal epithelial subtypes: 1.) ER+/PR+ cells, 2.) ER+/PR- cells, 3.) ER-/PR+ cells, and 4.) ER-/PR- cells (Kariagina *et al*., 2010; Arendt & Kuperwasser, 2015). Importantly, cells expressing hormone receptors (HR+; ER+ or PR+) rarely coexpress proliferative markers, while ER-/PR- cells do (Mallepell *et* al., 2006; Brisken *et* al.,1998). This is a result of steroid-regulated paracrine proliferation. However, in addition to these classic MG cell types, a HR+ cell has been described that is also positive for proliferative markers, due to steroid induced autocrine proliferation, and is very rare in the normal gland (Russo *et al*., 1999; Shoker *et al*., 1999; Clarke *et al*., 1997; Sivaraman *et al.,* 2001; Arendt & Kuperwasser, 2015). The MG epithelial landscape, or the proportion of these different

cells present during a given time, is dynamic and changes during different stages of development under steroidal direction (Sreekumar, *et al*., 2015). For example, following puberty, the percentage of ER+ cells is greatly reduced, but in pregnancy, ER+ and PR+ cells are present in high number (Macias *et al*., 2012). An aberrant proportion of these cells is consistent with disease, such as in ER+ BC, where abnormally high percentages of ER+ and PR+ cells make up the gland in addition to proliferating ER+ and PR+ cells (Tarulli *et al*., 2015; Russo *et al*., 1999; Shoker *et al*., 1999; Clarke *et al*., 1997; Sivaraman *et al.,* 2001). Interestingly, the population of proliferating HR+ cells have been implicated in BC risk (Obr & Edwards, 2012). Supporting this, high-risk undifferentiated structures of the MG (such as the TEB) possess higher percentages of autocrine proliferating cells than differentiated structures (Russo *et al*., 1999). This population also increases with age (Arendt & Kuperwasser, 2015), which, in itself, is a risk factor for BC (Fenton *et al*., 2012). Finally, this population can be modulated, which has been shown in a rodent model of pregnancy and carcinogenesis, in that pregnancy significantly reduced the proportion of HR+ autocrine cells in the gland (Sivaraman, et al., 2001). Collectively, these findings suggest that the MG epithelial landscape is indicative of BC risk, and can be modified by reproductive events. In this regard, no study to date has investigated the effect of precocious puberty on the MG epithelial landscape, which could provide insight into the mechanism by which this event increases BC.

Manganese-Induced Precocious Puberty Rat Model

While strong evidence supports the association between early puberty and BC risk, this has been very difficult to investigate due to the lack of a strong *in vivo* model to study this relationship. While many methods exist that induce precocious puberty in mammals (Wright *et al*., 2002; Laws *et al*., 2000; Kim *et al*., 2002), the majority of these studies use EDCs or

hormonally induced methods that can peripherally disrupt the endocrine system, and also have a direct effect on the developing MG. Epidemiological evidence has shown that over 97% of girls presenting with precocious puberty are diagnosed with idiopathic central precocious puberty (Cisternino *et al*., 2000). Central precocious puberty, also known as true precocious puberty, is defined as an early activation of the hypothalamic-pituitary-gonadal (HPG) axis, also termed hypothalamix-pituitary-ovarioan (HPO) axis in females, and is gonadotropin-dependent. Thus, given the clinical presentation of precocious puberty in the human population (Cisternino *et al*., 2000; Abreu *et al*., 2013), and the epidemiological evidence linking precocious puberty and BC risk (Day *et al*., 2017; Clavel-Chapelon & Gerber, 2002; Kvale, 1992), it is vital to utilize a model of central, not peripheral, precocious puberty that also closely models human MG development to investigate this association.

In recent years, our group has charaterized a model of Mn- induced central precocious puberty using Sprague-Dawley female rats (Pine *et al*., 2005). Mn is an essential trace element that is nutritionally required for normal physiological processes in mammals including reproduction (Keen *et al*., 1999; Smith *et al*.,1944; Boyer, *et al*., 1942). While very high exposures to Mn can be toxic, a deficiency can result in altered reproductive function, and is therefore necessary at normally physiological levels (ATSDR, 2012). Previously we showed that daily exposure to a low, but moderately elevated dose of 10mg/kg $MnCl_2$ during peripubertal development results in accumulation of the element in the reproductive centers of the hypothalamus, the preoptic area (POA) and medial basal hypothalamus (MBH) (Pine *et al*., 2005; Srivastava *et al*., 2013). This accumulation resulted in activation of upstream genes that regulate prepubertal GnRH secretion, rheb and mTOR, consequently resulting in activation of Kiss1 and GnRH (Srivastava *et* al., 2013), and the subsequent release of LH, FSH, and E_2 (Pine

et al., 2005). This central activation ultimately resulted in a moderate advance in the age of pubertal onset in Mn-treated females. Importantly, treatment with the mTOR inhibitor, Everolimus, blocked this Mn-induced advancement in pubertal onset (Srivastava *et al*., 2016), therefore confirming that Mn-induced precocious puberty (MnPP) is centrally driven.

Given the association between central precocious puberty and breast cancer risk, we investigated the effect of MnPP on MG development. Thus, using the same model, we exposed Sprague-Dawley females to 10 mg/kg $MnCl_2$ from PND 12 until PND 29 via gastric gavage. Upon completion of dosing, we collected prepubertal MGs at PND 30, and adult MGs at PND 120, 90 days after Mn exposure. Importantly, we observed a significant, 2-fold increase in serum E_2 in Mn-treated females at PND 30 when compared to controls, which was consistent with past work and epidemiological findings in human precocious puberty (Pine *et al.,* 2005; Prété *et al*., 2008). Using MG whole mount analysis, we determined that MnPP resulted in a significant increase in MG ductal differentiation, as indicated by the increased presence of postovulatory structures in the prepubertal gland. Specifically, there was a significant decrease in the mean number of undifferentiated TEBs, while the total number of differentiated alveolar buds (AB), lobular type 1 structures (Lob1) and terminal ducts (TDs) were increased when compared to controls. Collectively, these observations were consistent with that of a more mature MG (Russo *et* al., 1982; Russo & Russo, 1996), suggesting that MnPP resulted in precocious MG development. Immunohistochemistry revealed that the advanced morphological changes observed in MG whole mounts of Mn-treated females correlated with a significant increase in the proliferative marker, Ki67 at PND 30. This increase in proliferation coincided with an increase in phosphorylation of ERK ½ (pERK) in mammary epithelial cell (MEC) lysates from Mn treated females, which given its mitogenic implications (Meloche & and Pouysségur, 2007), supported

our proliferative phenotype. Importantly, we observed no accumulation of Mn in prepubertal MGs from Mn-treated animals at this time. This suggests that our observations were a result of Mn-induced central activation of the HPG axis, ultimately resulting in elevated circulating E_2 and precocious pubertal MG development.

In order to investigate if MnPP-induced precocious MG development had a lasting impact on MG growth and development, we collected adult virgin MGs at PND 120, 90 days after the last Mn treatment. It is important to note that by this time, serum E_2 levels were not different from controls. Despite this, cellular proliferation was sustained in PND 120 MGs from MnPP females, while proliferation had returned to low, normal levels in PND 120 MGs. This abnormally high level of proliferation resulted in aberrant MG growth and adult hyperplasia, as was indicated by histological observations of reactive stroma, disorganized luminal epithelial growth and ductal filling. Supporting this hyperplasic phenotype, we observed a significant increase in phosphorylation of Akt (pAkt), together with a significant increase in expression of ERα and the cell cycle regulators AP2α and p53 in MEC lysates from Mn-treated females at PND 120. While the mechanism explaining the switch from increased pERK1/2 expression in the prepubertal gland to activated pAkt in the adult gland are unknown, this change could possibly suggest a switch in the proliferative mechanism utilized in these glands, a common change observed in neoplastic transformation (Obr & Edwards, 2012; Tarulli *et al*., 2015). Supporting this, Akt is highly implicated in survival signaling pathways in cancer, can be induced by E_2 to stimulate proliferation (Lee *et al*., 2005), and is a marker of poor prognosis in BC (Veeriah *et al*., 2014). Furthermore, the simultaneous increases in ERα, AP2α and p53 expression suggest deregulation of the gland and are clinically expressed in ERα+ BC (Turner *et al*., 1998; Bhargava *et al*., 1994). While AP2α and p53 are normally protective, AP2α has been

shown to limit the regulatory effects of p53 on the cell cycle (Stabach *et al*., 2006). Additionally, p53 expression has also been demonstrated to increase ERα expression in MCF-7 breast cancer cells (Shirley *et al*., 2009). Collectively, these changes support our hyperplasic phenotype and suggest that these glands are in the early steps of pre-neoplastic transformation.

Collectively, our results suggest that a precocious rise in serum E_2, due to Mn-induced central precocious puberty, accelerates pubertal MG development resulting in altered cell fate and adult hyperplasia. We suggest that this fate may due to increased responsiveness to E_2 in the adult gland, thus increasing transformational risk with each subsequent menstrual cycle. Furthermore, the changes we observed due to early puberty are in line with what is seen in the human population, in that this change is centrally mediated (Abreu *et al*., 2013), and we observe an early rise in serum E_2 (Prété *et al*., 2008). Furthermore, using the rat in our model is beneficial in that both the human and rat mammary gland generally have a similar ductal-lobular organization, and like the majority of human BC, mammary neoplasms in the rat are predominantly hormone-dependent (Russo *et al*., 1996; Russo *et* al.,1990). Thus, our *in vivo* MnPP model may prove useful in investigating the relationship between precocious puberty and breast cancer risk.

CHAPTER III

MATERIALS AND METHODS

Animals and Housing

Adult Sprague Dawley rats were housed in the Texas A&M University lab animal facility and University of Texas Rio Grande Valley lab animal facility under controlled conditions of light (lights on: 0600 h; lights off: 1800 h), temperature (23°C), and with ad libitum access to food (Harlan Teklad 2016 rodent chow; Harlan Laboratories, Indianapolis, IN) and water throughout all experimental procedures. The diet contained 91 mg/kg Mn and 197 mg/kg of iron (Fe) based on analysis carried out at the Heavy Metal Analysis Laboratory, Department of Veterinary Integrative Biosciences, College of Veterinary Medicine, Texas A&M University. All procedures were approved by the University Institutional Animal Care and Use Committee (IACUC) at both Texas A&M University (TAMU) and the University of Texas Rio Grande Valley (UTRGV) and were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Experiment 1: Does Manganese Act as an Estrogen?

1.1 Experimental Design

The ER+ MCF7 human breast cancer cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). MCF7s were maintained in Dulbecco's Minimal Essential Medium (Corning Inc., Corning, NY) supplemented with 5% FBS and 1% Pen-Strep antibiotic in a humidified environment at 37°C and 5% CO_2. Cells at approximately 70%

confluency were seeded into 6-well dishes, transfected with an estrogen response element upstream of a luciferase reporter gene, and subjected to treatment with various concentrations of $MnCl_2$, E_2 as a positive control, or DMEM as a negative control. The concentrations of Mn chosen for this experiment were based on a dose conversion of normal human dietary Mn intake within estimated safe and adequate dietary intake (ESADDI) ranges as reported by the Agency for Toxic Substances and Disease Registry (ATSDR, 2012). All doses were based on the 3-5% Mn absorption rate of a 70 kg individual, and are represented in Table 1. 10 nM E_2 treatment was used as a positive control for canonical ER stimulation, as this dose of estradiol has been proven in stimulation of MCF7 cells (Furuya *et al*., 1989; Lu & Serrero, 2001).

1.2 Transfection

The day before transfection, MCF7s were seeded at 500,000 cells per well in 6-well dishes in complete medium. 3 hours prior to transfection, the cells were washed with phosphate buffered saline (PBS) and cultured in serum-free media without antibiotics. The cells were then co-transfected with 250ng pGL2 3X-ERE-TATA-luc and 100ng of pGLK in Opti-MEM using Lipofectamine 2000 (Invitrogen, Carlsbad, California) for 24 hours according to the manufacturer's instructions. The cells were then treated and prepared for luciferase reporter assay as described below. The pGL2 3X-ERE-TATA-luc plasmid that contains the luciferase reporter gene under the control of three estrogen response elements (ERE) was a gift from Donald McDonnell (Addgene plasmid #11354) (Hall, 1999).

1.3 Luciferase Reporter Assay

24 hours after transfection, the stably transfected cell line, MCF7-3X-ERE-TATA-luc, was washed with Dulbecco's PBS and media was changed to estrogen-free conditions: phenol red-free DMEM containing 5% dextran-coated charcoal-stripped fetal bovine serum for 3 hours.

Cells were then treated in triplicate with either 10nM E_2 (positive control), DMEM (control), 10 nM Manganese Chloride ($MnCl_2$), 25 nM $MnCl_2$, 50 nM $MnCl_2$, 100 nM $MnCl_2$, 250 nM $MnCl_2$, or 1μM $MnCl_2$ prepared in estrogen-free media for 24 hours.

Following treatment, luciferase reporter assays were carried out using Promega Dual Luciferase Reporter Assay Kit (Promega, Madison, WI) according to the manufacturer's instructions with the following modifications. Cells were washed once with PBS before adding 250μL of chilled passive lysis buffer to each well. Cells were then lysed with a cell scraper, pipetted up and down, and taken through two rapid freeze-thaw cycles. 20μL of each cell lysate was then transferred to a clean tube containing 100μL of LAR II reagent and mixed carefully by pipetting. Firefly reading was immediately taken using the 20/20n luminometer (Turner Biosystems, Sunnyvale, CA) and recorded. Immediately after reading, 100μL of Stop and Glo reagent was added to the sample, vortexed briefly, and Renilla reading was quickly taken and recorded. Luciferase activity is expressed as the Firefly to Renilla ratio:

$$Luciferase\ Activity = \frac{Firefly}{(Renilla/10)}$$

1.4 Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM), and were analyzed by ANOVA and Tukey-Kramer post test. Probability values <0.05 were considered statistically significant. Statistical analysis was done using the IBM PC program INSTAT software, while PRISM software was used to graph the results (GraphPad, San Diego, CA, USA).

Experiment 2: Does Post-Pubertal Mn Exposure Effect MG Development?

2.1 Experimental Design

Sprague Dawley rats from our colony were bred and allowed to deliver their pups normally at the University of Texas Rio Grande Valley lab animal facility. Each litter was culled

to 10-11 pups while maintaining 5-6 females in each litter. Half of the female pups from each dam were designated as saline-treated (controls), while the other half were designated as Mn-treated. On postnatal day (PND) 21, pups were weaned and separated with 3 to 4 females per cage.

Starting on PND 27, female rats where checked daily for vaginal opening (VO), an external indicator of first ovulation in rodents. As illustrated in Figure 3, once VO was observed, females were dosed daily via gastric gavage with either 10 mg/kg $MnCl_2$ or an equal volume of saline for a total duration of 18 days. Upon completion of 18 days of dosing, between PND 45 and 55 (referred to throughout as PND 50), half of the females in each treatment group were sacrificed via decapitation, and trunk blood and mammary glands were harvested and prepared for further analysis as described below. The remaining females in each group remained housed under normal conditions for 90 additional days, and trunk blood and mammary glands were collected between PND 135 and PND 145 (referred to throughout as PND 140). An illustration of this dosing regimen can be observed in Figure 3. All females were confirmed to be in the diestrus phase of the estrous cycle by observing vaginal cytology and minimal intraluminal uterine fluid on the day of sacrifice.

2.2 Mn Tissue Metal Analysis

Whole blood, MGs, liver, preoptic area, medial basal hypothalamus, and ovaries from both PND 50 and PND 140 females were weighed and collected in tubes that had been washed of all contaminants by soaking in a 4% nitric acid bath for a minimum of 24 hours. All tissues were subsequently lyophilized in a Labconco Freezezone 6 freeze dry system (Kansas City, MO) and dry weight was recorded. All samples were subjected to acid digestion using US EPA Method 200.3 (US EPA, 1996). Briefly, samples were digested in an acid solution with a

maintained ratio of 9 parts nitric acid (HNO_3) to 1 part hydrochloric acid (HCl) per 1 gram of dry sample or 5 grams of wet sample for approximately 2 hours at 95 ± 5°C on a hot block. The samples were allowed to cool to room temperature before adding 4 mL of 30% hydrogen peroxide (H_2O_2) and returning to 95 ± 5°C for a minimum of 1 hour until effervescence ceased completely, the sample became clear, and was near dryness. If the sample was not clear, 1 mL of 30% H_2O_2 was added again until the solution became clear or a total of 10 mL had been added. The samples were cooled once again to room temperature and then diluted to 5 mL with ultrapure water. Quality controls included water only samples, method blanks, and spiked Mn only samples. All samples were analyzed using the Perkin Elmer AAnalyst 800 Atomic Absorption Spectrometer (Shelton, Connecticut). Three replicates per sample were analyzed with the addition of a 0.1% Pd and 0.06%$Mg(NO_3)_2$ matrix modifier solution; 20 µL of sample was added together with 5 µL of matrix modifier solution. A correlation coefficient of 0.998 or better was obtained for each standard curve performed in all analyses.

2.3 Vaginal Cytology

Starting on the first day of dosing (the day of VO), vaginal smears were collected daily throughout the duration of dosing in order to monitor estrous cycling in both PND 50 and PND 140 post-pubertal groups. Briefly, approximately 100-200µL of double distilled water was gently inserted into the vagina of female rats with a glass dropper and gently mixed. The sample was then removed and placed on a glass slide for assessment of the relative ratio of nucleated epithelial cells (proestrus; Figure 5A), cornified squamous epithelial cells (estrus; Figure 5B), and leukocytes (diestrus 1 and 2; Figure 5 C and D) present in vaginal smears, and the stage was recorded.

Upon completion of dosing, the total number of completed estrous cycles was determined for each female. Additionally, the average length of each cycle, the total number of days spent in estrus, and the average length of estrus was determined. The mean number of each parameter per group was quantified and statistical analysis between groups was carried out as described below.

2.4 Serum Hormone Analysis

Trunk blood was collected from control and treated animals and allowed to coagulate at RT for 20 minutes before being centrifuged at 3,000XG for 30 min. The supernatant (serum) was removed, aliquoted in 100uL volumes, and stored at -80°C for later analysis. Serum E_2 was determined using a mouse/rat estradiol ELISA assay from Calbiotech (Spring Valley, CA) according to manufacturer's instructions. The assay sensitivity was 3pg/ml.

2.5 Mammary Gland Whole Mount and Morphological Analysis

MG whole mounts were processed as previously described (Dearth, 2014; de Assis, 2010; Reilly, 2014; Williams, 1983). Briefly, on PND 50, right inguinal MG #4 was collected, immediately spread onto slides, and incubated in Carnoy's fixative, composed of 25% glacial acetic acid (Sigma-Aldrich, St. Louis, MO) and 75% absolute ethanol (EtOH) (Sigma-Aldrich, St. Louis, MO), for 72 hours at room temperature (RT). The slides were then washed in 70% EtOH for 1 hour followed by distilled water for 30 minutes. MGs were then incubated in Carmine Alum stain for 4 days at RT, until the lymph nodes were stained through. The MGs were then dehydrated through graded ethanols, starting with 70% EtOH for 1 hour, 95% EtOH for 1 hour, and finally 100% EtOH for 1 hour. Next, the MG whole mounts were cleared of lipids with xylene for 48 hours at RT until transparent. The tissues were stored indefinitely in methyl salicylate and analyzed using the Leica EZ4D microscope.

MG whole mounts were quantified by counting the total number of terminal end buds (TEBs), alveolar buds (Abs), terminal ducts (TDs), and lobular type 1 (Lob1) structures within the C Zone, a 1 mm perimeter of the MG epithelial tree opposite of the nipple, previously characterized as the zone containing the most actively growing terminal ductal structures (Russo, 1996). Each of the above named structures was counted in each MG analyzed, with a total of 4 MGs analyzed per group. The mean number of each structure per group was quantified and statistical analysis between groups was carried out as described below.

2.6 Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). Differences between saline-treated control and Mn-treated groups were analyzed using the unpaired Student's t test, assuming random sampling. Probability values <0.05 were considered statistically significant. Statistical analysis was done using IBM PC INSTAT software, while PRISM software was used to graph the results (GraphPad, San Diego, CA, USA).

Experiment 3: Does MnPP Alter Steroidal Regulation of the MG Epithelial Landscape?

3.1 Experimental Design

Sprague Dawley rats from our colony were bred and allowed to deliver their pups normally at the Texas A&M lab animal research facility. Each litter was culled to 10-12 pups while maintaining 5-6 females in each litter. Half of the female pups from each dam were designated as saline-treated (controls), while the other half were designated as Mn-treated (MnPP). On PND 21, pups were weaned and separated with 3 to 4 females per cage.

As illustrated in Figure 3, the experimental regimen to induce central precocious puberty in female rats was utilized as has been previously described (Srivastava, 2013; Dearth, 2014; Srivastava, 2016). Briefly, starting on PND 12, females were dosed daily via gastric gavage with

either 10 mg/kg $MnCl_2$ or an equal volume of saline until PND 29 for a total duration of 18 days. On PND 30, half of the females in each treatment group were sacrificed via decapitation, and mammary glands were harvested and prepared for further analysis as described below. The remaining females in each group remained housed under normal conditions until sacrifice at PND 120, at which point MGs were collected and prepared for immunohistochemical (IHC), immunofluorescence (IF), and western blot (WB) analysis as described below. All females collected at PND 30 were confirmed to be in the late juvenile phase of development (anestrous) by confirming no vaginal opening and little to no detectable intraluminal uterine fluid at collection. All adult PND 120 females were confirmed to be in the diestrus phase of the estrous cycle by observation of vaginal cytology and minimal intraluminal uterine fluid the day of sacrifice.

3.2 Histological Analysis

Inguinal MG #4 was collected from each female, placed in cassettes, and fixed overnight in 4% paraformaldehyde in PBS and sent to the Histology Core at Texas A&M University for paraffin embedding. Multiple serial sections of each MG were obtained at a thickness of 5μm, placed on slides, deparaffinized using xylene, and then gradually rehydrated using a series of graded ethanols. Following rehydration, antigen retrieval was achieved by incubation of slides for 20 minutes at 100°C in a water bath in either sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.00) for IHC, or Tris-EDTA buffer (10 mM Tris base, 1 mM EDTA solution, 0.05% Tween 20, pH 9.00) for dual immunofluorescence. The slides were then washed three times in ultrapure water and once in PBS for 5 minutes each. Endogenous peroxidase activity was blocked by a 10-minute incubation in 3% H_2O_2 diluted in methanol. The slides were then washed in PBS and blocked for 1 hour in normal goat blocking serum antibody from ABC

Elite Kit (Vector Laboratories, Burlingame, CA) followed by an overnight incubation with primary antibody at 4°C with gentle shaking. Refer to Table 1 for a complete list of primary antibodies used.

3.2a Immunohistochemistry

Following incubation with primary antibody (Table 2), the slides were washed three times for 10 minutes each in PBS and incubated in biotinylated goat anti-rabbit secondary antibody from ABC Elite Kit (Vector Laboratories) for 1 hour at RT with gentle shaking. Excess secondary antibody was removed by three 10-minute washes in PBS. IHC slides were then incubated in avidin peroxidase for 30 minutes followed by a short incubation in 3'-3'diaminobenzidine (DAB) solution (Vector Laboratories) for the chromogenic reaction. All slides were then washed with water and counterstained in hematoxylin (Fisher Scientific, Hampton NH), dehydrated with graded ethanols, cleared with xylenes overnight, and finally cover slipped with permount. All IHC images were taken with the LeicaIC50 microscope and camera.

To determine the percentage of MG epithelial cells that stained positive for ER or PR at PND 120, three serial sections per animal per treatment group were analyzed. Three randomly selected 4mm^2 areas were analyzed in each section. The number of positively stained (brown) ductal epithelial cells relative to the total number of ductal epithelial cells (brown + purple) per section was averaged per animal and compared between groups.

Images of ERα and PR protein expression patterns in human breast ductal carcinoma in situ (DCIS) were provided by The Human Protein Atlas (available at http://www.protein atlas.org) (Uhlen *et al*., 2015).

3.2b Double Immunofluorescence Labeling

Following incubation with PCNA primary antibody (Table 2), the slides were washed three times for 10 minutes each in PBS and incubated with Alexa Fluor 488 goat anti-mouse secondary antibody (1:1000; EMD Millipore, Temecula, CA) for 1 hour at RT. Given the sensitivity of fluorophores to light, slides were protected from light at each step. Refer to Table 2 for a complete list of antibodies and dilutions used. Following incubation with secondary antibody, IF slides were washed in PBS and incubated overnight with the second primary antibody (either ERα or PR, Table 2) at 4°C with gentle shaking. The slides were washed three times for 10 minutes each in PBS and incubated with Alexa Fluor 594 goat anti-rabbit secondary antibody (1:1000; ab150084, Abcam, Cambridge, UK) for 1 hour at RT. Following three 10-minute washes in PBS, slides were counterstained for 6 minutes in 1.43 μM DAPI, washed briefly in PBS, and then coverslipped with Diamond Superfade mountant (Thermofisher, Waltham, MA). All images were taken with the Fluoview FV10i Confocal Microscope (Olympus).

To determine the percentage of MG epithelial cells that were positive for ER, PCNA, ER+PCNA, PR, or PR+PCNA, 2-4 serial sections per animal per treatment group were analyzed, with an average of 2700 cells counted per MG. The number of positively stained cells (red, green, or red+green) relative to the total number of MG epithelial cells (blue) per duct was averaged per animal and then compared between treatment groups.

3.3 Western Blot Analysis

3.3a Mammary Epithelial Cell Isolation

Enriched epithelial cell lysates were obtained by adapting previously described methods for mammary epithelial cell isolation (Pullan *et al*., 1996; Rudolph *et al*., 2009). Briefly, MG

numbers 3, 4 and 5 were harvested with all lymph nodes removed, and immediately placed in 10 mL of chilled mammary epithelial cell (MEC) digestion buffer containing 100 U/mL Hyaluronidase and 2 mg/mL Collagenase A in Ham's DMEM/F12 medium (Corning Inc., Corning, NY). The MGs were diced in solution with surgical scissors for approximately 2 minutes (until pieces were <1mm) and digested for 1-2 hours at 37°C with shaking at 180 rpm until tissue pieces were mostly dissolved. After digestion, the cell suspension was brought up to 35 mL with ice-cold F12 media and centrifuged at 800 rpm for 3 minutes at 4°C. The first pellet was saved, while the supernatant was transferred to a separate tube, and centrifuged for 10 minutes at 1500 rpm. The new supernatant was discarded, and the new pellet was combined with the first, original pellet. The new combined pellet was washed 3 times by centrifuging in 35 mL of Ham's F12 for 5 minutes at 1000 rpm, supernatant discarded, and then stored at -80°C for further analysis.

3.3b MEC Homogenization and Immunoblot Analysis

Isolated MEC fractions were homogenized on ice in 1% Igepal CA-630, 20 mM Tris-HCl, pH (8.0), 137 mM NaCl, 2 mM EDTA, 10% glycerol, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.25% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). The homogenates were incubated on ice for 30 minutes and centrifuged at 12,000G for 15 minutes at 4°C. The concentration of total protein in the resulting supernatant was determined by the BSA assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a standard. 60 μg of protein in Laemmli sample buffer containing 25 mM Tris-HCl, pH 6.8, 1% SDS, 5% β-mercaptoethanol, 1mM EDTA, 4% glycerol and 0.01% bromophenol blue were resolved through 10% SDS-PAGE gels and subsequently transferred onto polyvinylidene difluoride (PVDF) membranes. Following transfer, membranes

were blocked with 5% nonfat dried milk diluted in 0.1% Tween 20 in PBS (pH 7.4) (PBST) for 3 hours and subsequently incubated at 4^0C overnight with primary antibodies (Table 2) with gentle shaking. After incubation, membranes were washed in PBST and then incubated with secondary antibodies (1:50,000, anti-rabbit from Santa Cruz Biotech, Sc-2004; or anti-mouse from Santa Cruz Biotech, Sc-2005 at 1:50,000) for 2 hours at RT with gentle shaking. Following washing, protein was detected via chemiluminiscence (Western Lightning Plus-ECL, PerkinElmer, Shelton, CT) and quantified with NIH Image J software version 1.43 (National Institutes of Health, MD). Membranes were then stripped with Re-Blot Plus kit (EMD Millipore, Temecula, CA) and reprobed with β-actin for normalization.

CHAPTER IV

RESULTS

Experiment 1: Effect of Mn ERE Activation *in vitro*

Previously we showed that a low, supplemental dose of Mn during peripubertal development can moderately advance pubertal onset in female rats through a centrally regulated mechanism (Pine, 2005; Srivastava, 2013; Srivastava, 2016), resulting in E_2-driven advanced MG development and adult hyperplasia (Dearth, 2014). However, several metals have been shown to have varying levels of estrogenic activity (Choe *et al*., 2003). Although metal analysis suggested no accumulation of Mn in the MG following treatment, in order to confidently conclude that MnPP-advanced MG development is due to the actions of MnPP-induced E_2 secretion and not Mn, we asessed the estrogenicity of Mn. As expected, 10 nM E_2 treatment resulted in a significant increase in luciferase reporter activity ($p<0.001$) when compared to DMEM-treated controls (Figure 2). This was not surprising as E_2 is the natural ligand for ER, which in canonical ER signaling would subsequently bind to an ERE to induce transcriptional activity. Importantly, as shown in Figure 2, none of the tested Mn concentrations significantly effected luciferase reporter activty, suggesting that Mn, within human-relevant physiological ranges (Table 1), posseses no estrogenic properties.

Experiment 2: Effect of Post-Pubertal Mn on HPG Axis and MG Development

Multiple lines of evidence have suggested that extending a woman's reproductive years, and consequently increasing her cumulative exposure to E_2, increases her risk for the

development of breast cancer (Brisken, 2015; Chavez-MacGregor *et al*., 2005). However, recent evidence has suggested that the timing or age at exposure to E_2 may play a larger role in BC risk than previously thought (Hamajima, et al., 2012). In this regard, puberty has been established as a critical window of susceptibility in MG development (Dearth *et al*., 2014; Reilly *et al*., 2014; Fenton *et al*., 2013). Because of the discordance in theories (cumulative E_2 exposure vs. E_2 exposure during a critical developmental window), we assessed whether post-pubertal Mn exposure could augment the HPG axis and subsequently impact MG development after puberty.

2.1 Effect of Post-Pubertal Mn on Mn Tissue Levels

Prepubertal Mn treatment has been shown to result in accumulation of Mn in the reproductive center of the hypothalamus in female rats (Pine, 2005). Because of this, we assessed whether Mn treatment in older, post-pubertal, females could result in accumulation of the element in various tissues. Metal analysis revealed no accumulation of Mn in the reproductive centers of the hypothalamus (preoptic area or medial basal hypothalamus), ovaries, uterus, or mammary gland of post-pubertal exposed females at either PND 50 or PND 140. However, Mn levels were increased in the blood of Mn-treated females compared to controls at PND 50 ($p<0.01$) (Table 3; Figure 4A). It was not surprising to see an increase of Mn in the blood given that it had been only 24 hours since the last dose of Mn had been administered. This accumulation of blood Mn was not sustained, and had fallen by PND 140 (Figure 4B).

2.2 Effect of Post-Pubertal Mn on the Estrous Cycle

Because changes in cell typology of the vaginal wall reflect underlying endocrine events (McLean *et al*., 2012), we next assessed the effect of post-pubertal Mn exposure on estrous cycling by vaginal cytology (Figure 5 A-D). The average estrous cycle length in both saline-treated controls and Mn-treated females was not different (Figure 5E), and similarly, there was

no difference in the total number of estrous cycles in either treatment group (Figure 5F). Furthermore, there was no difference in the total number of days spent in the estrus stage (Figure 5G), or the average length of each estrus in either control or Mn-treated females (Figure 5H). Thus, post-pubertal Mn exposure has no effect on the estrous cycle of female rats.

2.3 Effect of Post-Pubertal Mn on Serum E_2

Previously we showed that prepubertal Mn treatment in female rats results in a precocious release of the normal hormonal milieu that drives pubertal development, resulting in precociously elevated serum E_2 (Dearth, 2014). Thus, we assessed whether post-pubertal Mn exposure could augment this same process, resulting in elevated serum E_2. Figure 6A shows a significant elevation in serum E_2 levels in PND 50 Mn-treated females (4.98 pg/mL; $p<0.01$) when compared to controls (2.64 pg/mL). As seen in Figure 6B, by PND 140, the level of serum E_2 was no longer significantly different between Mn-treated females (5.06 pg/mL) and age-matched controls (5.68 pg/mL).

2.4 Effect of Post-Pubertal Mn Exposure on MG Ductal Differentiation

Because we previously observed that a precocious elevation of serum E_2 during prepubertal development augments MG development (Dearth *et al.,* 2014), we assessed the effect of a post-pubertal elevation in serum E_2 could impact MG development. As seen in Figure 7, the Mn-induced post-pubertal elevation in serum E_2 was not sufficient enough to stimulate MG growth. Specifically, whole mount analysis revealed no difference in the number of terminal ductal structures between saline-treated controls or Mn-treated females at PND 50 (Figure 7A-D). This supports our hypothesis that while Mn treatment may still centrally augment the hypothalamic-pituitary-gonadal axis, the post-pubertal MG is naturally less sensitive to circulating estrogens than the prepubertal gland, and is therefore at decreased risk for BC

following insult. Thus, given the dynamic nature of the MG, these data support the hypothesis that the timing of E_2 exposure may be of greater importance in BC risk than length of exposure.

Experiment 3: Effect of MnPP on Steroid-Regulated MG Proliferation

A switch in the steroidal regulation of luminal epithelial cell proliferation has been observed in hyperplasias of the breast and in BC (Obr & Edwards, 2012; Shoker *et al*., 1999;), where the proportion of proliferating ER+ and PR+ cells that make up the pre-neoplastic breast or tumor is disproportionately large when compared to normal breast epithelium (Clarke *et* al.,1997; Shoker *et al*., 1999; Russo *et al*., 1999; Kariagina *et al*., 2010; Tarulli *et al*., 2015). Furthermore, the proportion of these cells correlates with BC risk (Obr & Edwards, 2012). The percentage of these cells has been investigated following different reproductive stages including early adulthood, pregnancy (Russo *et* al., 1999; Sivaraman *et al.*, 2001) and post menopause (Arendt & Kuperwasser, 2015), demonstrating that the MG epithelial landscape changes in response to these life events. However, the population of proliferating HR+ MG epithelial cells has never been investigated in regard to precocious puberty. Therefore, we characterized the effect of MnPP on steroid-regulated proliferation in the prepubertal and adult virgin MG.

3.1 Effect of MnPP on MG Steroid Receptor Expression at PND 120

Recently we showed that MnPP results in a significant increase in ER protein expression in adult virgin MGs of female rats (Dearth *et al*., 2014). Due to this previously observed change, we investigated the distribution of ER and PR in the adult MGs of precociously developed females. Figure 9A and B depict representative IHC images confirming the previously observed elevation in estrogen receptor (ER) expression in MnPP MGs (9B) when compared to controls (9A) at PND 120. Additionally, representative IHC images show a clear increase in PR expressing luminal epithelial cells of MnPP MGs when compared to controls at PND 120 (Figure

9 D, E). The expression of both hormone receptors in the precociously developed rat adult rat MG is similar to what is observed in the human breast (Figure C, F) in ductal carcinoma *in* situ (DCIS). Interestingly, ER+/PR+ hyperplasia of the breast commonly results in DCIS (Hartmann *et al*., 2015). The human breast sections were stained with CAB000037 ER antibody (Figure 9E) and CAB055100 PR antibody (Figure 9F), and were provided by The Human Protein Atlas (available at http://www.proteinatlas.org).

3.2 Effect of MnPP on E_2-Regulated Protein Expression

Given that MnPP-advanced MG development is E_2-regulated, we investigated the expression of known ER-regulated proteins important in pubertal MG development and regulation of proliferative mechanisms. WB analysis revealed a significant increase ($p<0.05$) in protein expression of the paracrine and autocrine proliferative mediator AREG in isolated MEC lysates from Mn-treated females at PND 30 (Figure 10A and C). Similarly, there was a significant increase in protein expression of the ER co-regulator, FOXA1 ($p<0.05$) (Figure 10A and D), and c-myc ($p<0.05$) in MEC lysates from Mn-treated females at PND 30 (Figure 10A and E). There was no change, however, in protein expression of PR isoforms A or B (Figure 12, A and C), sp1 (Figure 12A and D), EREG (Figure 12A and E), or p27 (Figure 12A and F) in MEC lysates at PND 30.

3.3 Effect of MnPP on Steroid-Regulated MG Ductal Proliferation at PND 30

In the normal MG, proliferation is regulated by the circulating levels of estrogen and progesterone. Therefore, we examined the relationship between steroid receptor expression and proliferation in the prepubertal rat MG and the effect precocious puberty had on this relationship. As depicted in Figure 11A , IF staining confirmed our previously reported (Dearth, 2014) more than 2-fold increase ($p<0.01$) in the percentage of PCNA+ proliferating luminal epithelial cells in

the MGs of MnPP females at PND 30 (Figure 11B). Confirming results revealed by WB analysis, there was no difference in the percentage of PR+ luminal epithelial cells in prepubertal MGs of either treatment group (Figure 11B). We next examined the expression of co-localized PR+/PCNA+ ductal epithelial cells in order to characterize the normal size of this cell population in the prepubertal MG. As depicted in Figure 11A, PR+ proliferating cells comprised only 2.04% of total ductal cells in the normal prepubertal MG at PND 30. Interestingly, the size of this cell population increased significantly ($p<0.05$) to 5.39% in the MGs of Mn-induced precocious MG development (Figure 11B).

3.4 Effects of MnPP on MG Protein Expression at PND 120

The expression of proteins elevated at PND 30 was not congruent in PND 120 MGs. In addition to the previously reported increase in ER expression, WB analysis revealed a significant increase ($p<0.01$) in protein expression of PR isoform B, but not isoform A, in isolated MEC lysates from Mn-treated females at PND 120 (Figure 12B and C). There was a significant increase ($p<0.01$) in protein expression of sp1 (Figure 12B and D), and a concomitant decrease in expression of the cell cycle regulator, p27 ($p<0.05$) in MEC lysates from MnPP females at PND 120 (Figure 12B and F). MGs of MnPP females also exhibited increased protein expression ($p<0.05$) of EREG when compared to controls at PND 120 (Figure 12B and E), but not AREG (Figure 10B and C), FOXA1 (Figure 10B and D), or C-Myc (Figure 10B and E).

3.5 Effect of MnPP on Steroid-Regulated MG Ductal Proliferation at PND 120

Given the persistent proliferation and hyperplasia previously observed in MnPP MGs at PND 120, we examined the relationship between steroid receptor expression and proliferation in adult virgin MGs of normal (control) and precociously developed (MnPP) female rats. Representative IF images show confirmation of previous IHC and WB results suggesting an

increase in both ER (Figure 14 A,B), and PR expression (Figure 13A,B) in PND 120 MnPP MGs, similar to that observed in human DCIS (Figure C and E). Additionally, IF images confirmed the previously observed sustained proliferation at PND 120, depicting a greater intensity of nuclear PCNA staining in MnPP adult MGs when compared to controls (Figure 13A; Figure 14A). Specifically, the mean percentage of ER+ (63.57%; $p<0.01$), PR+ (18.27%; $p<0.01$), and PCNA+ (46.47%; $p<0.001$) luminal epithelial cells was increased by more than 2-fold in MnPP adult glands when compared controls (Figure 13 B and Figure 14B).

Similar to previous reports (Sivaraman, 2001), a population of ER+ proliferating cells already exists in the normal adult virgin MG at PND 120, and comprised approximately half (4.99%) of total proliferating cells in the gland (9.27%; Figure 14A and B). Importantly, the percentage of dual labeled ER+ proliferating cells was significantly higher ($p<0.001$) in MnPP PND 120 MGs (Figure 14 B). Specifically, ER+ proliferating cells comprised 79.2% of all proliferating cells in MnPP glands, while they comprised only 41.67% in control glands at PND 120. On the contrary, dual labeled PR+ proliferating cells were rarely observed in the normal virgin MG at PND 120 (Figure 13 A,B), making up only 0.99% of total ductal epithelial cells (Figure 14 B), and approximately 10% of the already rarely proliferating cells at this stage of development. Representative IF images show a marked increase in the number of dual labeled PR+ proliferating cells in MnPP MGs at PND 120 when compared to controls (Figure 13 A). This is further demonstrated by a seven-fold increase ($p<0.01$) in the mean percentage of MG luminal epithelial cells staining positive for both PR and PCNA in MnPP rats at PND120 (7.22%; Figure 13 B) when compared to age-matched controls (0.99%; Figure 13 B).

CHAPTER V

DISCUSSION

While multiple epidemiological studies have implicated early or precocious puberty as a direct causal link in BC (Day *et al*., 2017; Berkey *et al*., 1999; Kelsey *et al*., 1993; Titus-Ernstoff, *et al*., 1998), no study to date has provided a mechanistic explanation for this link. This is important because the average age of pubertal onset in girls has consistently decreased over the past several decades, with 95% of cases being idiopathic (Herman-Giddens, *et al*., 1997; Parent *et* al., 2003). In recent years, our group has characterized an intriguing model of central precocious puberty in female rats, identifying Mn as a natural activator of the pubertal process (Pine, 2005; Srivastava, 2013; Srivastava, 2016). Using this model, we recently demonstrated that Mn-induced precocious puberty (MnPP), through a precocious rise in serum E_2, induces precocious MG development resulting in persistent proliferation and adult hyperplasia (Dearth, 2014). Thus, in the current study we utilized our MnPP model to address this link. Specifically, we demonstrate that precocious puberty alters steroidal regulation of proliferation in the prepubertal MG, altering the MG epithelial landscape and consequently sensitizing the gland to proliferative stimulus and malignant transformation. We further highlight puberty as a critical developmental window, demonstrating that the timing of E_2 exposure is of more importance in precocious puberty-related BC risk than the exposure itself.

Prior to ovulation, growth of the prepubertal MG is primarily hormone-independent, despite the expression of ER and PR in the gland (Saji *et al.*, 2000). Although the exact mechanism is unknown, inappropriate activation of these receptors has been shown to result in aberrant MG development (Bern *et al*., 1987; Fenton, 2006), a phenomenon we have previously observed due to precocious activation of the HPG axis (Dearth *et al.*, 2014). During pubertal development, E_2 is the primary proliferative stimulus in the MG, inducing ER transcriptional activity and dictating development primarily through paracrine-mediated proliferation (Brisken *et* al., 1998; Mallepell *et al*., 2006). In the present study, using a dual labeling IF approach, we characterized this steroid-mediated paracrine mechanism by demonstrating that only 2% of total mammary epithelial cells co-express both steroid receptors (PR) and the proliferative marker PCNA in the normal prepubertal MG of female rats. This finding is consistent with the percentage of proliferating PR+ cells observed (2.6%) in the MG of young, 45 day old, female rats, although these MGs were not prepubertal (Sivaraman, *et al*., 2001). Thus, the proliferating PR+ cell population is very small in the prepubertal MG, and seems to remain this way in the young post-pubertal gland. Importantly, when MG development was precociously induced in Mn-treated females, the percentage of proliferating luminal epithelial cells was significantly increased by 2-fold when compared to normally developing prepubertal glands. Surprisingly, despite no change in the expression of ER or PR in these MGs, the percentage of dual-labeled PR+ proliferating luminal epithelial cells had also increased by more than 2-fold (5.39%) when compared to controls. Interestingly, the protective effect of pregnancy has been shown to produce a similar, but opposite, result, in that the total percentage of HR+ cells did not change after pregnancy, but the total percentage of proliferating HR+ cells was significantly decreased (Sivaraman, *et al*., 2001). We therefore suggest that early puberty may alter the normal balance

of epithelial cell type in the developing MG, thereby changing the way the gland responds to its surrounding environment and increasing susceptibility to BC. The overall baseline level of proliferation observed in our adult virgin MGs was higher than reported elsewhere (Russo *et al*., 1999; Sivaraman, *et al*., 2001), but remains consistant with our previous work (Dearth *et al*., 2014). These differences may be attributed to differences in the method of detection, i.e. BrDU labeling and/or ^{3}H-thymidine radio-labeling vs PCNA and Ki67 labeling.

It is important to note that this modification in the prepubertal MG cellular makeup coincided with a 2-fold increase in serum E_2 (Dearth *et al*., 2014), suggesting that these changes were steroid-induced. Supporting this, we observed a significant increase in FOXA1 protein expression in Mn-treated females at PND 30, an ER co-regulator essential for hormone responsiveness in both normal MG development and BC (Bernardo *et al*., 2010). Importantly, increased FOXA1 expression has been shown to augment ER transcriptional activity, consequently amplifying normal ER function (Hurtado *et al*., 2011). Given this, we observed a significant increase in expression of ER target proteins, c-Myc and AREG, both of which play important roles is normal pubertal MG proliferation and BC when aberrantly expressed (McBryan *et al.,* 2008; Rose-Hellekant *et al*., 2006). Of special interest, AREG is a vital paracrine factor secreted from HR+ cells to induce proliferation in neighboring HR- cells in direct response to E_2 stimulus (McBryan *et al.*, 2008). While the proliferative actions of AREG are primarily paracrine in fashion, AREG has been suggested to act in an autocrine manner to stimulate proliferation of HR+ cells (Ma *et al*., 2001; Willmarth & Ethier, 2006; Kariagina *et al*., 2010). This is compliments our previous work showing that Mn-induced precocious MG development correlated with a significant increase in proliferation and phosphorylation of ERK½ (Dearth *et al.*, 2014), a response consistently seen following AREG induced proliferation

(McBryan *et al*., 2008). These data suggest that the overexpression of AREG observed in precociously developed MGs could account, at least in part, for the increased percentage of proliferating HR+ luminal epithelial cells observed in Mn-treated females at PND 30, ultimately resulting in an altered MG epithelial landscape. Importantly, we suggest that these effects in the MG are due to an early rise in serum in E_2 due to Mn-stimulated precocious activation of the HPG access and not just E_2 exposure in general. Supporting this, a Mn-induced post pubertal rise in serum E_2 had no effect on MG development. Furthermore, we determined that Mn does not possess estrogenic properties and have previously shown that Mn does not accumulate in the MG. Thus, a precocious elevation in serum E_2 due to early puberty may sensitize the MG to further cycling hormones by amplifying ER action via FOXA1, resulting in overexpression of steroid induced proliferative mediators c-Myc and AREG, and altered steroidal regulation of classic proliferative mechanisms in the prepubertal gland.

Consistent with our previous reports, the robust proliferation observed in prepubertal MGs of Mn-treated females was sustained at PND 120, while proliferation had naturally slowed in control glands. Previously we showed that this sustained proliferation resulted in MG hyperplasia in 20% of Mn-treated females, as was indicated by histological observation of reactive stroma, disorganized luminal epithelial growth, and ductal filling (Dearth *et al*., 2014). Clinically, a high percentage of human hyperplasia lesions are HR+ (Hartmann *et al*., 2015). In this regard, histological analysis revealed a significant increase in the percentage of both ERα and PR expressing luminal epithelial cells in Mn-induced precociously developed MGs compared to controls at PND 120, a phenotype clinically seen in human ER+ BC (Lange & Yee, 2008). Specifically, we observed a significant increase in protein expression of PR-B, but no change in PR-A at PND 120. Supporting our proliferative phenotype, P_4 has been shown to

induce proliferation in the MG via PR-B either directly or in a paracrine fashion (Lange and Yee, 2008). In the normal MG, P_4-induced proliferation is largely paracrine mediated, and is carried out by secretion of paracrine factors. We observed a significant increase in protein expression of one such factor, EREG, which is primarily stimulated by P_4 (McBryan *et al.*, 2008) and to a lesser extent by E_2 (Kariagina *et al*., 2010). Interestingly, the overexpression of both AREG and c-Myc observed at PND 30 was not significant at PND 120, suggesting that the primary stimulus of paracrine proliferation at this time point was P_4 via stimulation of EREG. This was not surprising given that in the normal adult MG, P_4 is the primary proliferative stimulus, producing the highest levels of proliferation in the breast during the luteal phase of the menstrual cycle when P_4 is highest (Lydon, *et al*., 1995; Kariagina *et al*., 2007; Brisken, 2013). Supporting the possibility of a facilitatory role of P_4 in the hyperplasic phenotype in precociously developed adult MGs, we observed a significant increase in expression of Sp1 in MGs of Mn treated females at PND 120. Sp1 is required by PR for transcriptional activation of multiple target genes including cell cycle regulators (Dressing *et al*., 2009), and is a marker of poor prognosis in BC (Beishline & Azizkhan-Clifford, 2015). It is important to note that while sp1 is necessary for many PR downstream effects, it has also been shown to mediate ERα transcriptional activity (Kim *et al*., 2003). Thus, our data suggest that the previously observed hyperplasic phenotype of precociously developed adult MGs is due, in part, to pathological proliferation as a result of aberrant expression of both ERα and PR.

A hallmark of pre-malignant hyperplasia and breast cancer is an increase in the percentage of proliferating ER+/PR+ cells, where steroid-regulated proliferation shifts from paracrine-mediated to autocrine-mediated growth, and directly correlates with breast cancer risk (Shoker *et al.*, 1999; Russo *et al.*, 1999; Obr *et al.*, 2012; Kariagina *et al.*, 2010). Although the

primary mechanism of this shift is unknown, the small population of proliferating HR+ cells normally found in the MG has been suggested as a potential source of ER+ BC (Russo *et al*., 1999). Consistent with other findings (Sivaraman, *et al*., 2001), while we observed a substantial decrease in the percentage of proliferating cells in control MGs from PND 30 to PND 120, approximately half of all proliferating cells in the adult virgin gland were also ER+, while one tenth were PR+. These data suggest that the normal population of proliferating ER+ cells is normally increased with age, as has been shown in the post-menopausal human breast, despite an overall reduction in proliferation (Shoker *et al*., 1999; Arendt & Kuperwasser, 2015). However, this population of cells has never been characterized as a result of precocious puberty. In this regard, MnPP adult MGs were significantly more proliferative than controls at PND120, and displayed a significant increase in the total percentage of both dual labeled proliferating ER+ luminal epithelial cells and proliferating PR+ luminal epithelial cells. Furthermore, 79.34% of all proliferative cells were also ER+, and 19.79% were also PR+, suggesting that this increase was not simply due to an overall increase in proliferation, but to a change in the primary mechanism of steroid-regulated proliferation. This result is significant as it provides, for the first time, a mechanistic explanation by which early puberty may predispose the MG to malignant transformation.

While further studies will be required to elucidate a mechanism explaining this shift in steroid-regulated cellular proliferation, given the tight association of ER and PR with the cell cycle (Dressing *et al*., 2009), these changes could be attributed to deregulation of cell cycle control. Supporting this, we previously showed that MnPP resulted in increased expression of AP2α and p53 in PND 120 MGs, an association known to mitigate the ability of p53 to adequately regulate the cell cycle (Stabach *et al*., 2006). Sp1, inducible by both ER and PR, also

has known associations with p53, and has been shown to counteract its inhibitory actions (Ohlsson *et* al., 1998). We also observed a significant decrease in the cell cycle inhibitor, p27 in MnPP females at PND 120. Interestingly, p27 has been suggested to block autocrine proliferation in PR-B expressing cells (Kariagina *et al*., 2007), while a loss of p27 in PR-positive breast cancer has been predicted to result in a bypassing of normal cell-cycle controls (Musgrove *et al*., 1998). Collectively, these data support a role of steroid-induced cell cycle deregulation in the premalignant phenotype observed in the MG of precociously developed female rats.

In conclusion, using our novel MnPP model, we clearly demonstrate that precocious puberty alters steroidal regulation of proliferation in the prepubertal MG by augmenting ER-regulated proliferative pathways, resulting in an altered MG epithelial landscape that is more sensitive to cycling E_2 and P_4. These pubertal changes persist in the adult MG, resulting in a steroid-induced predominance of autocrine regulated cellular proliferation and premalignant transformation.

TABLES AND FIGURES

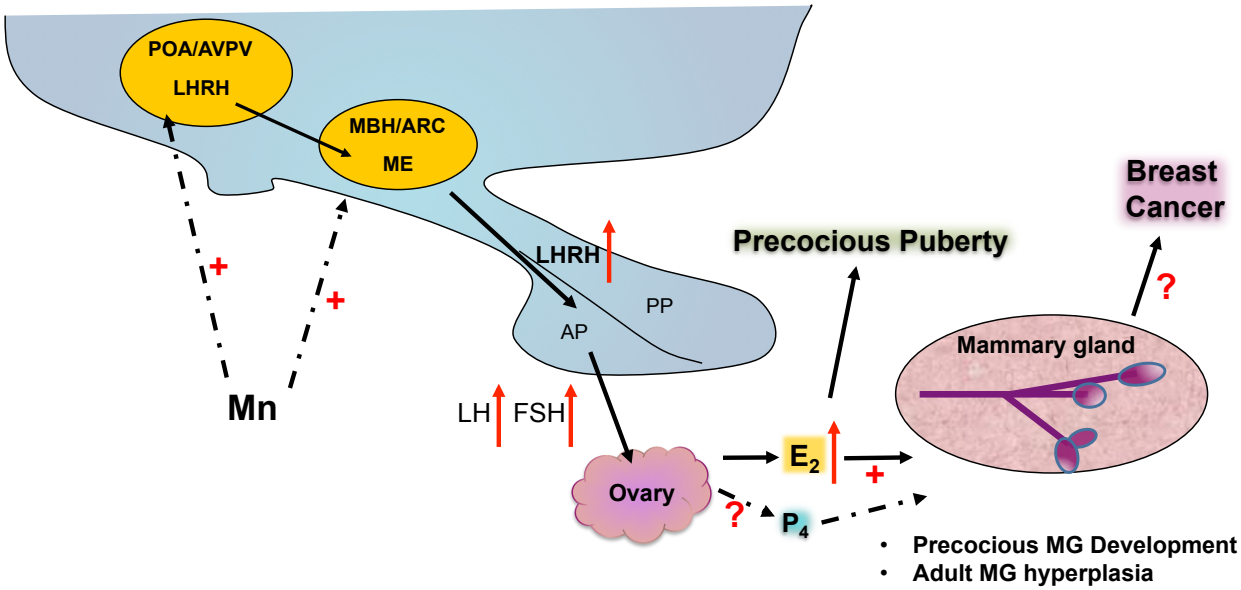

Figure 1. MnPP Model

A simplified summary of our previous findings describing our Mn-induced precocious puberty rat model, its application in mammary gland development, and potential role in breast cancer.

Relation Between Dose and Dietary Mn Intake	
Dose ($MnCl_2$)	**Human Daily Intake Range (mg)**
25 nM	6.93 – 11.54
50 nM	27.71 – 46.18
100 nM	138.54 – 230.89
1 μM	277.07 – 461.79

Table 1.Relation Between Dose and Dietary Mn Intake

The range of $MnCl_2$ chosen for this Experiment 1 was based on the ESADDI range and lowest observed adverse effect level (LOAEL) of 11mg as reported in the ASTDR Toxicological Profile for Manganese, 2012. Note that while average intake of Mn is between 3mg and 5mg, it has been considered safe to take in as much as 11mg. Certain diets and lifestyles have been reported to cause as high as 20 to 30mg daily intake of Mn (Hope *et al*., 2006). The numbers above are based on a 70 Kg individual with a 3-5% absorption rate of Mn.

Table 2. Antibody Table

Protein Target	Name of Antibody	Manufacturer, Catalog Number,	Species Raised in; Monoclonal or Polyclonal	Dilution Used	Application
AREG	Anti-Amphiregulin	Bioss, bs-3847R	Rb; polyclonal	1:750	WB
β-Actin	Anti-β-Actin	Sigma-Aldrich, A1978	Ms; monoclonal	1:50,000	WB
c-Myc	Anti-c-Myc (D84C12)	CST, 5605	Rb; monoclonal	1:500	WB
EREG	Anti-Epiregulin (C-9)	SCBT, sc-376284	Ms; monoclonal	1:500	WB
ER α	Anti-Estrogen Receptor α	EMD Millipore, 06-935	Rb; polyclonal	1:200	IF
ER α	Anti-Estrogen Receptor α [SP1]	Abcam, Ab16660	Rb; monoclonal	1:100	IHC
FOXA1	Anti-HNF-3 alpha/FoxA1	Novus Biol., NBP2-45269	Ms; monoclonal	3 µg/ µL	WB
p27	Anti-p27 Kip1 (D69C12)	CST, 3686	Rb; monoclonal	1:500	WB
PCNA	Anti-PCNA [PC10]	Abcam, Ab29	Ms; monoclonal	1:200	IF
PR	Anti-Progesterone Receptor [SP2]	Abcam, Ab16661	Rb; monoclonal	1:200	IF; IHC
sp1	Anti-Sp1	EMD Millipore, 07-645	Rb; polyclonal	1:500	WB

Table 2. Antibody Table

List of primary antibodies used in this study, along with their dilutions, company and catalog, and application. Abbreviations: CST: Cell Signaling Technology; SCBT: Santa Cruz Biotechnology; Novus Biol.,: Novus Biologicals; Rb: Rabbit; Ms: Mouse; WB: Western Blot; IF: Immunofluoresence; IHC: Immunohistochemistry.

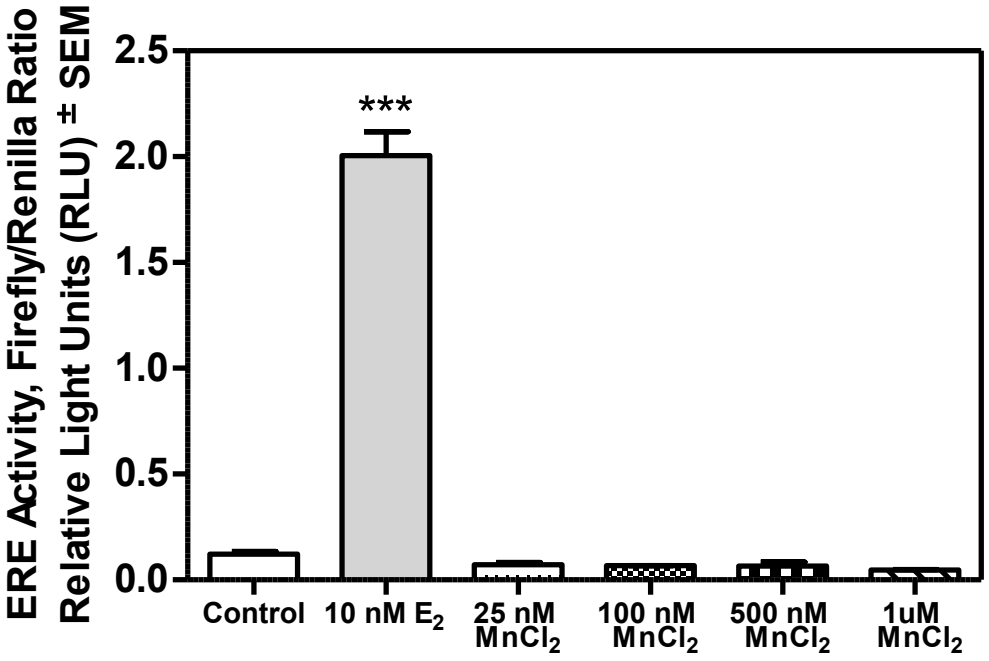

Figure 2. Estrogenicity of Manganese

As expected, luciferase reporter assays measuring ERE activity demonstrated that a 24 hour stimulation with10nM E_2 activates estrogen response elements (ERE) in MCF7 cells, while physiologically relevant concentrations of $MnCl_2$ did not. These data suggest that Mn does not possess estrogenic properties. Values represent the mean ± SEM in each bar. Statistics were determined with one-way ANOVA and Tukey-Kramer post-test. ***$p<0.001$; N=3 for each group; each N is a mean of 3 replicates.

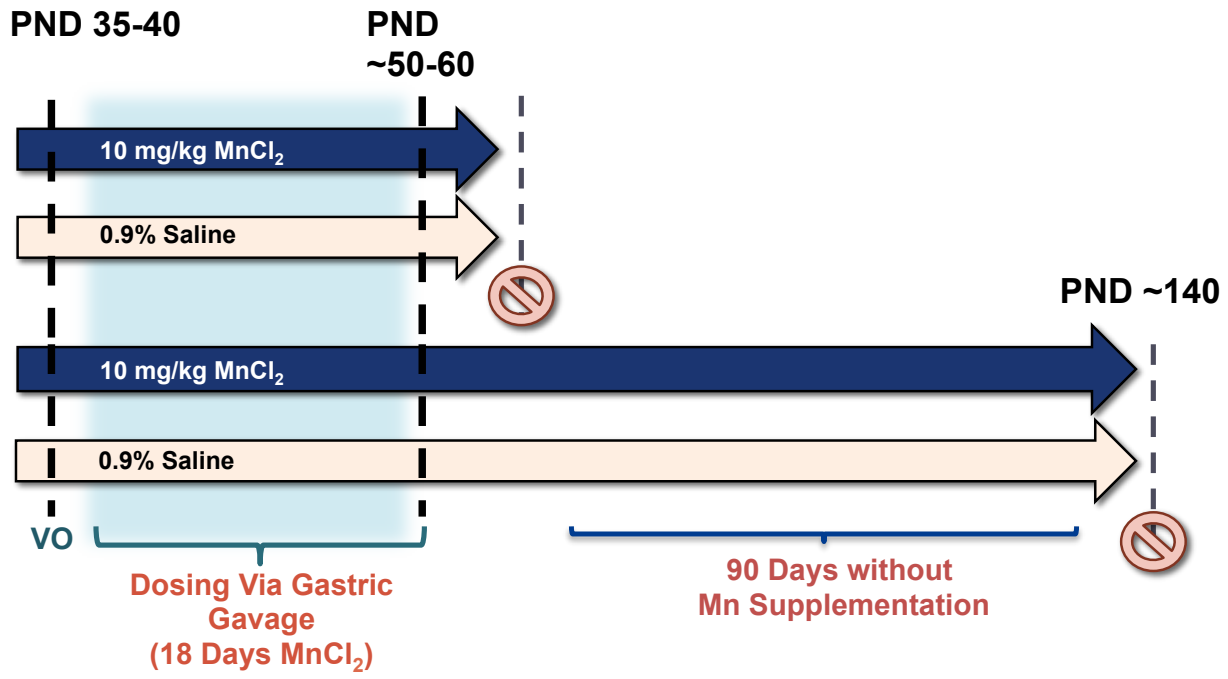

Figure 3. Post-Pubertal Mn Experimental Design

In short, females were monitored for vaginal opening (VO) starting on PND 27. Starting on the day of VO, females were dosed via gastric gavage with either 0.9% Saline (Control) or 10 mg/kg $MnCl_2$ for 18 days. On the 19th day, half of the females in each group were sacrificed, and serum and tissues were collected. The rest of the females remained housed in normal conditions for 90 additional days post-treatment, and were subsequently sacrificed.

		µg Mn/g Dry Tissue Weight ± SEM	
Tissue	**Group**	**PND 50**	**PND 140**
Blood	*Saline-Treated*	0.5198 ± 0.044	0.4448 ± 0.228
	Mn-Treated	1.0996 ± 0.1858 **	0.3683 ± 0.091
Liver	*Saline-Treated*	7.8858 ± 0.676	6.486 ± 0.169
	Mn-Treated	9.687 ± 1.563	6.321 ± 0.120
Ovaries	*Saline-Treated*	0.8691 ± 0.087	0.6546 ± 0.249
	Mn-Treated	1.0801 ± 0.1139	0.8732 ± 0.1887
Uterus	*Saline-Treated*	0.9190 ± 0.130	2.6232 ± 0.636
	Mn-Treated	1.2055 ± 0.185	2.2352 ± 0.586
Mammary Gland	*Saline-Treated*	0.1592 ± 0.032	0.0896 ± 0.017
	Mn-Treated	0.1136 ± 0.029	0.0910 ± 0.009
MBH	*Saline-Treated*	2.5947 ± 0.138	2.5935 ± 0.171
	Mn-Treated	2.6158 ± 0.119	2.3149 ± 0.080
POA	*Saline-Treated*	2.1488 ± 0.1159	2.1193 ± 0.095
	Mn-Treated	2.1498 ± 0.1489	2.484 ± 0.269

Table 3. Mn Content in Blood and Tissues of 50- and 140-Day Old Females

Daily $MnCl_2$ exposure caused a significant increase in the Mn content in blood in Mn-treated females at PND 50, but not in tissues. Values represent the mean ± SEM. Data was analyzed by student's t-test. N=10 and 9 for controls and Mn-treated, respectively at 50 days; N=9 in both treatment groups at 140 days. **$p<0.01$.

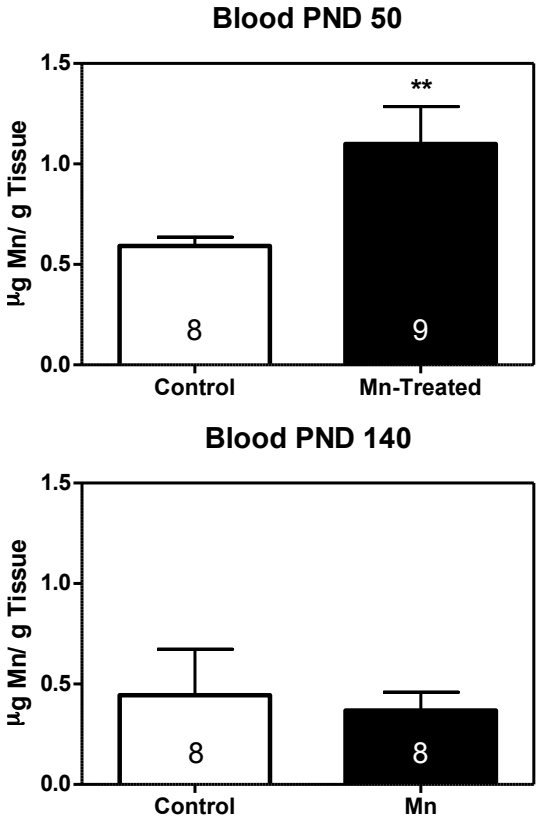

Figure 4. Mn Blood Content Following Post-Pubertal Exposure

Daily $MnCl_2$ exposure caused a significant increase in the total Mn content in blood in Mn-treated females at PND 50. The amount of Mn returned to normal levels by PND 140. Values represent the mean ± SEM. Data was analyzed by student's t-test. **$p<0.01$. N=8 and 9 for controls and Mn-treated females, respectively, at 50 days; N = 8 in both treatment groups at 140 days.

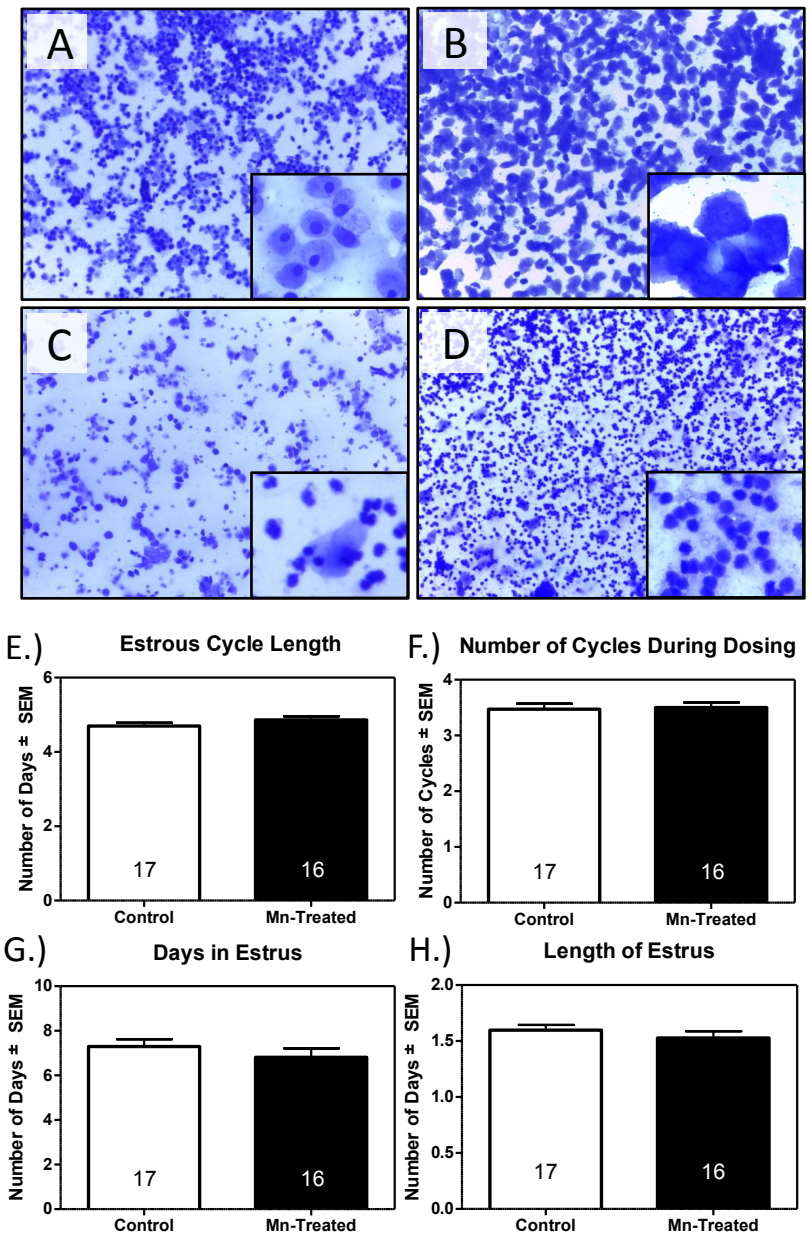

Figure 5. Effect of Post-Pubertal Mn on the Estrous Cycle

Images show respective crystal violet-stained vaginal smears for proestrus (A), estrus (B), diestrus 1 (C), and diestrus 2 (D). There was no difference between either treatment group in length of the estrous cycle (E), total number of cycles during dosing (F), total days spent in estrus (G), or length of estrus (H). Values represent the mean ± SEM. Data was analyzed by student's t-test. N is noted within bars.

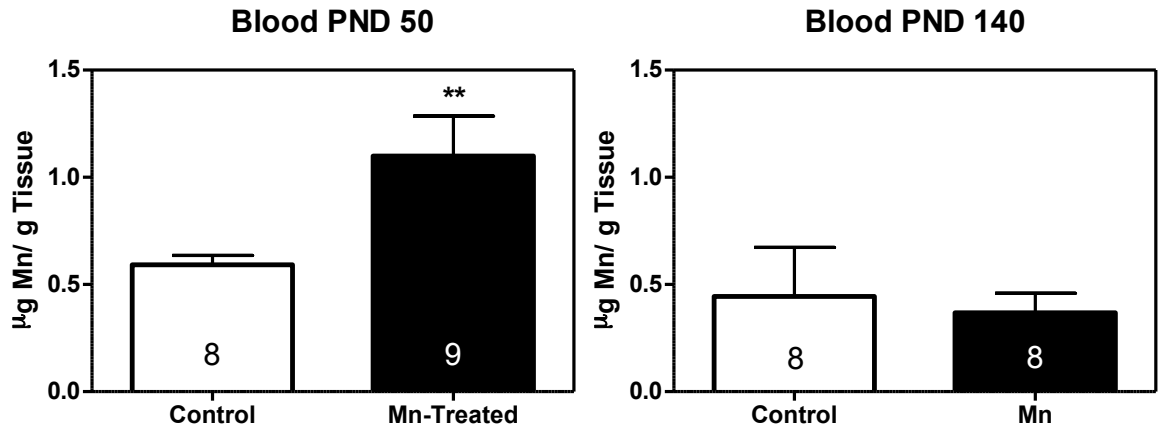

Figure 6. Effect of Post-Pubertal Mn Exposure on Serum Estradiol

Chronic post-pubertal exposure to $MnCl_2$ resulted in a significant increase in serum estradiol levels at PND 50 (A). However, this difference leveled off by PND 140 and was no longer significant. Values represent the mean ± S.E.M. Data was analyzed by student's t-test; **$p<0.01$. N is noted within bars.

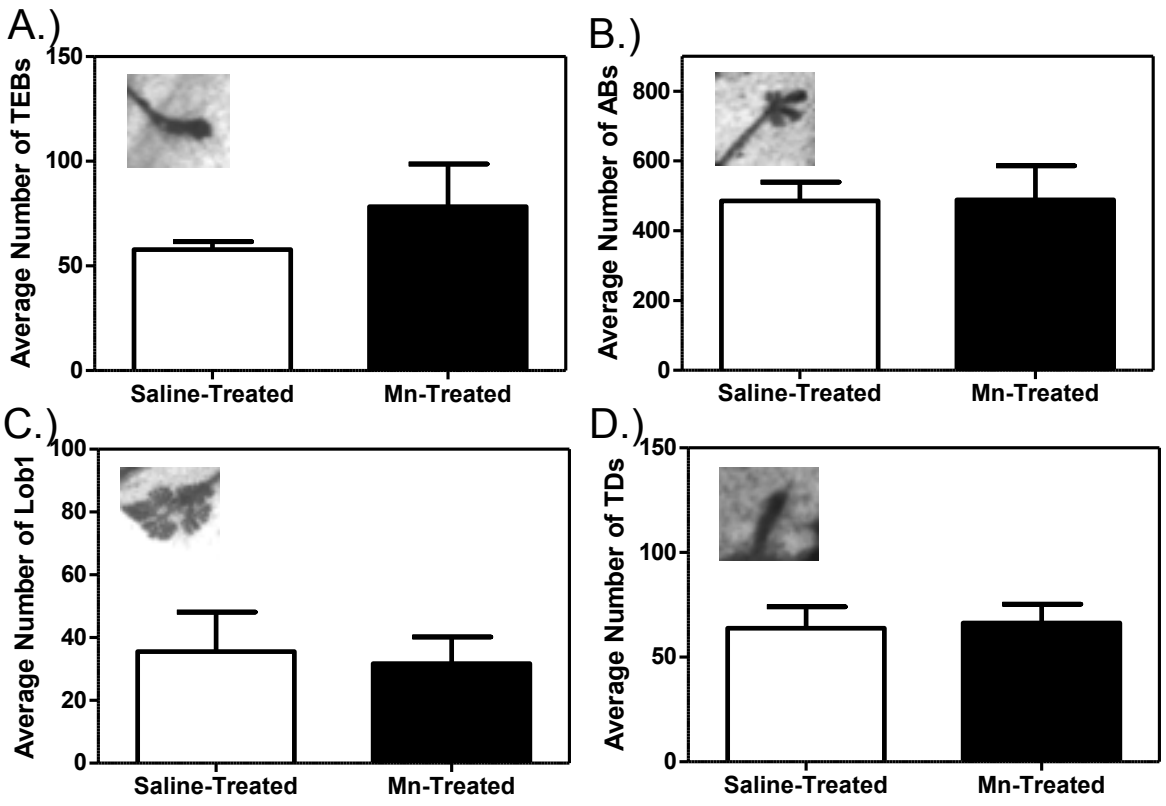

Figure 7. Effect of Post-Pubertal Mn on MG Ductal Differentiation

Chronic exposure to post-pubertal $MnCl_2$ had no effect on MG ductal differentiation. Specifically, Mn treatment resulted in no difference in the total number of TEBs (A), ABs, Lob1s (C), or TDs (D) when compared to controls. Values represent the mean (± SEM); N=4 for each group. Data was analyzed by student's t-test. Abbreviations: TEBs: terminal end buds; ABs: alveolar buds; Lob1: lobular type 1; TD: terminal ducts.

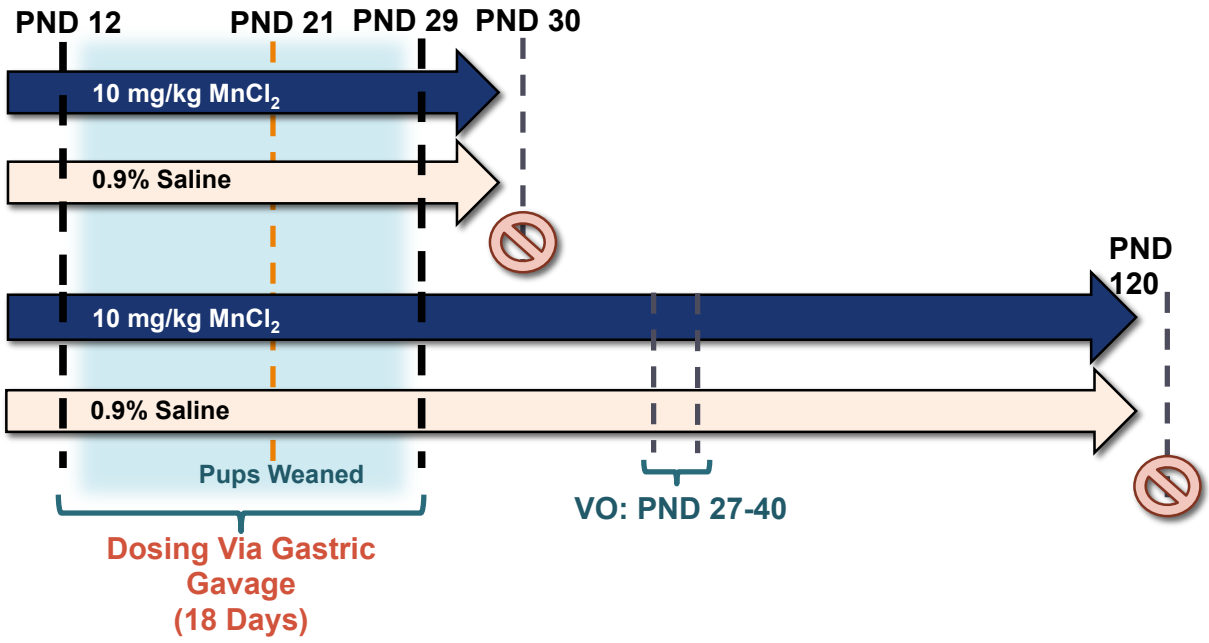

Figure 8. **MnPP Experimental Design**

Briefly, Sprague Dawley female rats were dosed daily via gastric gavage with either 10 mg/kg $MnCl_2$ or equal volume of 0.9% saline (control) starting on PND 12 through PND 29. We have shown previously that 10mg/kg $MnCl_2$ is the minimum effective dose that results in female precocious puberty. Females were sacrificed on either PND 30 or PND 120, at which point inguinal mammary gland #4 was collected from each female and processed for IHC, IF, or WB. Mn-induced precocious puberty was confirmed by monitoring vaginal opening (VO) starting at PND 27. At the time of harvest, PND 30 females were confirmed to be anestrous, while PND 120 females were confirmed to be in diestrus.

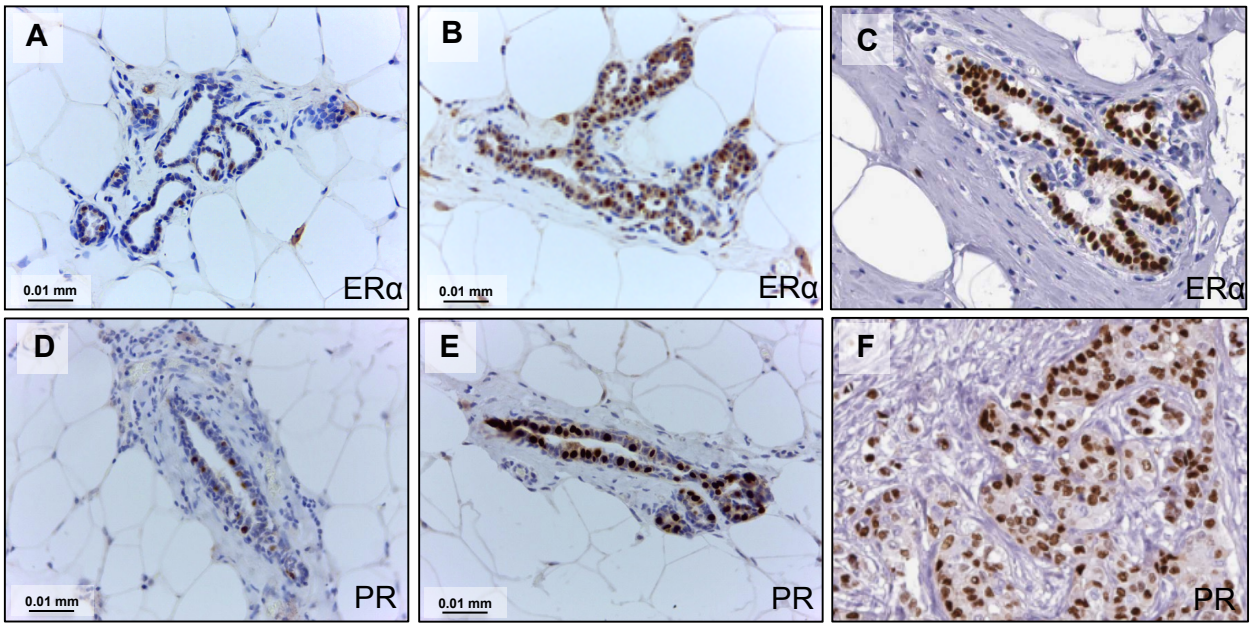

Figure 9. Expression of ER and PR in the Hyperplasic Rat and Neoplastic Human MG

IHC Analysis of Steroid Receptor Expression is PND 120 MGs. MnPP results in increased expression of ER and PR at PND 120. Representative images depict an increase in ERα positive cells in MnPP females (B) when compared to saline-treated controls at PND 120 (A). Additionally, an increase in PR-Positive cells was observed in MnPP females at PND 120 (E) when compared to controls (D). ER+ DCIS in a 55-year-old female (E) resembles ER expression levels in MnPP induced rat MG hyperplasia (B). Lobular carcinoma in a 51-year-old female shows strong positivity for PR+ (F), which although more advanced, maintains a level of PR+ expression similar to that of MnPP-induced rat MG hyperplasia (E).

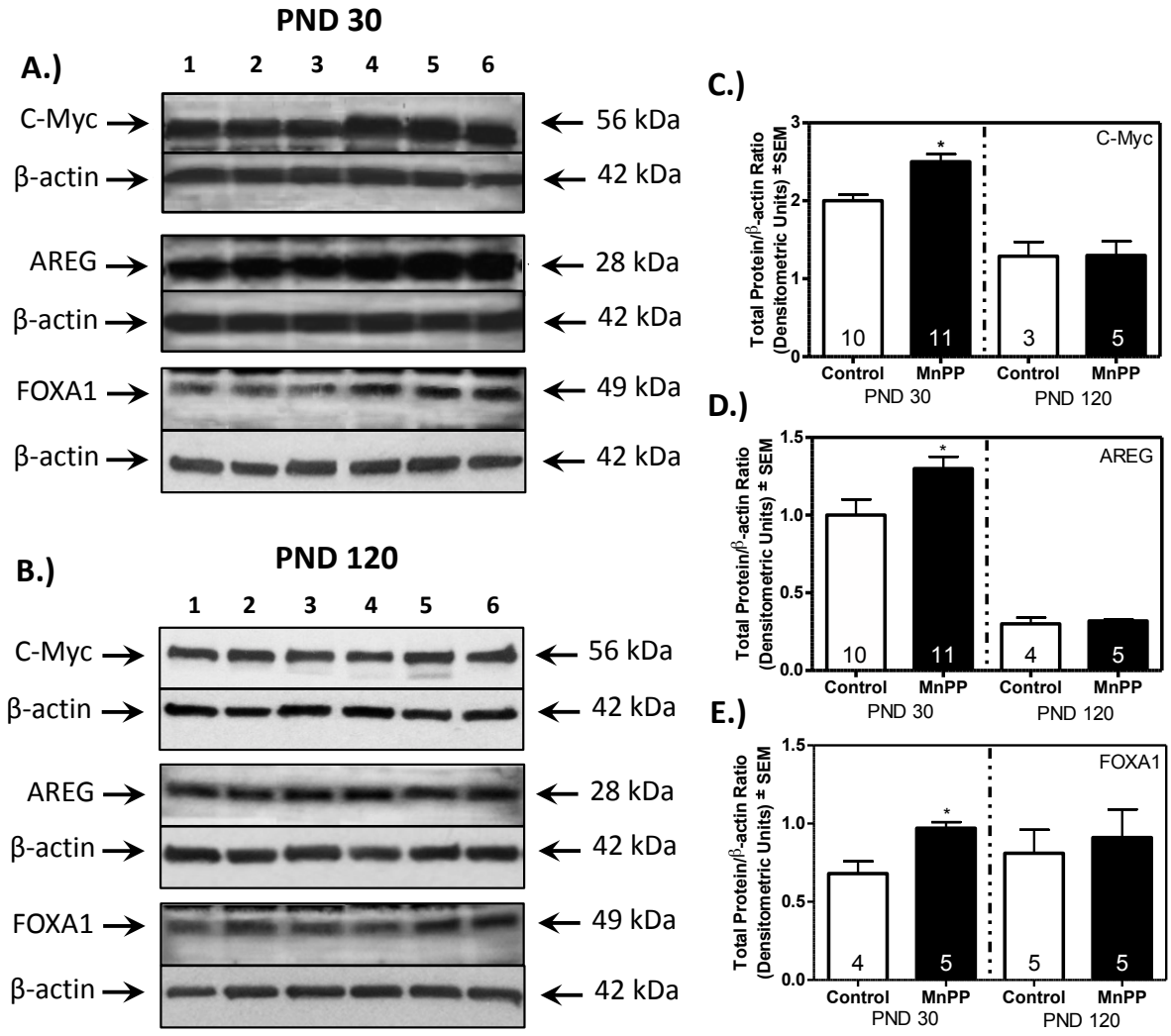

Figure 10. Effect of MnPP on E_2- Regulated Protein Expression

Representative images show that MnPP (lanes 1-3) resulted in increases expression of AREG, c-Myc, and FOXA1 in epithelial cell lysates from PND 30 prepubertal MGs when compared to controls (4-6) (A), but not at PND 120 (B). Bar graphs indicate changes in the mean (±SEM) protein expression from PND 30 to PND 120 of c-Myc (C), AREG (D), and FOXA1 (E). N is noted within bars. Data was analyzed by student's t-test. *$p<0.05$.

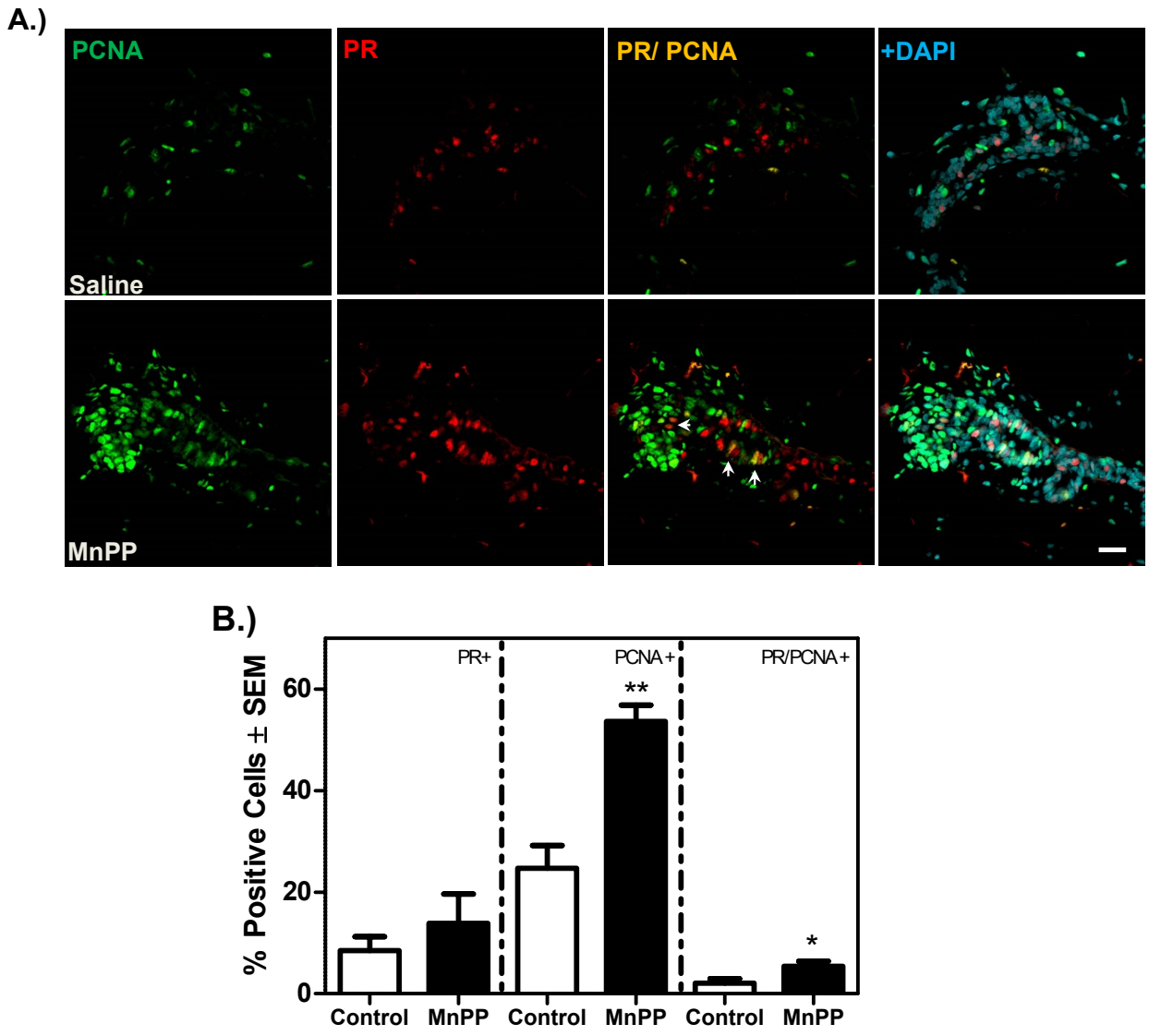

Figure 11. Distribution of PR+ and PCNA+ Cells in MnPP Gland at PND 30

Representative IF images (A) show PCNA (Green), PR (red), and dual labeling (A) cells expressed in MGs of PND 30 saline-treated and MnPP females. Graph (B) depicts mean (± SEM) percentage of each cell type comprising the gland at PND 30. Cells were counted from 2-4 serial sections from each animal, where an average of 3100 cells was counted per animal. White arrows indicate dual-labeled cells. Data was analyzed by student's t-test. Scale Bar = 20µM. **p<0.01; ***p<0.001; Scale Bar = 20µM; N=5 for control; N=4 for MnPP.

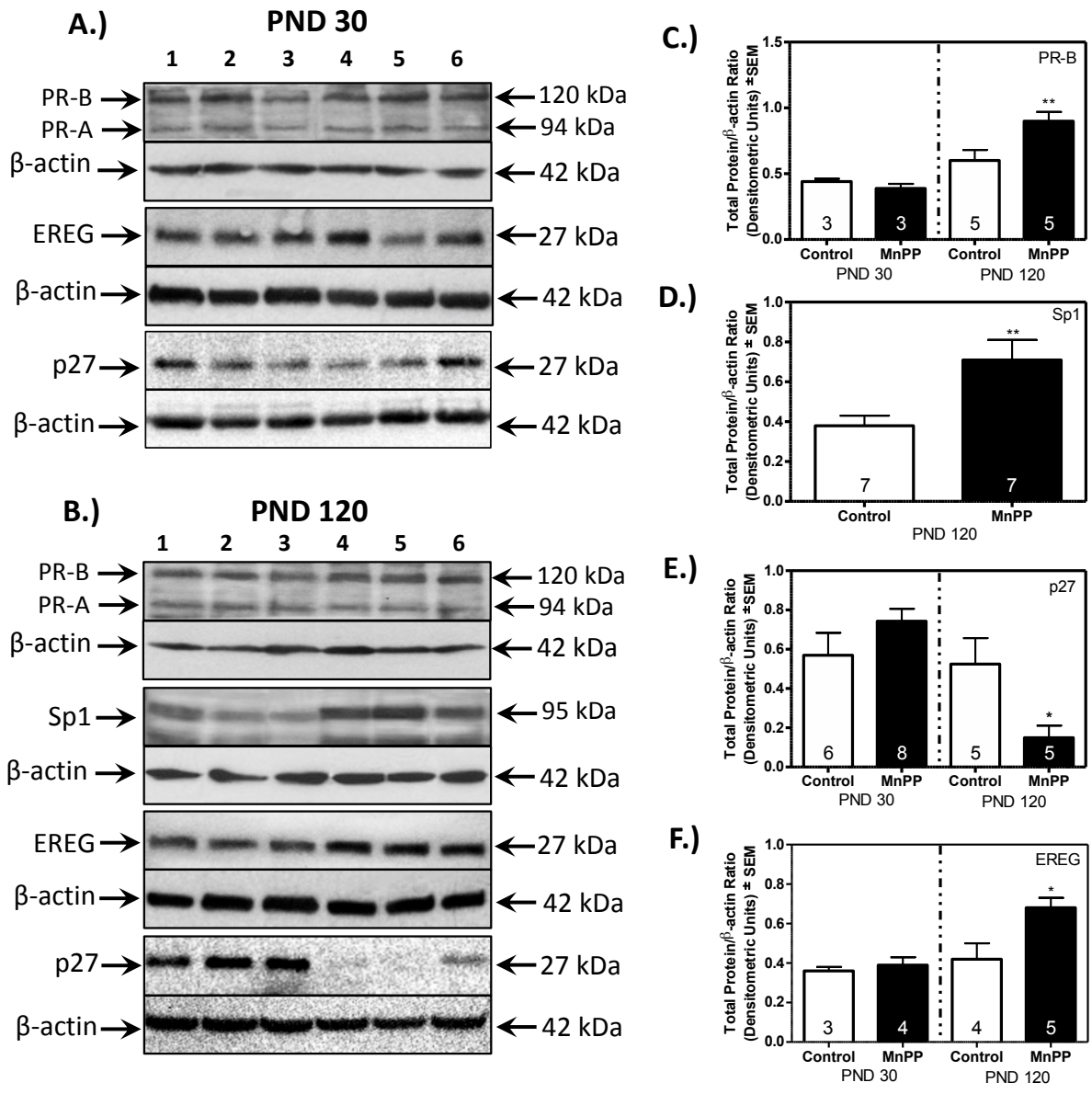

Figure 12. Effect MnPP on PR-Mediated Protein Expression

Representative western blots show that MnPP (lanes 1-3) result in increased expression of PR-B, sp1, and EREG in epithelial cell lysates from PND 120 MGs when compared to controls (4-6) (B), but not at PND 30 (A). Furthermore, MnPP results in a decrease in p27 at PND 120 (B) but not at PND 30 (A). Bar graphs indicate changes in the mean (±SEM) protein expression from PND 30 to PND 120 of PR-B (C), sp1 (D), p27 (E), and EREG (F). N noted within bars. Data was analyzed by student's t-test. *$p<0.05$; **$p<0.01$.

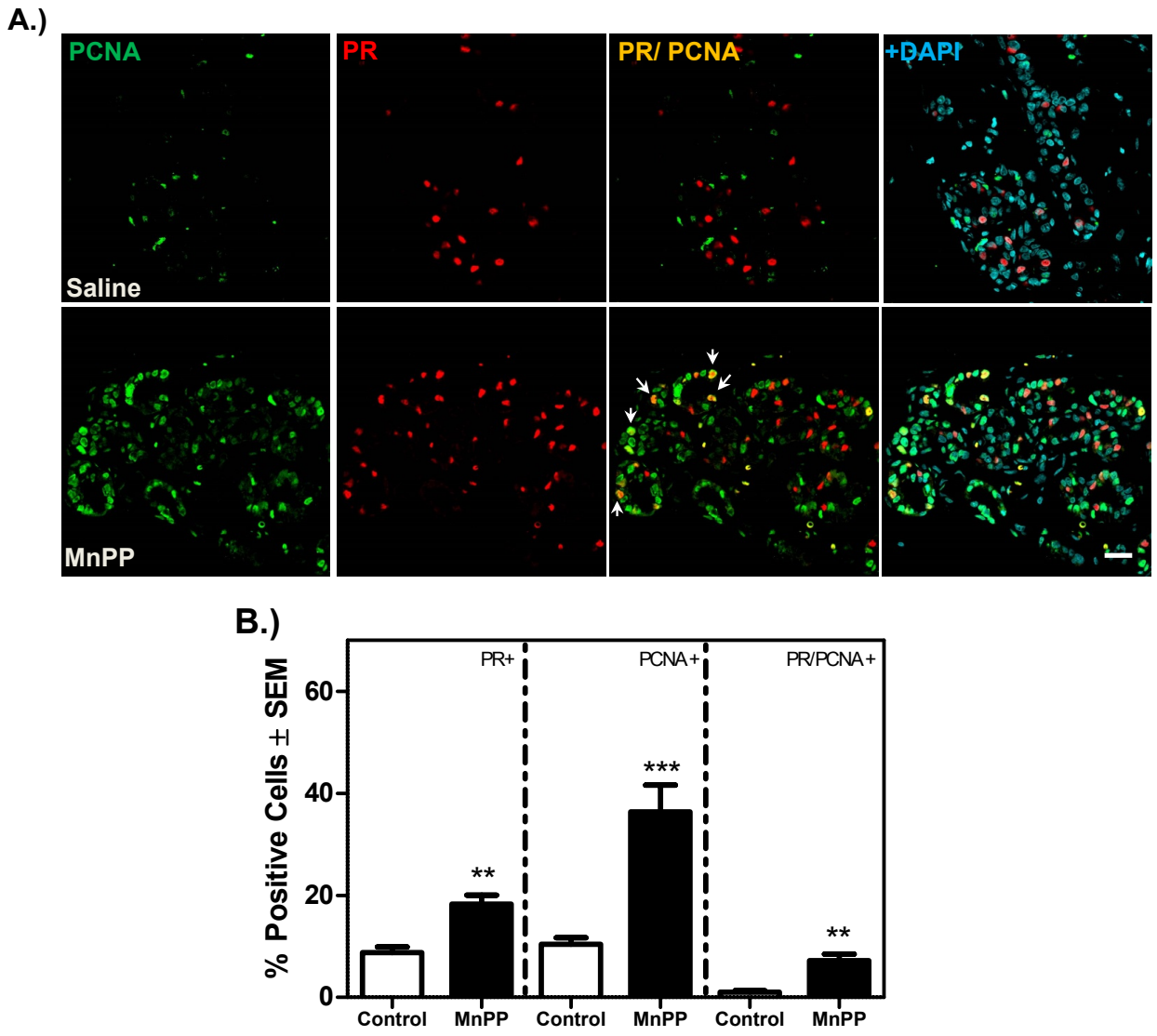

Figure 13. Effect of MnPP on Distribution of PR+ and PCNA+ Cells in the Adult Virgin MG

Representative IF images (A) show PCNA expressing (green), PR expressing (red) and dual labeling in MGs of PND 120 saline-treated and MnPP females. Graph (B) depicts mean (± SEM) percentage of each cell type comprising the gland at PND 120. Cells were counted from 2-4 serial sections from each animal, where an average of 1900 cells was counted per animal. White arrows indicate dual-labeled cells. Data was analyzed by student's t-test. Scale Bar = 20µM. **p<0.01; ***p<0.001; Control N=5; MnPP N=4.

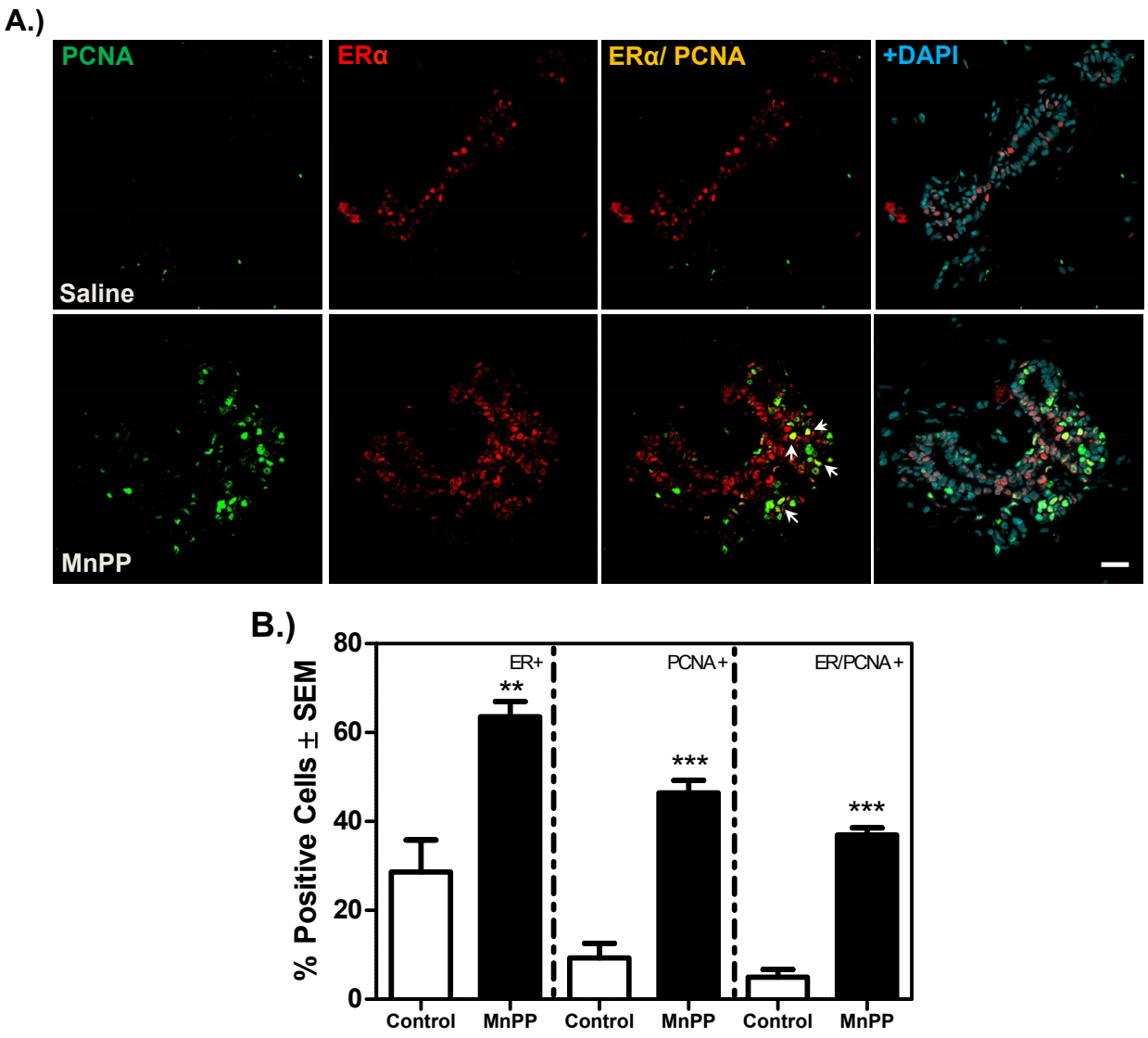

Figure 14. Effect of MnPP on Distribution of ER+ and PCNA+ Cells in the Adult Virgin MG

Representative IF images (A) show PCNA-expressing (green), ERα-expressing (red) and dual labeling in MGs of PND 120 saline-treated and MnPP females. Graph (B) depicts mean (± SEM) percentage of each cell type comprising the gland at PND 120. Cells were counted from 2-4 tissue sections from each animal, where an average of 3000 cells was counted per animal. White arrows indicate dual-labeled cells. Data was analyzed by student's t-test. Scale Bar = 20μM. **p<0.01; ***p<0.001; Control N=5; MnPP N=4. Scale Bar = 20μM. **p<0.01; ***p<0.001.

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BIOGRAPHICAL SKETCH

Alina Marie Hamilton began working in the laboratory of Dr. Robert Dearth in September of 2011, as an undergraduate. She was accepted into the UTPA Research Initiative for Scientific Enhancement (RISE) program in 2013. In 2014, she held a summer research internship in the laboratory of Dr. W. Les Dees at Texas A&M University. The following year, she obtained her Bachelor's degree in biology with a minor in chemistry from the University of Texas Pan American in May of 2015.

She began graduate study in the summer of 2015, and held a graduate assistantship with the UTRGV RISE Program during her tenure. Alina went on to earn her Master of Science degree in Biology from the University of Texas Rio Grande Valley in July of 2017, and will begin working on her doctorate at the University of North Carolina at Chapel Hill in the fall of 2017. She maintains a permanent mailing address at 1762 Summer Park Lane, San Antonio TX, 78213.