REPRESSOR OF ESTROGEN RECEPTOR ACTIVITY (REA) IS A GENE DOSE-DEPENDENT COREGULATOR PROTEIN AFFECTING ESTROGEN SIGNALING AND CELL SURVIVAL

BY

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DISSERTATION
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Repressor of Estrogen Receptor Activity (REA) is an evolutionarily conserved protein with established roles in multiple, essential cellular processes including transcription, mitochondrial biogenesis and replicative senescence. Previous reports suggest that REA is a multifunctional protein with important biological activity. However, despite increasing information, the physiological functions and regulatory mechanisms of REA are still incompletely understood. To gain insight into the role of REA in estrogen responsive tissues, we investigated its function in both the uterus and mammary gland by conditional deletion of the REA gene, because the conventional knockout of REA is embryonic lethal. To this end, we generated REA$^{f/f}$ mice and crossed them with PR-Cre knock-in mice as well as WAP-Cre transgenic mice, thereby creating conditional REA knockouts under the control of either the progesterone receptor or whey acidic protein promoter. We found that complete REA loss of function resulted in severe defects in both uterine and mammary gland development and functional activities. REA deletion in the uterus resulted in infertility due to severely compromised uterine development and function. Ablation of REA in the mammary gland resulted in impaired mammary gland ductal and alveolar morphogenesis, leading to reduced body size and growth of the offspring nursed by females lacking both REA alleles. These observations demonstrate that REA is critical for normal uterine and mammary gland function. By contrast, 50% reduction of REA via heterozygous inactivation enhanced estradiol (E2) response in both the uterus and mammary gland. Heterozygous REA mutant mice treated with E2 developed abnormally large uteri due to increased E2-mediated uterine epithelial cell proliferation and increased fluid imbibition. Additionally, mammary ductal elongation during puberty was accelerated in heterozygous mice. Our findings indicate that REA possesses dose-
dependent activity, showing a positive modulatory role in heterozygous REA animals and a negative modulatory role in homozygous REA animals. These studies in animals with conditional loss of both or only one allele of REA highlight the importance of the correct gene dosage of REA for normal uterine and mammary gland function.
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CHAPTER 1

ROLE OF ESTROGEN ACTION IN UTERINE AND MAMMARY GLAND PHYSIOLOGY

1.1. INTRODUCTION

*Molecular mechanisms of estrogen action*

The ovarian steroid hormone estrogen (E) is a key modulator of normal mammalian reproductive functions. It profoundly impacts the growth and development of female reproductive organs such as the uterus, ovary and mammary gland (8, 19).

The biological effects of estrogen are mediated through the estrogen receptor (ER) proteins which bind the hormones, dimerize, and regulate the transcription of estrogen-responsive genes. ER exist as two isoforms, ERα and ERβ, which are members of the nuclear receptor superfamily of ligand-activated transcription factors (22, 44). Although these two subtypes of ER differ with respect to tissue distribution and functional activities, both isoforms share a similar structure (28, 40). ERs contain an N-terminal activation function (AF-1), a DNA binding domain (DBD), and a C-terminal activation function (AF-2) which contains the ligand-binding domain (LBD) (12).

Although there are several proposed mechanisms of ER action, traditionally, two main categories are designated, namely, classical or genomic pathway and non-genomic pathway. In the classical mechanism of ER action, upon hormone stimulation, ERs dissociate from chaperone protein, dimerize, and bind to DNA sequences known as estrogen response elements (EREs) which are located in the promoters of target genes (40). Estrogen-dependent transcriptional regulation through ERE pathway involves a variety cofactors that function with liganded ERs to modify histones, alter chromatin structures, and recruit the RNA polymerase II (polII) transcriptional machinery (24). Many coactivators bind directly to agonist-activated AF-2 of ER
through short α-helical “LXXLL” motifs called NR boxes (14, 52). Coactivators include following: (1) histone-modifying enzymes complexes that contain number of the steroid receptor coactivator (SRC) family of proteins, (2) chromatin remodeling complexes such as SWI/SNF, and (3) mediator complexes such as TRAP and DRIP (52).

It is known that approximately one third of ER-regulated genes do not contain EREs in the promoters (43). These genes are regulated by ERE-independent genomic mechanism by which ER indirectly binds to other response elements through interactions with other transcription factors including activating protein 1 (AP1), stimulating protein 1 (SP1), or NF-κB (43, 51, 54).

Estrogen can also exert its effects through very rapid non-genomic mechanisms and are believed to be mediated through extranuclear ER. This mechanism is frequently associated with the activation of protein kinase cascades: phospholipase C (PLC)/protein kinase C (PKCs), Ras/Raf/MAPK, phosphatidylinositol 3 kinase (PI3K)/AKT and cAMP/protein kinase A (PKA) (11, 29, 31).

The role of estrogen action in uterine functions

The uterus is a major estrogen-responsive organ and plays a central role in female reproduction. During the proliferative phase of the menstrual cycle, 17β-estradiol (E2) stimulates the rapid growth of the uterine endometrium, including both epithelial and stromal cells (48). In rodents, the uterus responds to cyclical changes in estrogen and progesterone levels for preparation of implantation and for pregnancy to be established and maintained (18, 19, 42). During pro-estrous stage, a surge in the blood estrogen concentration results in the well characterized physiological and biochemical changes of the uterus. These responses have been
divided into two events that occur early, within the first hours after E2 surges, and subsequent responses that follow up to 24 h after E2 elevation (18, 33). Early uterine responses to estrogen include estrogen receptor occupancy, transcription of early phase genes such as \( c-fos \), water imbibition, hyperemia, eosinophil infiltration, and calcium influx. The later phase responses include the transcription of late phase genes such as \( lactoferrin \), increase in uterine wet weight, further accumulation of immune system cells, the development of the epithelial layer into columnar secretory epithelial cells, cellular hypertrophy and hyperplasia, resulting in growth of the uterus (18, 55). These physiological effects of estrogen in the uterus are mediated via its cognitive nuclear receptor, estrogen receptor alpha (ER\( \alpha \)) (8). In the mouse uterus, ER\( \alpha \) expression begins to appear in the stroma on postnatal day (P) 1 and is detectable maximally on P10, whereas the expression of epithelial ER\( \alpha \) is delayed and reaches a peak around P16. Thus, a full biological response to estrogen in terms of maximum increases in uterine weight is not possible until at least postnatal day 16, and can be observed only after the animal approaches weaning age (P19-21) (8, 57).

The role of ER\( \alpha \) in normal uterine function was largely elucidated through knockout of ER\( \alpha \) (\( \alpha \)ERKO) in mice. The \( \alpha \)ERKO mice have hypoplastic uteri but contain all three definitive uterine compartments, the outer myometrium, endometrial stroma, and epithelium. However, the uteri have a reduced proportion of stroma, with a sparse distribution of uterine glands (23). This suggests that ER\( \alpha \) expression may not be essential for uterine organogenesis itself, but it is required for normal uterine growth and maturation.

Estrogens also play a particularly critical role at the time of implantation. There is a limited time period, so called the window of implantation, when the uterus becomes favorable to blastocyst acceptance and implantation (30). In mice, the uterus becomes receptive on day 4 of
pregnancy and proceeds to the refractory state on day 5 (46). Estrogen levels within a very narrow range determine the duration of this window of uterine receptivity. It has been shown that this period of uterine receptivity shortens greatly if estrogen levels exceed this narrow range, because higher estrogen levels induce a refractory state of the uterus that is accompanied by aberrant expression of implantation-related genes (30).

Estrogen regulation of mammary gland development

The mammary gland is a specialized organ of female mammals, which enables them to supply their offspring with all essential nutrients via the milk produced in the gland. Two main compartments constitute the mammary gland: the epithelium, which consists of ducts and alveolar cells; and the stroma, which is also called the mammary fat pad. Each fat pad has an exterior nipple to which the primary epithelial duct is connected to allow the release of milk during lactation and at least one major lymph node (15, 49).

The majority of mammary gland development takes place postnatally beginning with the onset of puberty and remains undifferentiated until pregnancy and lactation (8, 15, 49). Development of the mammary gland is divided into five distinct stages: embryonic, pre-pubertal, pubertal, mature adult and pregnancy/lactation. Each developmental stage is tightly regulated by an orchestration of ovarian steroid hormone and growth factor action (4, 15, 26, 49). Throughout the recurrent estrous cycles, the epithelial ductal network and branching increase, while in pregnancy and lactation, alveolar units proliferate and differentiate into milk-secretory cells (15, 16). The ovarian steroid hormones, estrogen and progesterone, are key mediators of ductal morphogenesis and are mitogenic for mammary epithelial cells (15). These physiological effects of hormones are mediated through the estrogen receptor (ER) and progesterone receptor (PR), both members of the nuclear receptor superfamily of ligand-activated transcription factors (22).
Of the two estrogen receptors (ERα and ERβ), ERα is believed to be the primary receptor for mammary gland development and function.

After birth and until puberty, the mammary gland remains rudimentary. At onset of puberty, the mammary gland develops rapidly in response to changes in circulating hormone levels (16, 49). Ductal elongation during puberty occurs through cap cell proliferation at the terminal end buds (TEBs) of each individual duct, while maintaining close contact with the stromal fat pad. Estrogen has been shown to directly stimulate the formation of TEBs and promote proliferation of the mammary ductal epithelium (8, 9). However, the outermost proliferating cap cells do not express ER, suggesting the existence of indirect estrogen action in mammary gland development (8, 9). More recently, the Brisken lab performed a series of transplantation experiments to demonstrate that estrogen facilitates epithelial proliferation and morphogenesis through a paracrine mechanism (32). When ERα-null epithelial cells were mixed with the wild-type epithelial cells and transplanted into the cleared fat pads of wild-type mice, ERα-null epithelial cells proliferated when wild-type cells were in close proximity, suggesting that paracrine factors secreted by neighboring cells induce proliferation of ERα-negative epithelial cells (32). Several paracrine factors are known to regulate hormone-induced proliferation and morphogenesis including Wnt-4, receptor activator of NF-κB ligand (RANKL), growth hormone and insulin growth factor II (53). With regard to estrogen action, amphiregulin (AREG), a member of the epidermal growth factor receptor (EGFR) family of ligands that binds exclusively to ErbB1, is known to be the major paracrine mediator of ductal morphogenesis (7, 26).

The role of ERα in mammary gland development was also largely elucidated through αERKO mice. Mammary glands of αERKO mice were normal before puberty, however, after the
onset of puberty, a rudimentary ductal structure remained and the ducts failed to invade the mammary fat pad (8). More recently, Khan and colleagues developed whey acidic protein (WAP) driven Cre-mediated conditional ERα knockout mice in an attempt to address the role of ERα in mammary gland development during pregnancy and lactation (13). They demonstrated that the ablation of ERα in mammary epithelium during late pregnancy and lactation, when the alveolar epithelium has begun to differentiate, results in a loss of ductal side-branching and lobuloalveolar structures, ductal dilation, and decreased proliferation of alveolar progenitors (13). However, the mechanism by which estrogens regulate mammary gland development during pregnancy and lactation remains unclear.

The role of coregulators in modulating nuclear receptor-mediated actions

The state of receptor activation and inhibition is modulated not only by the nature of the hormonal ligand but also by a delicate balance between coactivators and corepressors (22, 34). Coactivator proteins assemble into several dynamic, multiprotein complexes that constitute the SRC/p160 family of proteins, CREB binding protein (CBP) and/or p300, and other factors that are recruited in a temporally ordered fashion (6, 34) and up-regulate nuclear receptor activity, at least in part, through enhanced histone acetyltransferase activity. ATP-dependent chromatin remodeling complexes, such as SWI/SNF, and the TRAP-DRIP-ARC (mediator) complex, which act sequentially or combinatorially, also enhance gene transcription by facilitating RNA polymerase II recruitment to promoters (5, 21, 50, 52). Corepressors act in an opposite manner to repress gene transcription, primarily through their interaction with un-liganded nuclear receptors or recruitment of histone deacetylases to form a repressive chromatin state (14, 34, 50). Among
corepressors, the best fully characterized are NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) (50).

To date, approximately 285 coregulators are reported in the literature, and those are frequently associated with numerous physiological functions and pathological conditions (27). Development of coregulator knockout animal models has greatly facilitated our understanding of their physiological impact at the organism level. Among 285 coregulators, 92 knockout mouse models have been documented so far. Of these 92 knockout mice, 53 were embryonic lethal, suggesting the overall importance of coregulators in developmental biology, whereas a wide spectrum of phenotypes were observed for others (27). Knockout models of the steroid receptor coactivator (SRC) family-SRC-1, SRC-2, and SRC-3-have revealed a great deal about coregulator biology in a whole animal context. Knockout of these coregulators results in a wide range of defects, including reproductive (uterine and mammary gland) and metabolic defects (27, 38, 41, 56). Deletion of the corepressor, RIP-140/Nrip-1, resulted in infertility due to anovulation and NCoR knockout was embryonic lethal, with defects in erythrocyte and thymocyte production and central nervous system defects (17, 27). These knockout animal models demonstrate that coregulator proteins are vitally important for normal physiological function, and are also broadly implicated in many pathological states and will be of great interest in clinical medicine.

Estrogen receptor-selective corepressor, REA

ER-selective corepressor REA was previously identified from yeast two-hybrid screening through its ability to interact preferentially with the liganded ER, and it was shown to repress ER transcriptional activity (37). The REA gene is located in chromosomal region 12p13 in humans and chromosome 6 in mice and encodes a 34-kDa protein that is the first ER-selective
coregulator to be identified (1, 37). REA interacts directly with ER by in vitro GST pull-down assays, and this interaction is hormone dependent (10, 37). REA does not directly affect the ability of ER to bind DNA, but rather competes with coactivators (SRC-1 or -3), thereby interfering with functional coactivator recruitment to ER (10). In addition to binding ER, REA has been shown to associate with diverse cellular proteins to regulate transcription such as HDAC, a histone deacetylase and the ovalbumin upstream promoter transcription factors I and II (COUP-TFI, COUP-TFII) and enhancer of zeste homolog 2 (EZH2), a chromatin modifying polycomb group histone methyltransferase known to be involved in chromatin compaction and transcriptional repression (20, 25).

The regions of REA that is required for interaction with ER, and for its repressive activity were previously mapped (10). The regions of REA required for its interaction with ER and for its repression of ER activity are different. The repressive activity requires two regions of REA, encompassing amino acids 19-49 and 150-174, while REA binds to ligand binding domain (AF-2) of ER through a region encompassing amino acids 175-198 (10).

Additionally, REA has also been shown to exist in a complex with prohibitin (PHB), which has 50% amino acid sequence identity and 60% similarity, in the mitochondrial inner membrane (36). The function of these mitochondrial protein complexes has long been implicated in diverse cellular processes such as mitochondrial biogenesis, cell death, and senescence (2, 3, 35, 45).

The physiological function of REA as a significant modulator of ER activity in vivo was previously established by the conventional REA knockout mouse model. Genetic disruption of both alleles of REA (REA
\(^{-/-}\)) resulted in embryonic lethality, suggesting the importance of REA in developmental biology. However, REA heterozygous animals (REA
\(^{+/−}\)) with deletion of single
REA allele were viable, and exhibited haploinsufficiency that enhanced estrogen function and activity in both the uterus and mammary gland (39, 47).

1.2. REFERENCES


CHAPTER 2

COREULATOR REA GENE DOSAGE IS CRITICAL FOR UTERINE DEVELOPMENT AND FERTILITY

2.1. ABSTRACT

Although the effectiveness of hormone-receptor complexes is known to depend on coregulator partner proteins, little is known about the roles of coregulators in uterine development, early stages of pregnancy, and implantation. Because conventional genetic deletion of the coregulator, repressor of estrogen receptor activity (REA) was embryonic lethal, to define the roles of REA in post-embryonic stages and in a tissue-specific manner, we have generated REA conditional knockout mice by cre-loxP recombination in which REA function was abrogated only in progesterone receptor (PR)-expressing tissues. We have examined the role of REA in uterine development and functional activities and found that REA shows gene dose-dependent activity. Conditional homozygous mutant mice (REA\textsuperscript{d/d}) developed to adulthood and showed normal ovarian function, but females were infertile due to severely compromised uterine development and function characterized by cell cycle arrest and apoptosis, resulting in failure of implantation and decidualization. By contrast, mice heterozygous for REA (REA\textsuperscript{f/d}) had a very different phenotype with estradiol treatment resulting in hyperstimulated, abnormally large uteri showing increased proliferation of luminal epithelial cells and enhanced water permeability associated with up-regulation of aquaporins. Associated with uterine over-stimulation by hormone, these heterozygous mice showed a subfertility phenotype with reduced numbers and sizes of litters. These findings highlight that REA is a multifunctional protein with key roles in uterine development and regulation of estrogen receptor activities required for fertility that are distinguishable on the basis of REA’s gene dose-dependent effects.
2.2. INTRODUCTION

Nuclear receptors act as ligand-activated transcription factors mediating the actions of many hormones and some non-hormonal ligands. The state of receptor activation and suppression is modulated not only by the nature of the ligand but also by delicate balance between coactivators and corepressors in a cell (7, 12, 18, 28). Repressor of Estrogen Receptor Activity (REA) was initially identified as a coregulator of the Estrogen receptor (ER) that repressed the activity of estrogen (32). Our prior conventional genetic disruption of both alleles of REA resulted in embryonic lethality (38), suggesting that REA has fundamentally important cellular functions. Interestingly, however, heterozygous (REA+/−) animals, which contained one-half the normal level of REA, developed normally, but their uteri and mammary glands showed enhanced responsiveness to estrogen (34, 38), indicating that REA also had a crucial function as a modulator of estrogen receptor activity. To be able to address the role of REA in uterine development and function, we have generated mice with conditional tissue-selective deletion of REA.

REA, also known as prohibitin 2, is a highly conserved protein (1). In addition to binding to the ER, REA has been shown to interact with cellular proteins associated with chromatin remodeling and transcriptional repression, such as ovalbumin upstream promoter transcription factors I and II (COUP-TFI, COUP-TFII), enhancer of zeste homolog 2 (EZH2), a chromatin modifying polycomb group histone methyltransferase and histone deacetylase 1 (HDAC1) (16, 21, 22), and to compete with coactivators such as steroid receptor coactivator-1 (SRC-1) for binding to chromatin of estrogen-regulated genes (32). In addition to its role as a brake on estrogen-ER regulated gene expression in the nucleus, REA/PHB2 has also been demonstrated to exist in a complex with prohibitin (PHB) in the mitochondrial inner membrane (31), and these
mitochondrial protein complexes have been implicated in mitochondrial biogenesis, cell death and senescence (2, 29, 31, 37). These findings imply that REA may have pivotal roles in several key cellular processes. However, despite accumulating data, the physiological functions that REA controls during development remain elusive.

In the present study, to circumvent the embryonic lethality we encountered in conventional knockout mice and to enable the study REA function in post-embryonic tissues, we have generated conditional REA knockout mice (REA\(^{dd}\)) by cre-loxP recombination in which REA function was abrogated only in progesterone receptor (PR)-expressing cell lineages. Our findings in animals with conditional loss of both alleles of REA or only one allele of REA highlight the importance of the correct gene dosage of REA for optimal uterine development, growth and function. Additionally, this knockout mouse model is the first to enable definition of the complete loss of REA function phenotype and is also currently the only available model for delineating the functional role of REA as a nuclear receptor coregulator in vivo.

2.3. MATERIALS AND METHODS

*Generation of conditional REA knockout mice*

All animals were maintained in accordance with the NIH Guide for Care and Use of Laboratory Animals, and all procedures described here were approved by the University of Illinois and Baylor College of Medicine Institutional Animal Care and Use Committees. Adult female C57BL mice were purchased from Harlan Co. (Indianapolis, IN).

The mouse BAC library clone 284H12 (Invitrogen, Carlsbad, CA) was prepared by alkaline lysis method and this DNA was used as the template for mouse REA genomic DNA to generate a targeting vector. The targeting vector was designed to flank the REA gene exons 2
through 6, which are required for REA repressive activity and for interaction with ER. Two loxP sequences were introduced into REA intron1 and intron6. Frt-floxed neomycin-resistance gene was inserted between intron 6 and the 5'-loxP site. The targeting vector contained flanking 5.2 kb (5'-targeting arm) and 3.0 kb (3'-targeting arm) mouse REA and neighboring sequences for homologous recombination. In addition, the herpes simplex virus thymidine kinase (HSV-TK) gene was located outside of the REA sequence and served as a negative drug-selection marker. The sequence of the targeting vector was verified by a fluorescent dye-terminator sequencing method using the BigDye DNA Sequencing Kit (PE Applied Biosystems, Foster City, CA). The targeting vector was linearized by I-CeuI digestion and purified by phenol-chloroform extraction method for electrophoration to ES cells. ES cell work was carried out as previously described (20, 38).

Genomic DNAs from selected ES clones were digested with XbaI, followed by Southern blotting with 5’ and 3’ probes. The 5’ probe for Southern screening was generated by PCR using the BAC284H12 (Research Genetics, Inc) clone DNA as a template. A 459 bp 5’ probe was generated by PCR of this template using primers 5’-

CGGAATTCCCTATGGACTTGTGTGAGAAGCCG -3’ and 5’-

CGGGATCCCTCAGCCAACCTGGTGATTGTGAGAAGCCG -3’. In the same way, a 3’ probe, 375 bp, was generated using primers, 5’-CGGGATCCCGCATATATATGTGTGTACTCA-3’, and 5’-

CGGGATCCCGGTAGTGTGTGTACTCA-3’. Both 3’ and 5’ probes are designed to detect the external region of the targeted locus for selection of the correct insertion. ES cell lines showed a 22.4 kb fragment of the wild-type allele, but in targeted cell lines this altered locus was detected with 14 kb and 8.4 kb fragments as the result of the introduction of additional XbaI sites to the targeting vector (Figure 2.1). ES cells heterozygous for the targeted insertions were
microinjected into blastocysts donated from a C57BL/6J strain mouse and implanted into pseudopregnant female mice. Male chimeras were mated with C56BL/6J females.

Genotypes of REA\(^{\text{flox-frt-neo}^+/+}\) mice were determined by PCR. For PCR analysis, the REA\(^{\text{flox-frt-neo}}\) allele was detected by primer 1 (P1; 5’-TGGGCTGTCATATTCAAG-3’) and primer 2 (P2; 5’-GGTGGGATTAGATAAATGCC-3’), which bind to the neomycin resistant cassette of REA\(^{\text{flox-frt-neo}}\) allele. This primer pair amplifies a fragment 696 bp from the REA\(^{\text{flox-frt-neo}^+/+}\) mice, but not from wild-type mice. DNA was also amplified by using primer 3 (P3; 5’-CCCAGAACTTGAAAGCCTAGCTG-3’) and primer 4 (P4; 5’-GAGGTCATGGACAGGAGTTGG-3’) which bind to exon1 and 2, respectively. In this case, a 743 bp fragment was detected from the REA\(^{\text{flox-frt-neo}}\) allele and a 611 bp fragment was detected from the wild-type allele.

To generate REA\(^{\text{flox}^+/+}\) mice, REA\(^{\text{flox-frt-neo}^+/+}\) mice were crossed with mice expressing Saccharomyces cerevisiae enhanced FLP1 recombinase variant (FLPe) from the Gt(ROSA)26Sor gene (The Jackson Laboratory) to facilitate in vivo frt-neo deletion. Conversion of the REA\(^{\text{flox-frt-neo}^+/+}\) allele into a REA\(^{\text{flox}^+/+}\) allele was determined by PCR analysis. The REA\(^{\text{flox}}\) allele was detected using primers 3 and 4, which amplify a 743 bp fragment. The REA\(^{\text{WT}}\) allele was detected as a 611 bp fragment after amplification with primers 3 and 4. In addition, primer 5 (P5; 5’-GCAGGAACAGCGACAGAAGATTG-3’) and 6 (5’-TAGCAGCCCTTGGATGCTGAAG-3’) for another loxP site were also used for distinguishing REA\(^{\text{flox-frt-neo}}\) allele and REA\(^{\text{WT}}\) allele. The wild-type allele produced a 495 bp fragment and the REA\(^{\text{flox-frt-neo}}\) allele yielded a 901 bp fragment. The frt-neo deletion by FLP-mediated recombination was confirmed by PCR using primers 1 and 2, which amplify a 696 bp fragment from the neomycin resistant gene cassette. In this case, no signal was detected in REA\(^{\text{flox}^+/+}\) mice.
The female REA^{flox/+} mice were mated with the male PR^{cre/cre} knock-in mice (44) to generate the REA^{flox/+} PR^{cre/+} bigenic mouse model. To create the REA^{flox/flox} PR^{cre/+} (denoted REA^{dd}) mice for deletion of the REA gene in both alleles, several pairs of REA^{flox/+} PR^{cre/+} mice were mated.

Superovulation, implantation and decidualization analyses

The details of these methods have been previously described (19, 23, 33). Briefly, superovulation was induced in 24-day-old mice by administering 5 IU of pregnant mares’ serum gonadotropin intraperitoneally (EMD Biosciences, Inc., San Diego, CA), followed by 5 IU human chorionic gonadotropin (Sigma-Aldrich Co. St. Louis, MO) given intraperitoneally 48 hours later. Oocytes were flushed from the oviducts 24 h post human chorionic gonadotropin injection and counted.

Implantation sites at 5.5 days post-coitum (dpc) were visualized by an intravenous injection of 1% of Chicago Blue 6B (Sigma-Aldrich Co. St. Louis, MO) dye solution and the number of implantation sites was counted.

To induce decidual reactions, ovariectomized mice were primed with three once daily subcutaneous injections of 100 ng of 17β-estradiol (E2). After 2 days rest, mice were treated with daily subcutaneous injection of 1 μg of progesterone (P4) and 6.7 ng of E2 in 0.1 mL of sesame oil. One uterine horn was mechanically stimulated by an intraluminal injection of 50 μL of sesame oil 6 h after the third injection. The right horn was not stimulated. Daily injection of P4 and E2 were administered until day 5. Mice were sacrificed at 0 and 2 days after stimulation.

Isolation of uterine stromal cells and induction of decidualization in vitro
Uterine stromal cells were isolated as previously described (24, 42). Briefly, uterine horns of day 4 pregnant female mice were dissected longitudinally and cut into 5-6 mm pieces. The tissues then were washed with Hank’s balanced salt solution (HBSS) and digested with dispase (6 g/L) and pancreatin (25 g/L) for 1hr at RT, followed by 10 min at 37°C. The tissue-digestion mixture was gently mixed, and the luminal epithelial containing supernatant was discarded. The partially digested tissues were then washed once in HBSS and re-digested with collagenase (0.5 g/L) for 45 min at 37°C. Digested tissues were mixed until the supernatant become turbid with dispersed stromal cells. The cell suspension was filtered through a 70 µm-pore-size mesh (BD Biosciences) and centrifuged at 2,000 rpm for 5 min. Cells were then resuspended in DMEM-F12 with 2% FBS, and seeded in 6-well culture plate. Isolated uterine stromal cells were grown in the medium containing 1 µM P4 and 10 nM E2 for 96 h to induce in vitro decidual reactions.

Recombinant Adenovirus preparation

The Cre-expressing recombinant adenovirus was constructed using an AdEasy XL adenoviral vector system kit (Stratagene) according to the manufacturer’s protocols. Cre coding region containing nuclear localization signal (NLS) was amplified from PGK-Cre-bpA plasmids (43) obtained from Addgene (Addgene plasmid 115430), and REA coding region was retrieved from pCMV-REA (32) and cloned to pShuttle-CMV vector. The pShuttle-CMV-Cre plasmids and pShuttle-CMV-REA-Flag were linearized with PmeI restriction enzyme and then transformed into BJ5183-AD-1 cells. AdEasy-Cre plasmids were selected with PacI enzyme after homologous recombination in BJ5183-AD-1 cells. AdEasy-Cre plasmids then were amplified, linearized and transfected into human AD-293 cells. After transfection, cells were
cultured in DMEM media with 10% FBS until 80-90% of cells were detached from flasks. Adenovirus were collected and purified by CsCl gradient protocol as described previously (25).

Uterine stromal cells were isolated from day 4 pregnant mice and grown as described above. 5X10^5 cells were seeded in 6-well plates and cultivated in 5% CO₂ for 8 h at 37°C. After the cells were allowed to attach for 8 h, the unattached cells were removed by washing with HBSS and then incubated with either control adenovirus (AdCMV) or Cre adenovirus (AdCre) at different multiplicity of infection (MOI).

Uterine bioassays

Ovariectomized (8 week of age) or immature (21 day old) female mice were injected subcutaneously (s.c.) daily with E2 (0.5 µg/10g of body weight/day) for 4 days. Injections consisted of compound dissolved in DMSO and then diluted 1:10 in sesame oil. At 24 h after the last injection, animals were sacrificed by carbon dioxide sedation and cervical dislocation. Uteri were removed, washed in cold phosphate-buffered saline (PBS), and weighted after removal of associated fat and expression of any luminal fluid. One uterine horn from each uterus was stored for RNA isolation, and the other horn was fixed for histology.

Histology and immunostaining

Hematoxylin and eosin staining or immunohistochemistry was performed as previously described (19). In brief, the tissues were fixed in 10% buffered formalin phosphate for 24hr at room temperature, transferred to 70% ethanol, and then embedded in paraffin. 4 µm sections were subjected to hematoxylin and eosin staining or immunostaining using antibodies to REA (Millipore Co.), PR (DAKO Co.), p21 (BD Biosciences), or Caspase-3(R&D System).
For 5-bromo-2-deoxyuridine (BrdU) incorporation assay, mice were injected intraperitoneally with 30 µg/g of body weight BrdU (BD Biosciences, San Jose, CA) 2h prior to sacrifice. Uteri were fixed, embedded in paraffin and tissue sections were stained with BrdU antibody (BD Biosciences).

**Western blot analysis**

Immunoblotting was performed as previously described (38). Uterine stromal cell extracts were prepared using ice-cold lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1% SDS) supplemented with protease inhibitors (complete EDTA free; Roche, Indianapolis, IN) and phosphatase inhibitors (Phopho-stop; Roche). Protein concentrations were determined by the BCA protein assay system (Pierce, Rockford, IL). Proteins (20-50 µg) were separated on SDS-PAGE gels, transferred on nitrocellulose membranes and subjected to immunoblotting with anti-REA (Millipore, Co., Billerica, MA), anti-Cre (Covance Inc., Madison, WI) and anti-β-actin (Sigma-Aldrich, St. Louis, MO).

**RNA isolation and real-time PCR**

Total RNA was isolated from whole uterine tissue or uterine stromal cells using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed and analyzed by real-time PCR as described previously (38). Primers for the genes studies are as follows: REA forward (f), AGTGCTGCGTCCATTGTAA; REA reverse (r), TCTTCGGATCAACAGGGACAC; PRPf, CCAGCTCATGGACCTGAACAT; PRPr, GGAGTGATCCATGCACCCATA; BMP2f, AAAGCGTCAAGCCAAACACA; BMP2r, ACCCCACATCATGGAAGTCCA; Hoxa10f,
CACAGGCCACTTCGTTTCTT; Hoxa10r, TTTGTCCGCAGCATCGTAGAG; Hoxa11f,
ATTTTTGATGAGCGTGGTGCTCCCT; Hoxa11r, AGAAATCTGGACCCGAGACGT; Wnt4f,
TGCCAATACCAGTTCCGGA; Wnt4r, TCACCACCTTCCCAAGACAG; VDRf,
CATCTGCATTGTCTCCCCAGA; VDRr, TTGGATAGCGGTCCTGAAT; 36B4f,
CGACCTGGAAAGTCCAACTAC; 36B4r, ATCTGCTGCATCTGCTTG; EGFRf,
TGGATGAAGGGACATGGAGG; EGFRr, TGGACGGGCTGTTGAAGAA; HGFl,
GCAAGACAATGTCTTTCCAGCC; HGFr, CATGAACATCGTGGATGCCA; p21f,
TTCCGCACAGGAGCAAAGT; p21r, ATGAGCGCATCGCAATCAC.

*TUNEL staining*

Apoptosis was detected by using the In Situ Cell Death Detection Kit (Roche Applied Science) according to the manufacturer’s protocol. Briefly, deparaffinized and rehydrated sections were incubated at 37°C for 15 min with 20 µg/mL of proteinase K in 10 mM Tris-HCl (pH 7.6). Sections were washed twice with PBS and incubated for 1 h at 37°C with TUNEL reaction mixture in a humidified chamber. After the incubation, sections were rinsed three times with PBS and counterstained with DAPI.

**2.4. RESULTS**

*Construction of conditional targeting vector and generation of REA<sup>dd</sup> mice*

To circumvent the lethality we encountered previously in conventional REA knockout mice (38), we employed a Cre-loxP mediated recombination strategy to create conditional REA knockout mice. The targeting vector was designed (Fig. 2.1A) to eliminate REA exons 2 through 6, encoding amino acids 44 to 237, which are required for interaction with ER and repressive
activity of ER-target genes (6). Recombinase-responsive elements were introduced, including two \textit{loxP} sequences introduced into REA introns 1 and 6 and an \textit{frt}-floxed neomycin-resistance gene inserted between intron 6 and the 5\textsuperscript{′}-\textit{loxP} site. These elements were recognized and excised by mice expressing Cre and Flp recombinase. Positive ES cells harboring the targeted allele were confirmed by Southern blot screening and injected into blastocysts donated from a C57BL/6 strain and implanted into pseudopregnant female mice (Fig. 2.1B) \textit{REA}\textsuperscript{fl/neo+} chimeric animals were mated with Flpe mice (8) for the deletion of neomycin resistance gene cassette in vivo and as a result, \textit{REA}\textsuperscript{fl+} mice were generated. All animals were genotyped by PCR (Fig. 2.1C). To ensure the functional integrity of \textit{loxP} sequences of the targeted allele and to examine the efficiency of Cre-\textit{loxP} mediated deletion, we first tested Cre-mediated REA allele excision in vitro. Uterine stromal cells isolated from \textit{REA}\textsuperscript{fl} female mice were infected with adenovirus expressing Cre recombinase (AdCre) to delete the REA gene, and REA levels were then examined by qRT-PCR and protein immunoblotting. As shown in Fig. 2.1D, REA levels were markedly reduced by AdCre infection, demonstrating functional integrity of \textit{LoxP} sequences of targeted allele.

Because of our interest in delineating REA actions on fertility, we used mice expressing Cre recombinase under control of the progesterone receptor (PR) promoter (PR\textsuperscript{cre/+}) to delete REA in post-embryonic reproductive tissues (44). PR-Cre knockin (PR\textsuperscript{cre/+}) mice have been used successfully to eliminate targeted genes in uterine cells (5, 21, 23, 33). The \textit{REA}\textsuperscript{d/d} mice were finally generated by crossing of the PR\textsuperscript{cre/+} with \textit{REA}\textsuperscript{fl} mice. Ablation of REA alleles in uteri and mammary glands, PR-expressing tissues, was confirmed by genotyping PCR (Fig. 2.1E). Sequencing analysis of the PCR product from the null allele confirmed PR-driven Cre excised REA exons 2 through 6 (Fig. 2.1F).
Conditional homozygous (REA\textsuperscript{dd}) female mice are infertile - Analysis of ovulation and implantation in the uterus

Conditional REA homozygous mutants developed to adulthood without gross abnormalities, but female homozygous mutant mice were completely infertile. To examine the breeding capacity of conditional REA knockout mice, REA\textsuperscript{ff} and REA\textsuperscript{dd} females were mated with wild-type males and observed for 6 months (Table 2.1). No pups were born from REA\textsuperscript{dd} females over a 6-month period. In contrast, REA\textsuperscript{dd} males displayed normal fertility when mated with wild-type females. Because aggressive mating behavior has been reported in ER-knockout mice (4, 35), we examined this but, no abnormal sexual behaviors were observed in the mutant females and copulatory plugs were detected at the normal frequency, indicating normal mating behavior of homozygous REA knockout animals.

To further explore the cause of infertility, we examined the estrous cycle of REA knockout mice as a measure of the integrity of the hypothalamic-pituitary-ovarian reproductive axis. Examination of vaginal epithelial cytology demonstrated that females cycled normally (data not shown). Next, we investigated the possibility of ovarian dysfunction as a cause of infertility in REA\textsuperscript{dd} females. We first tested whether the ovary of REA\textsuperscript{dd} was responsive to exogenous gonadotropin treatment. As shown in Fig. 2.2A, the number of ovulated eggs was similar to that observed in control mice. In addition, histological studies on ovaries isolated from the superovulation treatment revealed normal follicle maturation and corpora lutea formation (Fig. 2.2B and C), further supporting that REA\textsuperscript{dd} have no obvious ovarian defects.

We next examined whether blastocysts could properly attach to the uterine luminal epithelium to initiate the implantation process. On the morning of pregnancy day 4-5 (4.5-5.5 dpc), females were injected by Chicago Blue B dye solution and implantation sites were counted.
As shown in Fig. 2.2D and E, no implantation sites were detected in the uteri of REA<sup>dd</sup> females, while the normal number of implantation sites was observed in the controls. Collectively, these results show that, while ovarian function is normal in REA<sup>dd</sup> animals, the uterus is completely non-receptive to blastocyst implantation.

**Uterine decidual response is impaired in REA<sup>dd</sup> females**

Successful implantation is followed by proliferation and differentiation of endometrial stromal cells into decidual cells (24, 42). We wished to examine whether decidualization, a subsequent step after implantation could occur in the uteri of REA<sup>dd</sup> animals. We examined uterine decidual response by artificial decidual stimulation, which can be induced in the absence of embryo implantation by an artificial stimulation to estrogen- and progesterone-primed uteri in rodents (26). REA<sup>f/f</sup> and REA<sup>dd</sup> female animals were ovariectomized and treated with a well-defined 13 day regimen of exposure to exogenous estrogen and progesterone (See Materials and Methods), and oil was infused into the lumen of the left uterine horn to initiate a decidual response, with the right horn not manipulated to serve as the control. As shown in Fig. 2.3A, the uteri of REA<sup>f/f</sup> animals exhibited a full decidual response accompanied with a dramatic increase in uterine size, as well as characteristic enlargement of decidual cell layers as seen by histology. By contrast, the stimulated uterine horn of REA<sup>dd</sup> mice failed to undergo a decidual response and expression of the decidualization marker genes BMP2, Wnt4, and PRP was significantly lower in the uteri of REA<sup>dd</sup> animals (Fig. 2.3C).

To investigate the specific requirement of REA in the uterine stroma for functional and morphological decidual reactions, we examined decidualization of uterine stromal cells in vitro after exposure to estrogen and progesterone. This well-characterized protocol allowed us to
examine the role of REA in the stromal compartment only, as well as enabling strict control of Cre-mediated loss of REA by adenoviral infection of cells (24, 42). Primary stromal cells isolated from uteri of day 4 REA^{f/f} pregnant mice were infected with either control adenovirus or AdCre to excise REA alleles. As shown in Fig. 2.3D and E, AdCre effectively depleted mRNA and protein expression of REA and this Cre-mediated REA ablation in stromal cells led to a significant reduction of the stromal differentiation markers, prolactin-related protein (PRP), Hoxa10 and Hoxa11, whereas the expression of other genes not associated with decidualization such as vitamin D receptor (VDR) and GAPDH remained unaltered. These results demonstrate that REA is critical factor for successful decidualization both in the uterus in vivo and in uterine stromal cells in vitro.

REA^{dd} females have uterine developmental defects and altered expression of developmental genes

In REA^{dd} conditional knockout mice, we examined the role of REA in uterine development as a critical precursor for successful adult uterine function. The mature uterus is comprised of the endometrium and myometrium and development of the endometrial glandular epithelium as well as differentiation of endometrial stroma and myometrium from the mesenchyme occurs during the neonatal stages after birth and is established by postnatal day 15 (PND15) (3, 9, 47). As indicated in Fig. 2.4A, the uterine morphology in mature REA^{dd} animals resembled that of an immature uterus; uterine diameter was dramatically smaller because of reduced stroma, and reduced luminal and glandular epithelial volume was evident. Thus, we speculated that REA^{dd} female infertility might be the result of abnormal neonatal uterine development and we therefore investigated this.
We collected uteri from different stages of postnatal development and analyzed uterine gene expression and histological phenotypes. These evaluations revealed that uteri from REA mutants had a morphology similar to that of wild-type animals until PND10, but an abnormal uterine phenotype became apparent by PND14 (Fig. 2.4B). To further test whether this abnormal uterine development was the result of loss of REA, we examined the REA expression level during postnatal development. Genotyping revealed that targeted REA exons were excised by PR-driven Cre activity as early as PND5 (Fig. 2.4C). Depletion of REA mRNA and protein expression was also confirmed by qRT-PCR and immunohistochemistry (Fig. 2.4D and E). Of note, loss of REA expression was observed in the luminal epithelial beginning at PND5 and REA loss was extended to the stromal compartment after PND5 and this loss was observed until PND14 (Fig. 2.4E). Consistent with this age- and tissue-specific loss of REA, as shown in Fig. 2.4F, PR expression was only detected in the epithelial at PND5 with stromal expression occurring between PND5 and 10. Therefore, PR-dependent, Cre-mediated loss of REA was well correlated with the endogenous PR expression. These results indicate that loss of REA expression leads to abnormal uterine development and adversely affects uterine receptivity and pregnancy maintenance.

We also examined the expression of several genes implicated in the regulation of postnatal uterine development (9, 14) including bone morphogenic protein 2 (BMP2), Hoxa10, epidermal growth factor receptor (EGFR) and hepatocyte growth factor (HGF). As shown in Fig. 2.5A, expression of these genes was significantly down-regulated in the uteri of REA<sup>dd</sup> mice.

*Neonatal uterine cell proliferation is decreased and apoptosis is increased in conditional homozygous REA mutant mice*
Our findings of uterine hypoplasia in conditional homozygous REA mutant mice led us to investigate cell proliferation and apoptosis. The RNA and protein expression level of the cyclin-dependent kinase inhibitor, p21, was significantly increased in the uteri of REA<sup>dd</sup> mice compared to that of wild-type (Fig. 2.5B and C) and this elevated p21 expression closely corresponded with the tissues in which the expression of REA was lost (Fig. 2.5D). It is important to note that increased expression of p21 was detected only in the luminal epithelial cells at PND10, but its expression was extended to stroma by PDN14. The pattern of increased expression of p21 inversely correlating with the spatial and temporal expression pattern of REA (Fig. 2.5D) implies that increased p21 expression in cells lacking REA may result in cell cycle arrest and impede normal uterine growth. To examine cellular proliferation, specifically S-phase activity of the cell cycle, REA<sup>ff</sup> and REA<sup>dd</sup> at 5, 10 and 14 days of age were injected with BrdU 2 h prior to sacrifice and uteri were collected. As shown in Fig. 2.6A, REA mutant uteri exhibited decreased BrdU incorporation indicating that DNA replication was impaired in uteri of REA<sup>dd</sup> mice.

We also assessed DNA fragmentation, one of the hallmarks of apoptosis, by TUNEL analysis on wild-type and conditional homozygous mutant uteri at 14 days of age. We observed markedly increased apoptotic cell death in the REA<sup>dd</sup> uterine epithelium and stroma, as evidenced by increased fluorescence (Fig. 2.6B). We next examined activation of the apoptosis-promoting executioner caspase-3 in the wild-type and REA-null uterine cells by immunohistochemistry. Because caspase-3 is known to be cleaved from 32 kDa of procaspase-3 to 18 kDa of caspase-3 to be active, we examined the level of the cleaved form of caspase-3 (39). As shown in Fig. 2.6C, positive cells for active caspase-3 were present only in the REA<sup>dd</sup> uterine
Collectively, these data suggest that the hypoplasia seen in the REA \textsuperscript{dd} uterus is likely the result of a combination of increased G1-S-phase arrest and increased apoptosis.

\textit{Conditional REA heterozygous (REA} \textsubscript{ff} \textit{) animals show enhanced estrogen responsiveness}

To test the hypothesis that REA function might be gene dose-dependent, we generated conditional heterozygous REA animals (REA \textsubscript{ff}) by crossing REA \textsubscript{dd} and wild-type animals and first examined the early postnatal uterine development of these animals. As opposed to the severely impaired development we observed in uteri of homozygous REA mice, uterine morphology and gene expression during postnatal development were found to be similar in REA \textsubscript{ff} and REA \textsubscript{fd} (Fig. 2.S1). We next performed uterine bioassays to examine the REA dosage effect on uterine response to estrogen stimulation. Immature REA \textsubscript{ff}, REA \textsubscript{fd} and REA \textsubscript{dd} animals were treated for 4 days with E2 or control vehicle, and uterine response was monitored by assessing gross morphology, histology, and uterine weight gain. Strikingly, uteri with conditional loss of one allele of REA (REA \textsubscript{fd}) exhibited a very different uterine phenotype from that of conditional homozygous mutant (REA \textsubscript{dd}) mice. The uterine morphology of conditional REA heterozygous females was similar to that of the wild type mice (data not shown), however, treatment with E2 resulted in markedly larger and fluid-filled uteri (Fig. 2.7A). Of note, the lumen area of REA \textsubscript{fd} animals was extremely large as compared to wild-type or homozygous animals. Also, the estrogen-induced uterine weight increase was significantly greater in REA \textsubscript{fd} animals compared to wild type or homozygous animals (Fig. 2.7B).

To investigate if this exaggerated estrogen-induced uterine weight gain was a result of increased uterine cell proliferation, REA \textsubscript{ff}, REA \textsubscript{fd} and REA \textsubscript{dd} mice were treated with E2 for 4 days and then injected with BrdU at 2 h prior to sacrifice. As shown in Fig. 2.7C, the majority of
BrdU positive cells was seen in luminal epithelial cells in response to E2 treatment, and the proportion of BrdU positive cells was significantly greater in REA<sub>fl/+</sub> animals vs. wild-type or homozygous animals. Thus, the data shown in Fig. 2.7 demonstrate that loss of REA results in distinct uterine phenotypes dependent on the dosage of the REA gene, such that the REA protein level significantly modulates estrogen actions in the uterus.

**REA gene dosage impacts uterine gene regulation**

Our in vivo findings demonstrate that loss of one REA allele enhanced estrogen activity in uterine tissues, whereas complete loss of both REA alleles impaired uterine cell proliferation and increased apoptosis. To determine whether uterine gene expression also depends on REA dosage, we monitored the expression of C3 and Lactoferrin since both are genes well known to be up-regulated by E2 in the uterus (38). As shown in Fig. 2.8A, estrogen increased C3 and Lactoferrin expression much more in the uteri of REA heterozygous animals than in wild type floxed animals.

Because the uteri of conditional heterozygous REA mutant animals treated with E2 were filled with large amounts of fluid, we examined whether the genes responsible for uterine water imbibition might be hyper-stimulated in the REA conditional heterozygous uteri. It has been shown that estrogen stimulates water imbibition in the uterine endometrium, in part, by water channel proteins termed aquaporins (AQPs) (17). To date, 13 isoforms of AQPs have been identified in mammals and specific AQP isotypes have been shown to be expressed in male and female reproductive tissues (15). To test the hypothesis that reduction of REA might enable hyper-stimulation of the expression of AQPs in uteri of conditional heterozygous REA mutants, we examined the expression of APQs reported to be expressed in the uterus (15). As shown in
Fig. 2.8B, among those tested, two AQPs (AQP1 and 2) were down-regulated and three AQPs (AQP 3, 4 and 5) were up-regulated by E2 in wild type (REA\textsuperscript{ff}) uteri. Interestingly, AQP4 stimulation in response to E2 was significantly greater in REA\textsuperscript{fd} animals vs. wild-type, and was greatly reduced in REA\textsuperscript{dd} uteri, suggesting that the enhanced fluid accumulation in the uteri of REA\textsuperscript{fd} animals might be associated with hyper-stimulation of this aquaporin.

*Conditional heterozygous (REA\textsuperscript{fd}) female mice show reduced fertility*

To further investigate if the uterine hyper-responsiveness to estrogen observed in conditional heterozygous females impacts reproductive capacity, REA\textsuperscript{fd} females were mated with wild-type males and observed for continuous breeding over 6 months. As presented in Table 2, REA\textsuperscript{fd} females showed a significant decrease in litter size compared to control mice (6.1±2.5 vs. 8.9±2.1 pups per litter, p<0.001) and had fewer numbers of pregnancies (4.3±1.8 vs. 6.4±0.5, p<0.001) over the 6-month period compared to control mice (Fig. 9).

2.5. DISCUSSION

Our findings reveal a key role of REA in cell proliferation, survival, and adenogenesis to enable proper uterine development and functional activities whereas conditional loss of both REA alleles in female mice resulted in infertility due to severely compromised uterine development. Perhaps more interestingly, we found that conditional loss of only one REA allele resulted in enhanced uterine response to estrogen and increased cell proliferation. This in vivo study thus indicates that the correct gene dosage and level of REA are crucial for optimal uterine function and that REA serves as a brake to appropriately control estrogen-ER activity.
REA/PHB2 is an evolutionarily conserved protein and has increasingly been found to be involved in diverse cellular processes including transcription, mitochondrial biogenesis and replicative senescence (22, 31, 32, 37). Our lab previously identified REA in a yeast two-hybrid screen using ER as bait and characterized REA as a coregulator that repressed the transcriptional activity of the ER (6, 32, 34, 38). In addition to its role as a transcriptional coregulator, REA has been shown to exist in a protein complex with prohibitin1 (PHB1) in the mitochondrial inner membrane, and the mitochondrial REA/PHB1 complex appears to be important for maintaining mitochondrial integrity and biogenesis (29-31, 36, 37).

To enable insight into the cell- and tissue-specific physiological functions of REA, we generated REA conditional knockout animals in which REA function was abrogated only in progesterone receptor (PR)-expressing cell lineages. Although PR expression has been detected as early as the blastocyst stage, it has been shown that embryonic-derived PR is not required for embryonic survival, evidenced by PR knockout mice (13, 26). As expected, PR-driven Cre activity to conditionally remove REA alleles resulted in viable conditional REA knockout animals which developed to adulthood.

*Uterine developmental defects of homozygous REA knockout animals: Enhanced cell cycle arrest and altered uterine adenogenesis*

Normal uterine function is required for fertility, with reproductive health critically dependent on proper embryonic and perinatal development, as evidenced by exposure of neonates to endocrine disruptors resulting in adult infertility and abnormal estrous cycles (45). The uterus begins to develop from the Mullerian duct during embryonic developmental stages but it is not completed until after birth. Postnatal uterine development involves massive cell...
proliferation and differentiation to establish the mature endometrium and myometrium of the uterus (3, 9, 14, 47). We observed that the uteri of homozygous REA mutants displayed an immature, hypoplastic morphology. This uterine hypoplasia was associated with cell cycle arrest, as evidenced by elevated cell cycle inhibitor p21 expression and reduced BrdU uptake, and increased apoptosis of uterine epithelial and stromal cells. Loss of REA also resulted in alteration of a number of genes implicated in uterine development. Our histological examination of postnatal uteri showed a continuous increase in growth and size of the uterus until the age of 14 days, and this growth was positively correlated with increased levels of growth factors and growth factor receptors. The uterine defects seen in REA knockout animals may be, at least in part, mediated through a decreased level of EGFR. Moreover, these uteri contained notably fewer endometrial glands. The endometrial glands secrete or transport diverse cellular factors such as leukemia inhibitory factor and calcitonin required for the establishment of uterine receptivity and nourishing of the developing conceptus. Endometrial gland development from the luminal epithelium, termed adenogenesis, involves a series of morphogenic events: budding of glandular epithelium (GE) from the luminal epithelium, penetration of stroma by tubes of GE, coiling and branching of GE and it is a predominantly postnatal process. In the rodent uterus, uterine adenogenesis proceeds from days 9 through 15 (9). Interestingly, the window of time at which the altered uterine phenotype develops in REA knockout animals (between ages of 10 to 14 day) corresponds with the period of endometrial gland development. Of note, our observation that depletion of REA expression started from the luminal epithelium and caused cell death suggests that the early cell death of luminal epithelial cells in REA knockout animals likely contributes to the failure of successful uterine adenogenesis.
**REA function as a nuclear receptor coregulator**

Our prior in vivo study (38) of conventional REA heterozygous animals (REA<sup>+/−</sup>) along with the current studies with conditional tissue-specific REA homozygous (REA<sup>d/d</sup>) and conditional REA heterozygous (REA<sup>f/d</sup>) animals demonstrate that REA is a gene dosage-dependent coregulator able to profoundly impact steroid receptor function. Female REA heterozygous animals showed a greater uterine weight gain in response to E2 and this was due, in part, to increased E2-induced cellular proliferation, as evidenced by BrdU incorporation, providing strong evidence for REA being a coregulator that represses the activities of estrogen. Strikingly, treatment of REA<sup>f/d</sup> animals with E2 resulted not only in large but also very fluid-filled uteri. Our findings suggest that the exaggerated fluid accumulation seen in the uteri of REA<sup>f/d</sup> animals might be through deregulation of aquaporin expression, especially of interest because alterations in the expression and regulation of aquaporins have been strongly associated with reproductive disorders (15). The hyper-responsiveness to estrogen with 50% reduction of REA dosage also influenced female reproductive function and resulted in a subfertility phenotype. Both litter size and the frequency of pregnancies was significantly decreased in REA<sup>f/d</sup> animals, implying that tightly regulated estrogen responsiveness of the proper magnitude is critical for optimal fertility.

**REA is a protein with multiple functions in the uterus**

It is increasingly appreciated that many cellular proteins have more than one intracellular localization and carry out several functions. For example, the estrogen receptor is localized primarily in the nucleus but is also found at extranuclear locations, suggested to be cytoplasmic, mitochondrial, or membrane associated, and to have effects on gene expression and also on
activation of protein kinases and the regulation of energy metabolism (10, 27). Numerous connections have also now demonstrated between mitochondrial and nuclear activities, and evidence of mitochondrial-nuclear communications, the latter involving transcription factors and coactivators that regulate both nuclear and mitochondrial gene expression (40). Indeed, mitochondrial biogenesis requires a coordination of expression of nuclear and mitochondrial genomes-encoded proteins and therefore cross-talk between these cellular organelles (40).

Our findings provide evolving understanding of REA as a nuclear transcriptional coregulator, and also support a mitochondrial function for REA, as complete deletion of REA induced apoptosis and increased caspase-3 activation. Indeed, there is evidence for an REA complex with PHB1 in mitochondria (32, 36, 46), PHB1 was shown recently to act like REA to repress ERα activity (11). In Xenopus, however, the prohibitins have unique functions and Xphb2 cannot substitute for or rescue the activity of Xphb1 as a neural crest specifier in Xenopus development (41).

Our in vivo studies presented here provide new insight into REA functions and assist in understanding the key roles of REA in uterine development and maintenance of uterine function at maturity, supporting fertility, implantation and uterine decidualization. We have demonstrated that REA is clearly a negative regulator of estrogen activity, ensuring optimal physiological actions of estrogen by serving as a brake to keep estrogen-stimulated activities in check. Because of the impaired cell proliferation and increased apoptosis observed in uteri of conditional homozygous REA mice, it is not possible to examine coregulator functions when there is complete loss of REA. However, uteri of heterozygous conditional REA mice showed normal uterine morphogenesis and development, but enhanced responsiveness to estrogen that resulted in a subfertility phenotype, providing new insight into gene dose-dependent physiological roles
of REA in development. An important advantage of our REA conditional knockout animals is that this mouse model system has enabled examination of the function of REA as a coregulator in reproductive tissues and demonstrated both the critical mitochondrial and nuclear activities of REA.

2.6. REFERENCES


2.7. FIGURES, TABLES AND LEGENDS

FIGURE 2.1

A

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C

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Figure 2.1. Design of conditional targeting vector and strategy for disruption of the REA gene.

(A) The targeting vector contains positive (NEO), negative (TK) selection markers, two frt (indicated as yellow ovals) and two loxP sequences (indicated as triangles). Two loxP sites are located in between exons 2 and 6 in the REA gene and two frt sites flank a neomycin resistant gene cassette. The REA^floxfrt-neo^ allele was created by homologous recombination in ES cells and the REA^floxf/-^ allele was derived from a REA^floxfrt-neo^ allele through in vivo FLP-mediated recombination. Finally the PR-Cre mice were used to generate the conditional deletion of the REA gene by Cre-mediated excision in the Progesterone Receptor (PR) expressing cell lineage.

(B) Genomic DNA from ES cell clones was prepared and subjected to Southern blotting after XbaI digestion. The location of the 5’ and 3’ probe is indicated in (A). Wild-type allele (22.4 kb) and REA^floxfrt-neo^ allele (14 kb and 8.4 kb) are shown. (C) Genomic DNA isolated from mouse tail biopsies was genotyped by PCR using primer sets (P1, P2, P3 and P4) designed for genotyping PCR shown at left (D) REA protein and mRNA were analyzed by immunobloting or qRT-PCR, respectively in mouse uterine stromal cells after adenovirus-mediated Cre (AdCre) deletion. mRNA levels were normalized relative to 36B4, and fold change was calculated relative to control. Results are the average ± SD of at least three independent experiments. (E) Genomic DNA isolated from uterus, mammary gland, liver, kidney and heart was genotyped by PCR. (F) PCR product from P1 and P4 primers was purified, sequenced and mapped to the mouse genome by UCSC BLAT.
**Figure 2.2.** Early pregnancy events in REA\(^{dd}\) mice.

(A) Female mice for each genotype at age 24 day were subjected to superovulatory dose of the gonadotropins PMSG and hCG. Oocytes were then extracted from their oviducts and counted. (B) Follicular development of the ovary was assessed by histological examination of an ovary section after treatment with PMSG only for 48 hr. (C) Formation of Corpora lutea (CL) was assessed by histological examination after complete superovulation treatment. Note the presence of numerous mature follicles and CL (indicated as arrow) in wild-type and mutant ovaries. (D) Representative photographs of uteri from REA\(^{ff}\) and REA\(^{dd}\) at 5.5 dpc. Arrows indicates sites of implantation. (E) Implantation sites were visually counted by the localized retention of Chicago Blue dye.
FIGURE 2.3

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Figure 2.3. REA<sup>dd</sup> mice show impaired decidual response in vivo and in vitro.

(A) Representative gross anatomy and hematoxylin- and eosin-stained uterine sections from REA<sup>ff</sup> and REA<sup>dd</sup> mice after decidual stimulation. The left horn (L) was stimulated, and the right horn (R) was not stimulated (see Materials and Methods). Note the dramatic increase in size of the left horn after mechanical stimulation. (B) Detection of REA in the stimulated left horn by immunohistochemistry and qRT-PCR. Note high expression of REA in decidual zone. (C) Expression level of molecular markers for decidualization measured by qRT-PCR. (D) In vitro decidual response of uterine stromal cells. Mouse stromal cells from day 4 pregnant uteri were infected by mock or different MOI of AdCre, then REA mRNA and protein levels were analyzed by qRT-PCR and immunoblotting, respectively (E). (F) Molecular markers specific to stromal cells were monitored by qRT-PCR. The data are represented as mean±SD. *, P<0.05; **, P<0.01.
Figure 2.4. REA<sup>dd</sup> mice show abnormal uterine growth and maturation.

(A) Representative hematoxylin- and eosin-stained uterine sections from REA<sup>ff</sup> and REA<sup>dd</sup> adult mice (8 wks). The amount of stromal (S), endometrial glands (GE) and luminal epithelial tissue is minimal in mutant uterus. (B) Neonatal uterine morphology was examined by histological examination of uterine section of REA<sup>ff</sup> and REA<sup>dd</sup> mice at postnatal days 5, 10, 14 and 21 (PND4, 10, 14 and 21). (C) REA gene is excised in the uteri of REA<sup>dd</sup> from as early as age 5 days. REA deletion was confirmed by conventional genotyping. (D) REA mRNA level was monitored by qRT-PCR. Immunohistochemical detection of REA (E) and progesterone receptor (PR) (F) from PND 5, 10 and 14 uteri of REA<sup>ff</sup> and REA<sup>dd</sup> mice. The data are represented as mean±SD. *, P< 0.05; **, P< 0.01.
FIGURE 2.5

A

BMP2  Hoxa10

mRNA Fold Change

PND3  PND10  PND14  PND21  PND5  PND10  PND14  PND21

EGFR  HGF

mRNA Fold Change

PND3  PND10  PND14  PND21  PND5  PND10  PND14  PND21

B

p21

mRNA Fold Change

PND2  PND10  PND14  PND21

C

p21 staining

D

p21 staining  REA staining
Figure 2.5. Uterine developmental gene expression is altered in REA mutant mice.

(A) mRNA level of molecular markers (BMP2, Hoxa10, EGFR and HGF) implicated in uterine development are monitored by qRT-PCR. (B) The cell cycle inhibitor p21 mRNA expression levels are elevated in the uterus of REA<sup>dd</sup> mice, as measured by qRT-PCR and (C) p21 protein levels are elevated in the uterus of REA<sup>dd</sup> by immunohistochemistry. (D) Reduction of REA expression is correlated with increased p21 expression as measured by immunohistochemistry. The data are represented as mean±SD. *, P< 0.05; **, P< 0.01.
Figure 2.6. **REA<sup>dd</sup>** mutants have decreased neonatal uterine cell proliferation and increased apoptosis.

(A) Representative histologic sections of BrdU immunohistochemistry (See Materials and Methods) in **REA<sup>ff</sup>** and **REA<sup>dd</sup>** uterus at day 14 mice. Note decreased BrdU incorporation in the mutant uterus. (B) Representative fluorescence images of TUNEL staining in uterine sections from **REA<sup>ff</sup>** and **REA<sup>dd</sup>** uteri at day 14 mice. (C) Immunohistochemical detection of active-caspase-3 expression in the **REA<sup>ff</sup>** and **REA<sup>dd</sup>** uteri of day 14 mice. *, P< 0.05; **, P< 0.01.
Figure 2.7. Uterine growth and cell proliferation in response to estrogen is enhanced in REA heterozygous (REA<sup>f/d</sup>) mice

(A) Gross anatomy and hematoxylin- and eosin-stained uterine sections isolated from vehicle or E2 treated REA<sup>f/f</sup>, REA<sup>f/d</sup> and REA<sup>d/d</sup> mice. (B) Uterine weight was measured 24 h after the last injection and normalized to each animal’s body weight. (C) BrdU immunohistochemistry in uteri from REA<sup>f/f</sup>, REA<sup>f/d</sup> and REA<sup>d/d</sup> mice treated with E2 for 4 days. Uteri were collected 24 h after the last injection and mice were injected intraperitoneally with BrdU 2 h prior to sacrifice. (D) BrdU incorporation was represented by mean percentage of BrdU-positive cells/total cells monitored in at least 5-6 40X fields. **, P< 0.01.
Figure 2.8. Uterine estrogen regulated gene expression is enhanced in REA heterozygous mutant mice.

(A) mRNA levels of E2-up-regulated gene expression (complement C3, lactoferrin) monitored by qRT-PCR. (B) mRNA levels of Aquaporins known to be expressed in the uterus monitored by qRT-PCR. The data are represented as mean±SD. *, P< 0.05; **, P< 0.01.
**Figure 2.9.** REA<sup>fid</sup> mice show reduced fertility.

(A) Average of the total numbers of deliveries per dam over 6 months of breeding. (B) Average numbers of pups per litter. Data are mean±SD. **, P< 0.01.
FIGURE 2.10

Figure 2.10. Model for the relationship between REA gene dosage and ER activity.

Estrogen signaling was enhanced with 50% reduction of REA level (REA<sup>f/d</sup> mice), resulting in an increased uterine weight gain and enhanced E2-mediated gene expression with estrogen treatment. However, complete depletion of REA (REA<sup>d/d</sup> mice) caused cell cycle arrest and apoptosis, leading to the impairment of REA dependent function essential for ER action.
TABLE 2.1

<table>
<thead>
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<th>No. of:</th>
<th>Mean±SD</th>
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<td></td>
<td></td>
<td>Pups</td>
<td>Litters</td>
</tr>
<tr>
<td>REA&lt;sup&gt;f/f&lt;/sup&gt;</td>
<td>9</td>
<td>549</td>
<td>60</td>
</tr>
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<td>REA&lt;sup&gt;d/d&lt;/sup&gt;</td>
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</table>

<sup>a</sup>NA, not applicable
### TABLE 2.2

**TABLE 2.2. REA<sup>fr/d</sup> Females are Subfertile**

<table>
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<tr>
<th>Genotype</th>
<th>Sample size</th>
<th>No. of:</th>
<th>Mean±SD</th>
<th>Pups per litter</th>
</tr>
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<tr>
<td>REA&lt;sup&gt;ff&lt;/sup&gt;</td>
<td>7</td>
<td>403</td>
<td>45</td>
<td>8.9 ± 2.1</td>
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<tr>
<td>REA&lt;sup&gt;fr/d&lt;/sup&gt;</td>
<td>7</td>
<td>184</td>
<td>30</td>
<td>6.1 ± 2.5 **</td>
</tr>
</tbody>
</table>

**p-value: <0.001**
Figure 2.S1. REA<sup>ff</sup> mice show normal uterine growth and maturation.

(A) Neonatal uterine morphology was examined by histological examination of uterine sections of REA<sup>ff</sup>, REA<sup>fd</sup> and REA<sup>dd</sup> mice at postnatal days 5, 10, 14 and 21 (PND4, 10, 14 and 21). (B) mRNA level of BMP2, EGFR and p21 was examined by qRT-PCR. Data are mean±SD.
CHAPTER 3

REPRESSOR OF ESTROGEN RECEPTOR ACTIVITY (REA) GENE DOSAGE IS CRITICAL FOR MAMMARY GLAND MORPHOGENESIS

3.1. ABSTRACT

Estrogen receptor (ER) is a key regulator of mammary epithelial proliferation and ductal elongation during postnatal mammary gland development and is also strongly associated with breast tumorigenesis. Since ER-mediated transcriptional activity critically depends on coregulator proteins, altered coregulator expression has been implicated in breast cancer. Here, we investigate the consequences of reduction or loss of function of Repressor of Estrogen Receptor Activity (REA) by conditionally deleting REA at different stages of mammary gland development and show that REA has essential roles in the morphogenesis of the mammary gland. Notably, we find that heterozygosity and nullizygosity for REA result in very different mammary gland phenotypes. During puberty, mice homozygous null for REA show severely impaired mammary ductal elongation and morphogenesis, whereas mice that are heterozygous for REA display accelerated mammary ductal elongation and an increased number of terminal end buds. During pregnancy and lactation, mice with homozygous REA gene deletion show a loss of lobuloalveolar structures and increased apoptosis of mammary alveolar epithelium, leading to significant reduction of body weight of the offspring nursed by females lacking REA, whereas body weight of the offspring nursed by females heterozygous for REA was slightly higher than that of pups nursed by control mice. We also find that up-regulation of amphiregulin, the major paracrine mediator of estrogen-induced ductal morphogenesis in response to estradiol (E2), is greater in the mammary gland of REA heterozygous mice compared to control mice. Additionally, we demonstrate that reduction of REA level by RNA interference in breast cancer
cells enhances E2-mediated gene expression. Taken together, our findings reveal that REA is crucial for normal mammary gland development and morphogenesis, and possesses a dosage-sensitive positive modulatory role in the normal mammary gland and in ERα-positive breast cancer cells.

3.2. INTRODUCTION

The majority of mammary gland development takes place in distinct stages of postnatal development. Specifically, puberty, pregnancy, lactation and involution are all tightly controlled by the orchestrated action of ovarian steroid hormones and growth factors (5, 19, 28, 43). Throughout the recurrent estrous cycles, the epithelial ducts and branches increase, while in pregnancy and lactation, alveolar units proliferate and differentiate into milk-secretory cells (5, 19, 20). The ovarian steroid hormones, estrogen and progesterone, are key mediators of ductal morphogenesis and are mitogenic for mammary epithelial cells (1, 19). These physiological effects of hormones are mediated through the estrogen receptor (ER) and progesterone receptor (PR), both members of the nuclear receptor superfamily of ligand-activated transcription factors (24).

The role of ERα in mammary gland development was largely elucidated through knockout of ERα (αERKO) in mice. Mammary glands of αERKO mice were normal before puberty; however, after the onset of puberty, a rudimentary ductal structure remained and the ducts failed to invade the mammary fat pad, demonstrating that ERα is required for normal ductal elongation and outgrowth during puberty (6, 10, 14, 31). Although these animal models provided us invaluable insights into the biological function of ERα in mammary gland development of virgin mice, exploring the role of ERα in mammary glands beyond puberty was
not possible, due to the infertility of αERKO mice. More recently, Khan and colleagues developed whey acidic protein (WAP) driven Cre-mediated conditional ERα knockout mice in an attempt to address the unresolved role of ERα function in the mammary gland during pregnancy and lactation (15). They demonstrated that the ablation of ERα in mammary epithelium during late pregnancy and lactation results in a loss of ductal side-branching and lobuloalveolar structures, ductal dilation, and decreased the proliferation of alveolar progenitors (15). The observations in both conventional and conditional ERα knockout mouse models demonstrate that ERα profoundly impacts multiple developmental stages of mammary gland development—puberty, pregnancy and lactation.

It is now well established that the transcriptional activity of ER is modulated by a delicate balance between coactivators and corepressors (32, 49). Therefore, changes in the expression level of ER coactivators or corepressors can affect the transcriptional activity of the estradiol-ER complex and is shown to underlie various disorders of estrogen target tissues (16).

Repressor of ER activity (REA) was initially identified as an ER selective coregulator that repressed the activity of estrogen (36). Our prior heterozygous (REA+/−) animals displayed a haploinsufficiency phenotype in mammary gland development in which loss of one REA allele resulted in enhanced mammary ductal elongation during puberty and increased lobuloalveolar development during pregnancy, suggesting that REA is an important modulator of mammary gland development (38). Several other lines of study also suggest that levels of REA might be associated with breast carcinogenesis. REA expression levels were shown to be positively correlated with ER in breast cancer, but inversely correlated with tumor grade (50). Also, the F-box protein Skp2B, which is often overexpressed in breast cancers, interacts with REA resulting in degradation of the REA protein. Additionally, MMTV-Skp2B mice overexpressing Skp2B
developed mammary tumors (53). These findings suggest that altered levels of REA might be linked to breast tumorigenesis.

In this study, we sought to dissect the physiological roles of REA at distinct stages of mammary gland development. To this end, we generated two types of mammary-specific conditional REA knockout animals using a cre-loxP recombination strategy. In order to define the role of REA during puberty and after late pregnancy, two different Cre-expressing mice were used in this study: progesterone receptor (PR)-Cre knockin mice and whey acidic protein (WAP)-Cre transgenic mice, respectively. In our analyses of conditional REA mutant mice, we have found that heterozygosity and nullzygosity for REA resulted in opposite mammary gland phenotypes. Mice homozygous null for REA show impaired mammary ductal elongation and a loss of lobuloalveolar structures, whereas those lacking only one REA allele display an enhancement of mammary ductal outgrowth. The phenotypic features of REA heterozygous mutant mice are, in part, associated with hyper-stimulation of E2-mediated transcription. Our study reveals a critical role for REA gene dosage for normal mammary gland function.

3.3. MATERIALS AND METHODS

Generation of transgenic REA knockout mice

The female REA\textsuperscript{floxed/floxed} mice (Park et al. unpublished described in Chapter 2) were mated with male PR-Cre knock-in mice (51) to generate the REA\textsuperscript{floxed/floxed} PR\textsuperscript{cre/+} mice. Briefly, female homozygous REA-floxed mice (REA\textsuperscript{floxed/floxed}) were mated with male homozygous PR-Cre mice (PR\textsuperscript{cre/cre}) to produce offspring (REA\textsuperscript{floxed/+} PR\textsuperscript{cre/+}) that are heterozygous for both mutations. These heterozygous mice (REA\textsuperscript{floxed/+} PR\textsuperscript{cre/+}) were then bred with each other to produce conditional REA homozygous mutant mice that carried two REA floxed alleles and Cre gene. In
these mice, Cre-mediated excision of floxed REA led to a null mutation of this gene in PR-expressing tissues. To generate the REA\textsuperscript{flox/+} WAP-Cre bigenic mouse model, female REA\textsuperscript{flox/flox} mice were mated with male whey acidic protein (WAP)-Cre transgenic mice (30, 54). WAP-Cre transgenic mice were generously provided by the laboratory of Dr. Lothar Hennighausen (National Institutes of Health, Bethesda, MA). As a means to assess the efficiency of lactation in REA\textsuperscript{flox/flox} WAP-Cre dams, the body weight of fostered pups nursed by REA\textsuperscript{flox/flox} WAP-Cre dams were compared to the weights of pups nursed by REA\textsuperscript{flox/flox} dams. On postnatal day 2 (P2), litter size was normalized to the 6 pups.

\textit{Animals, hormone treatment, and tissue collection}

All animals were maintained in accordance with the NIH Guide for Care and Use of Laboratory Animals, and all procedures described here were approved by the University of Illinois Animal Care and Use Committee. Female mice were ovariectomized at 6 weeks of age and rested for 2 weeks for clearance of endogenous steroids. These mice were then injected subcutaneously (s.c) with 17β-estradiol (E2) (0.05 µg/g of body weight/day) in 0.1 mL of sesame oil for 5 days. For each treatment group, at least seven mice were used at each time point.

\textit{Whole-mount staining of mouse mammary glands}

Fourth inguinal mammary glands were excised, spread onto a glass microscope slide, and fixed in 4% paraformaldehyde for 2 h at 4°C. The samples were washed with PBS and stained in carmine alum solution overnight at room temperature. The samples were then dehydrated using stepwise ethanol concentrations and defatted in xylene overnight.
**Histology and immunostaining and BrdU incorporation assay**

Hematoxylin and eosin staining or immunohistochemistry was performed as previously described (25). In brief, the tissues were fixed in 10% buffered formalin phosphate for 24h at room temperature, transferred to 70% ethanol, and then embedded in paraffin. 4 µm sections were subjected to hematoxylin and eosin staining or immunostaining using antibodies to REA (Millipore Co.), PR (DAKO Co.), ER (Novocastra) or Caspase-3 (R&D System).

For 5-bromo-2-deoxyuridine (BrdU) incorporation assay, mice were intraperitoneally injected with 30 µg/g of body weight BrdU (BD Biosciences, San Jose, CA) 2h prior to sacrifice. Mammary glands were fixed, embedded in paraffin and tissue sections were stained with BrdU antibody (BD Biosciences).

**Western blot analysis**

Immunoblotting was performed as previously described (41). The fourth inguinal mammary gland was removed at the indicated developmental stage and snap frozen in liquid nitrogen. The protein lysates were prepared using ice-cold lysis buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 1% SDS) supplemented with protease inhibitors (complete EDTA free; Roche, Indianapolis, IN) and phosphatase inhibitors (Phopho-stop; Roche). Protein concentrations were determined by the BCA protein assay system (Pierce, Rockford, IL). Proteins (20-50 µg) were separated on SDS-PAGE gels, transferred on nitrocellulose membranes and subjected to immunoblotting with anti-REA (Millipore, Co., Billerica, MA), anti-phospho-STAT5 (Cell Signaling Technology) and anti-ERK2 (SantaCruz Biotechnology).
**RNA isolation and real-time PCR**

Total RNA was isolated from mammary glands at the indicated developmental stage using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. One microgram of total RNA was reverse transcribed and analyzed by real-time PCR as described previously (41).

**TUNEL staining**

Apoptosis was detected by using the In Situ Cell Death Detection Kit (Roche Applied Science) according to the manufacturer’s protocol. Briefly, deparaffinized and rehydrated sections were incubated at 37°C for 15 min with 20 µg/mL of proteinaseK in 10 mM Tris-HCl (pH 7.6). Sections were washed twice with PBS and incubated for 1 h at 37°C with TUNEL reaction mixture in a humidified chamber. After the incubation, sections were rinsed three times with PBS and counterstained with DAPI.

**Cell culture and RNA interference**

MCF-7 cells were grown in MEM (Sigma-Aldrich) supplemented with 5% calf serum (HyClone). At 6 days before E2 treatment, cells were switched to phenol red-free MEM containing 5% charcoal-dextran-treated calf serum. Medium was changed on 2 and 4 of culture and cells were then seeded in 60 mm dishes to be 60-70% confluent upon transfection. siRNA targeting firefly luciferase (siCONTROL Non-targeting siRNA #2, Dharmacon) or REA were transfected in OPTI-MEM media (Invitrogen) using Dharmafect (Dharmacon) to a final concentration of 50 nM. After 48 h of siRNA transfection, cells were treated for 4 h with 10 nM E2.
3.4. RESULTS

REA is widely expressed in the developing mammary gland

To investigate the biological function of REA on mammary gland development and morphogenesis, we generated REA conditional knockout mice by Cre-loxP recombination in which REA function was abrogated only in progesterone receptor (PR)-expressing mammary cells. We first examined the expression pattern of REA during mammary gland development. As shown in Fig. 3.1, almost all mammary cells were positive for REA expression. However, PR was only expressed in body cells and a subgroup of luminal epithelial cells during puberty, thus restricting loss of REA expression to the body cells within the terminal end buds (TEBs) and to a subgroup of luminal epithelial cells in the pubertal gland (Fig. 3.1). Genotyping confirmed the ablation of REA alleles in mammary glands of virgin mice (data not shown). As we previously found that REA\(^{f/f}\) PR\(^{Cre/+}\) mice were infertile (Park et al, unpublished), PR-Cre-mediated REA knockout animals were only used in order to examine the role of REA in mammary gland development prior to pregnancy. Of particular note, the number of mammary cells expressing PR was previously shown to be significantly reduced during pregnancy, lactation and involution (23). We also confirmed very low PR expression at lactation day 14 by immunohistochemistry (Fig. 3.1). Additionally, we assessed the pattern of ER\(\alpha\) expression because we are particularly interested in how REA impacts ER\(\alpha\) signaling during mammary gland development. As shown in Fig. 3.1, the expression pattern for ER\(\alpha\) was similar to that observed for PR in the pubertal mammary gland, but ER\(\alpha\) expression was quite high during late lactation period (lactation day 14).
Mammary ductal outgrowth is impaired in mice lacking both alleles of REA, but is enhanced in mice lacking only one allele of REA

ERα signaling is known to direct ductal elongation and branching, which occurs through the proliferation of TEBs (26). We wished to determine the contribution of REA to pubertal mammary gland development. To this end, we analyzed the status of ductal elongation and branching in the virgin mammary glands of REA<sup>f/f</sup>, REA<sup>f+/+PR<sub>Cre/+</sub></sup> and REA<sup>f+/+PR<sub>Cre/+</sub></sup> mice. Since normal ductal elongation begins around 4 weeks of age and ends by 10 weeks (21), number four inguinal glands were isolated and whole mounted at 4, 6, and 8 weeks of age. To assess the extent of ductal outgrowth, the distance from the lymph node to the end of the longest extended duct was measured. Although the gross morphology of glands from 4 weeks of age was similar in REA<sup>f/f</sup>, REA<sup>f+/+PR<sub>Cre/+</sub></sup> and REA<sup>f+/+PR<sub>Cre/+</sub></sup> mice (data not shown), differences in mammary ductal outgrowth of pubertal period mice (6-8 weeks of age) were apparent. As shown in Fig. 3.2A, mammary ductal morphogenesis was severely impaired in glands from mice homozygous null for REA. More interestingly, in comparison with glands from REA<sup>f/f</sup> mice, ductal morphogenesis was accelerated in glands from REA<sup>f+/+PR<sub>Cre/+</sub></sup> mice, as evidenced by longer ductal length in the absence of only one allele of REA. Similarly, the number of terminal end buds was significantly higher in glands from REA<sup>f+/+PR<sub>Cre/+</sub></sup> mice (19.8±4.0, n=7) than in glands from REA<sup>f/f</sup> mice (9.3±1.3, n=7) (P<0.01). Although the number of terminal end buds declined in both genotypes at 8 weeks of age, it was still significantly higher in glands from REA<sup>f+/+PR<sub>Cre/+</sub></sup> mice (12.8±2.1, n=8) than in glands from REA<sup>f/f</sup> mice (8.3±2.5, n=7) (P<0.01). After puberty, mice homozygous null for REA displayed even more severe defects in mammary morphogenesis (24 weeks of age). The mammary ductal network of REA<sup>f/f</sup> PR<sub>Cre/+</sub> mice had barely invaded the fat pad, and side branching was almost fully absent, while control REA<sup>f/f</sup> females exhibited
extensive branching and normal fat pad invasion (data not shown). These observations that mammary ductal outgrowth is accelerated by loss of one allele of REA, but inhibited by loss of both alleles, suggest that normal mammary gland development and morphogenesis is sensitive to REA gene dosage.

**Conditional deletion of the REA gene in the mammary gland during pregnancy and lactation**

Since REA^{ff} PR\textsuperscript{Cre/+} females are infertile, we were unable to explore the physiological function of REA during pregnancy and lactation with REA^{ff} PR\textsuperscript{Cre/+} mice. Thus, REA conditional knockout mice that carry a Whey Acidic Protein (WAP)-Cre transgene and REA floxed alleles (REA^{ff} WAP-Cre) were generated (Fig. 3.3A) (54). In the mammary gland, the WAP-Cre transgene was shown to be expressed exclusively in mammary epithelium during late pregnancy and through lactation and is activated by prolactin and Stat5 signaling (7, 11, 29). Because WAP is expressed in differentiating mammary epithelial cells, the use of the WAP-Cre transgenic mice allowed us to delete the REA gene only in differentiated cells and to examine whether REA is needed for the maintenance of differentiated mammary epithelium after it has undergone pregnancy-induced proliferation and differentiation (11, 45). As shown in Fig. 3.3B, ablation of the REA alleles was mammary gland specific among tested organs as examined by genotyping. Using qRT-PCR and immunoblotting, we also confirmed the deletion of REA in REA^{ff} WAP-Cre mammary tissues from late pregnancy (P18) to lactation (L2 and L14) (Fig. 3.3C-D). As shown in Fig. 3.3E, uniform REA protein expression was observed in REA^{ff} epithelium at L14, while about half of the epithelial cells were devoid of REA in REA^{ff} WAP-Cre mice.
Pups nursed by REA\textsuperscript{ff} WAP-Cre females displayed greatly reduced weight gain

Although the REA\textsuperscript{ff} WAP-Cre dams could support their litters during lactation, REA deficiency in the epithelium of the mammary glands profoundly impaired the normal growth of the offspring. All litters born to REA\textsuperscript{ff} WAP-Cre dams survived until the time of the weaning (postnatal day 21) but these pups exhibited low body weights (Fig. 3.4A and B). To carefully examine the time line of lactation failure of the REA\textsuperscript{ff} WAP-Cre mice, pup body weights were measured throughout postnatal development (P2-P20). On P2, the litter size was normalized to six pups. As shown in Fig. 3.4C and Table 3.1, the average body weight of 2-day-old pups nursed by REA\textsuperscript{ff} WAP-Cre dam was about 6\% less than that of pups from REA\textsuperscript{ff} females (1.28 g versus 1.36 g). The growth retardation of the pups was even more severe after day 7 (lactation day 7), with the average body weight of pups nursed by an REA\textsuperscript{ff} WAP-Cre mother being about 34\% less than that of pups nursed by control mice. Statistical analysis of this data is summarized in Table 3.1.

REA is required for the maintenance of differentiated lobuloalveolar structure

During pregnancy and lactation, marked changes occur in the mammary gland. Initial changes observed during pregnancy include an increase of ductal branching and formation of alveolar buds, and these alveolar buds progressively differentiate into individual alveoli that ultimately become milk-producing lobules during the second half of pregnancy (43). Following parturition, the secretory lobuloalveolar structures become more apparent with extended luminal space (2). To determine the impact of conditional REA ablation on mammary gland development, we examined the mammary gland morphology from REA\textsuperscript{ff} and REA\textsuperscript{ff} WAP-Cre females at multiple stages of pregnancy and lactation by hematoxylin-eosin staining. As shown
in Fig. 3.4C, the number of alveoli from the REA^{ff} mammary gland was greatly increased from day 8 (P8) to day 18 pregnancy (P18). Proteins (stained by purple/pink in hematoxylin and eosin-stained sections) and lipids begin to appear in the alveolar lumen and the alveolar epithelial cells, respectively. Fully differentiated alveoli are observed at L2 and are expanded further until the lactation is complete (Fig. 3.4C). Morphological changes in the REA^{ff} WAP-Cre mammary gland were similar to control mice until L2 (Fig. 3.4C). However, in the case of the REA^{ff} WAP-Cre mammary gland, at lactation day 14 (L14), the alveoli begin to collapse and adipocytes begin to reappear in the mammary gland (Fig. 3.4C). The epithelium also appears very disorganized in the REA^{ff} WAP-Cre mammary glands. After weaning of the pups, the mammary gland goes through a process of involution, requiring both apoptosis and tissue remodeling (42, 43). Interestingly, the histology of the mammary gland between REA^{ff} and REA^{ff} WAP-Cre during involution was very different. As shown in Fig. 3.4C, after 3 days of weaning, the REA^{ff} mammary glands still maintained the secretory alveolar structures with apoptotic bodies visible in luminal spaces, while most of the alveoli in REA^{ff} WAP-Cre mice were already collapsed. Thus involution seems to be dramatically accelerated in REA^{ff} WAP-Cre mice. These observations indicate that REA is required for normal lobuloalveolar development and maintenance of differentiated lobuloalveolar structures during pregnancy, lactation and involution.

**REA is required for Stat5-mediated gene expression, lactose synthesis and lipid secretion**

The observed growth retardation of offspring nursed by conditional REA knockout mice and abnormal lobuloalveolar structures in the mammary gland of mice lacking REA led us to investigate the content of milk produced by REA^{ff} WAP-Cre mice. Mouse milk contains about
12% protein (several casein proteins, α-lactalbumin, whey acidic protein, lactoferrin, secretory immunoglobulin A, and others), 30% lipid, and 5% lactose (2). We first examined the content of milk proteins in the REA^ff WAP-Cre mice at different stages of pregnancy and lactation. As shown in Fig. 3.4D, mRNA expression for major milk proteins, caseins and whey acidic protein (WAP), was dramatically up-regulated as lactation progressed in the control mice. However, expression of these genes was significantly dampened in the mammary gland of REA^ff WAP-Cre mice. We also found that expression of α-lactalbumin, an essential and limiting cofactor for lactose synthase (43) and butyrophilin, known to be involved in milk lipid secretion (43, 44), were greatly reduced in the mammary gland of REA^ff and REA^ff WAP-Cre mice (Fig. 3.5C). Of note, the significant reduction of the expression of these genes was observed at lactation day 14 (L14) which is approximately the same time we observed alveoli collapse in the conditional knockout mammary glands.

Because these milk protein genes have been shown to be regulated by prolactin (PRL) via phosphorylation of Stat5 (30, 39), we wished to determine if a diminished activation of Stat5 in the mammary gland of REA^ff WAP-Cre mice might be responsible for the compromised milk protein gene expression. To this end, levels of phosphorylated Stat5 were examined in the mammary gland of REA^ff and REA^ff WAP-Cre at L14 by immunoblotting. As shown in Fig. 3.4E, phosphorylation of Stat5 in the REA^ff WAP-Cre at L14 was markedly reduced compared to REA^ff mice. These data imply that deficiency of REA in the mammary gland contributes to an altered mammary morphogenesis, leading to compromised milk production.

*Pups nursed by REA^ff WAP-Cre females displayed increased weight gain*
Since we observed accelerated mammary ductal outgrowth in the REA^{f/+}PR^{Cre/+} mammary gland during pubertal period, we wished to examine the impact of loss of only one allele of REA on mammary gland development during pregnancy and lactation. To this end, pups body weight nursed by REA^{f/+}WAP-Cre females were measured and the gene expression involved in milk production was examined. As shown in Fig. 3.5A, the average body weight of 5-day-old pups nursed by REA^{f/+}WAP-Cre dam was about 16% more than that of pups from REA^{ff} females (2.66 g versus 3.09 g). Similarly, the average body weights of 7-, 9-, 11-, 13 and 14-day-old pups fostered by REA^{f/+}WAP-Cre females were 18% (3.64g versus. 4.32 g), 13% (4.77 g versus 5.42 g), 9% (5.89g versus. 6.46 g), 7% (6.92 g versus 7.47 g) and 8% (7.21 g versus 7.82 g) more than that of pups from control mice. Consistent with this body weight results, stimulation of the genes involved in milk production (caseins, α-lactalbume, butyrophilin, and Elf-5) was significantly greater in the mammary gland of REA^{f/+}WAP-Cre females (Fig. 3.5B), but these differences were apparent only in early lactation periods (lactation day 2 and 5). Statistical analysis of this data is summarized in Table 3.1.

Complete REA loss of function induces apoptosis of differentiated mammary epithelial cells

Because the REA^{ff} WAP-Cre mice displayed earlier alveoli collapse in the mammary gland, we wished to determine whether mammary epithelial cells in REA^{ff} WAP-Cre mice had a higher apoptotic rate. To do this, we examined expression of mRNA for cell cycle-regulatory proteins, p21, p27 and p53 by qRT-PCR. As shown in Fig. 3.6A, the cyclin-dependent kinase inhibitor, p21, was significantly increased in the mammary glands of REA^{ff} WAP-Cre compared to that of wild-type, while expression of the two other genes, p27 and p53, was not affected by REA deletion. In order to examine the extent of apoptosis in the wild-type and conditional REA
mutant, TUNEL analysis was used as a measurement of DNA fragmentation on mammary glands obtained from both REA$^{ff}$ and REA$^{ff}$ WAP-Cre mice at L14. We observed markedly increased apoptotic cell death in the REA$^{ff}$ WAP-Cre mammary, as evidenced by increased fluorescence (Fig. 3.6B). Similarly, we also examined the activation of the apoptosis-promoting executioner, caspase-3, in the wild-type and REA-null mammary glands by immunohistochemistry. As shown in Fig. 3.6C, positive cells for active caspase-3 were present only in the REA$^{ff}$ WAP-Cre mammary glands. Of note, most of the apoptotic bodies were present in luminal spaces. Taken together, these data indicate that loss of REA during late pregnancy and lactation results in an inability to maintain the differentiated mammary alveolar structure, possibly due to a combination of increase in G1-S-phase arrest and increased apoptosis.

 Reduction of REA enhanced estrogen responsiveness in the mouse mammary gland

Since we observed accelerated mammary ductal outgrowth in the absence of one allele of REA and because estrogen is known to be critical for this process, we hypothesized that REA represses estrogen-mediated mammary ductal outgrowth. Additionally, we found that the differences in mammary ductal outgrowth between REA$^{ff}$ and REA$^{ff}$ PR$^{Cre/+}$ mice were very apparent particularly during pubertal period (6-8 weeks of age). To test our hypothesis, we ovariectomized mice at 6 weeks of age and treated with estradiol (E2) or control vehicle for 5 days. As shown in Fig. 3.7A, ovariectomy at 6 weeks of age causes TEB regression and halts ductal elongation while treatment with E2 resumes TEB formation and ductal elongation. In an attempt to identify the gene responsible for accelerated mammary ductal outgrowth seen in the REA$^{ff}$/PR$^{Cre/+}$ mice, we examined the expression level of amphiregulin in the mammary gland
treated with E2 because amphiregulin is the major paracrine mediator of ductal outgrowth and directly regulated by E2 (8, 28). As shown in Fig. 3.7B, up-regulation of amphiregulin in response to E2 was greater in the mammary gland of REA heterozygous mice, suggesting that accelerated mammary outgrowth in the mammary gland of REA^{+/+}PR^{cre/+} animals is, in part, associated with hyper-stimulation of amphiregulin.

3.5. DISCUSSION

Our conditional REA knockout models, devoid of either one or both REA alleles, have uncovered a critical relationship between gene dosage of REA and mammary gland development and functional activities. Complete loss of both REA alleles severely impedes mammary ductal morphogenesis and maintenance of differentiated lobuloalveolar structures, whereas a reduction of REA dosage via single allele deletion enhances mammary morphogenesis. In the present study, we have demonstrated that the consequences of complete REA loss result in increased apoptosis of mammary epithelial cells as evidenced by DNA fragmentation and caspase activation. Loss of a single REA allele results in accelerated ductal morphogenesis and is at least partially due to enhanced estrogen responsiveness.

Repressor of Estrogen Receptor Activity (REA), also known as prohibitin2 (PHB2) is an evolutionarily conserved protein with established roles in multiple, essential cellular processes including transcription, mitochondrial biogenesis and replicative senescence (4, 9, 12, 18, 27, 33, 35, 38, 40, 41). Previous reports suggest that REA is a multifunctional protein, located in several sub-cellular regions—nucleus, plasma membrane, and mitochondria (35). Although the molecular function of REA is still not completely understood, REA activity in mitochondrial
functions has been considered to be critically linked to cell proliferation and mitochondrial morphogenesis (33-35). Our findings in animals with conditional deletion of both REA alleles agree with previously observed embryonic lethality of conventional REA knockout animals (41) and \textit{in vitro} studies that have shown that the deletion of REA impaired proliferation of mouse embryonic fibroblasts (MEFs) (33). In particular, Langer and colleagues recently have shown that REA controls cell proliferation and apoptosis by regulating small dynamin-like GTPase OPA1 (optic atrophy 1), a component of the mitochondrial fusion machinery (33). Silencing of REA resulted in a loss of OPA1 and altered cristae formation, which was restored by expression of a long form of OPA1(33). Consistent with these findings, we also observed a loss of long form of OPA1 in the mammary glands from REA$^{ff}$ WAP-Cre mice (data not shown). The phenotypic outcomes of complete loss of both REA alleles in the mammary gland- cell cycle arrest and increased apoptosis of mammary cells- could be partially associated with defects of mitochondrial functions of REA.

Female mice lacking only one allele of REA had a mammary gland phenotype very different from that of homozygous null REA mice. In virgin mice, 50% reduction of REA dosage resulted in accelerated mammary ductal elongation and an increased number of terminal end buds in a specific pubertal period (6-8 weeks of age), whereas the morphological differences from mammary glands at 15 weeks of age were not apparent. Although both estrogen and progesterone are key mediator of ductal morphogenesis, estrogen is generally considered to be responsible for ductal outgrowth and minor-branching, while progesterone leads to tertiary branching and alveolar development (1, 13, 19). Our observations with accelerated ductal outgrowth in the pubertal mammary gland suggest that REA might profoundly impact estrogen-mediated ductal morphogenesis. Furthermore, in an attempt to identify genes that are responsible
for this accelerated ductal elongation and are also regulated by estrogen, we ovariectomized
pubertal mice (6 weeks of age) and treated them with estradiol (E2) for 5 days. We found that
up-regulation of amphiregulin, the major paracrine mediator of estrogen-induced ductal
morphogenesis, was greater in the mammary glands of REA heterozygous mice compared to
control mice. In addition to this hyper-stimulation of amphiregulin by E2 in the absence of one
allele of REA, enhanced ER transcriptional activity was also demonstrated by our prior in vivo
ERE-luciferase activity assay in conventional REA heterozygous animals (REA+/−) where ER
transcriptional activity was much greater in REA+/−/ERE-Luc mice than in WT/ERE-Luc mice
(38). Thus, an important future goal in investigating the role of REA in mammary ductal
morphogenesis will be to identify the specific downstream targets that are altered in the
mammary gland of REA heterozygous mice by expression profiling.

The role of ERα in mammary gland development during pregnancy and lactation was
recently investigated by generation of conditional ERα knockout mice by use of WAP-Cre
transgenic mice (15). Because conventional ERα knockout mice (αERKO) were infertile, it was
not possible to explore the role of ERα beyond puberty. Conditional ERα knockout mice also
showed that ERα ablation in mammary epithelium during late pregnancy and throughout
lactation resulted in a loss of ductal side-branching and lobuloalveolar structures, ductal dilation,
and decreased proliferation of alveolar progenitors (15). Both of these conventional and
conditional ERα knockout mouse models demonstrate that ERα profoundly impacts pubertal as
well as pregnant and lactating mammary gland development.

Our analysis of mice that are heterozygous for REA only during late pregnancy and
lactation (REA+/−WAP-Cre) showed that the average body weight of the offspring nursed by
REA^{+/−}WAP-Cre mice was higher than that of pups nursed by control mice. Consistent with these body weight results, stimulation of the genes involved in milk production (caseins, α-lactalbumine, butyrophilin, and Elf-5) was significantly greater in the mammary gland of REA^{+/−}WAP-Cre females. However, distinct morphological differences between REA^{+/−} and REA^{+/+}WAP-Cre mice throughout pregnancy and lactation were not observed (data not shown), whereas our prior conventional REA heterozygous animals (REA^{+/−}) displayed histologically enhanced pregnancy-associated ductal and alveolar morphogenesis (38). The phenotypic differences between conditional and conventional REA heterozygous animals were possibly due to the differences of time- and cell-specific inactivation of one REA allele because the use of WAP-Cre transgenic mice allowed us to delete one allele only after mammary epithelium differentiation is complete and only in alveolar epithelial cells. Additionally, it is not clear whether observed phenotypic outcomes from REA^{+/−}WAP-Cre mice are also associated with altered estrogen signaling during pregnancy and lactation, because estrogen levels are known to be declined sharply after birth because of the loss of the corpus luteum. We also could not rule out the possibility that REA might enhance ER activities by estrogen-independent pathways during pregnancy and lactation.

Since ER-mediated transcriptional activity critically depends on coregulator proteins, altered coregulator expression has been implicated in breast cancer (17, 37). For example, one of well characterized coactivator protein for ERα, AIB-1/SRC-3, is shown to be overexpressed in 60% of primary breast tumors and transgenic mice in which AIB-1 is overexpressed in the mammary gland resulted in development of malignant mammary tumors (3, 17, 52). There are also several lines of studies suggesting that REA might be associated with breast carcinogenesis.
REA expression levels were shown to be positively correlated with ER in breast cancer, but inversely correlated with tumor grade (37). More recently, the F-box protein Skp2B, which is often overexpressed in breast cancers, was shown to interact with REA resulting in degradation of the REA protein (53). In the mammary gland of MMTV-Skp2B mice in which Skp2B is overexpressed, REA levels were found to be low and these mice develop mammary tumors (53). These findings suggest that altered levels of REA might be linked to breast tumorigenesis.

In the present study, we found that the number of terminal end buds (TEBs) was significantly increased in the mammary gland of REA heterozygous mice compared to that of control mice. TEBs are the structures in the mouse mammary gland that give rise to malignant mammary tumor upon exposure to a chemical carcinogen (22, 46, 48). Similar structures in a human breast, called terminal ductal lobular unit 1 (TDLU1), also known to be the sites of breast cancer initiation in women (47). Thus, it is possible that reduced level of REA in the mammary gland might enhance the susceptibility to chemically induced mammary tumorigenesis. Of note, enhancement of estrogen responsiveness seen in the mammary gland of heterozygous animals was also observed in ERα positive human breast cancer cells, evidenced by hyper-stimulation of E2-mediated up-regulated genes (pS2, PgR, GREB1, and SERPINB9) (data not shown). These findings suggest that some functional activities of REA are common in both the mouse mammary gland and breast cancer and REA might possess a potential role in human breast cancer.

In summary, our animal models with conditional loss of both or one REA allele provide the first in vivo evidence that REA is a multifunctional protein with a gene dosage-sensitive modulatory role in mammary gland development.
3.6. REFERENCES


3.7. FIGURES, TABLE AND LEGENDS

FIGURE 3.1

Figure 3.1. REA is expressed in the developing mammary gland.

Immunohistochemical detection of ER, PR and REA in virgin terminal end buds (TEBs) (6 weeks) and lactating alveoli (lactation day 14). The scale bars represent 200 µm.
FIGURE 3.2

A

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B

- **Ductal length (mm)**
  - 6 wk: REA^{+/}, REA^{+/} PR^{Cre/+}, REA^{+/} PR^{Cre/+}
  - 8 wk: REA^{+/}, REA^{+/} PR^{Cre/+}, REA^{+/} PR^{Cre/+}

C

- **Number of TEB**
  - 6 wk: REA^{+/}, REA^{+/} PR^{Cre/+}
  - 8 wk: REA^{+/}, REA^{+/} PR^{Cre/+}
Figure 3.2. Mammary ductal outgrowth is faster in mutant mice heterozygous for REA, but severely impaired in mice homozygous null for REA.

(A) Whole-mount staining of the number 4 inguinal mammary glands from REA^{ff}, REA^{ff} PR^{Cre/+} and REA^{ff} PR^{Cre/+} virgin mice at 6, 8 and 15 weeks of age. (B) Ductal length of the mammary glands at 6 and 8 weeks of age. Distance from the lymph node to the end of the longest extended duct was measured. The line in box plots represents the median length measured from eight mammary glands. (C) The number of terminal end buds per mammary gland was counted in wholemounts of number 4 inguinal glands at 6 weeks of age. *, P< 0.05; **, P< 0.01.
Figure 3.3. Conditional knockout of the REA gene during pregnancy and lactation.

(A) Schematic diagrams of the REA gene targeting strategy. (B) Genomic DNA isolated from uterus, ovary, mammary gland, liver, kidney and heart was genotyped by PCR. (C) REA mRNA and protein (D) were analyzed by qRT-PCR or immunoblotting, respectively in mammary tissues at different stages of pregnancy (P) and lactation (L). The data are represented as mean±SD. (E) Immunohistochemical detection of REA in REA<sup>ff</sup> and REA<sup>ff</sup> WAP-Cre mammary glands at L14. *, P < 0.05; **, P < 0.01.
FIGURE 3.4

A

C

D

E
Figure 3.4. REA is required for the maintenance of differentiated lobuloalveolar structure.

(A) Representative photograph of pups nursed for 20 days by REA^{+/−} or REA^{+/+} WAP-Cre mice.

(B) Box plots of pups body weight nursed by REA^{+/−} or REA^{+/+} WAP-Cre mice during postnatal development. Horizontal lines in the boxes represent the first, second (the median), and third quartiles. (C) Representative hematoxylin- and eosin-stained mammary gland sections from REA^{+/−} and REA^{+/+} WAP-Cre at different stages of pregnancy (P), lactation (L) and involution (I). Magnification, ×40. (D) qRT-PCR analysis of genes involved in milk protein expression (casein-α, casein-β, casein-κ and whey acidic protein (WAP)), lactose synthesis (α-lactalbumin) and milk lipid secretion (butyrophilin) at different stages of pregnancy (day 8 and 18) and lactation (day 2, 14 and 19). The data are represented as mean±SD. (E) Mammary gland lysates were analyzed by immunoblotting for REA and phosphor-Stat5. ERK2 served as a loading control. *, P< 0.05; **, P< 0.01.
Figure 3.5. Body weight gain of pups nursed by REA^{+/+} WAP-Cre females was increased and correlated with increased milk-related gene expression.

(A) Box plots of pups body weight nursed by REA^{+/+} or REA^{+/+} WAP-Cre mice during postnatal development. Horizontal lines in the boxes represent the first, second (the median), and third quartiles. (B) mRNA level of REA, casein-β, casein-κ, α-lactalbumin, butyrophilin and Elf-5 was examined by qRT-PCR at different stages of pregnancy (day 18) and lactation (day 2, 5, 9 and 14). The data are represented as mean±SD. *, P<0.05; **, P<0.01.
FIGURE 3.6

Figure 3.6. REA°/° WAP-Cre mutants have increased apoptosis of mammary epithelium. (A) The cell cycle inhibitor p21 levels are elevated in the mammary gland of REA°/° WAP-Cre mice, as measured by qRT-PCR. The data are represented as mean±SD. (B) Representative fluorescence images of TUNEL staining in mammary gland sections from REA°/° and REA°/° WAP-Cre mammary glands of lactation day14 mice. (C) Immunohistochemical detection of active caspase-3 expression in the REA°/° and REA°/° WAP-Cre mammary glands of lactation day14 mice. *, P< 0.05; **, P< 0.01.
Figure 3.7. Reduction of REA enhances E2-mediated transcription in mouse mammary gland.

(A) Whole-mount staining of mammary glands from vehicle (oil) or estradiol-treated REA^{ff} and REA^{ff} PR^{Cre/+} and REA^{ff} PR^{Cre/+} mice. 6 week old mice were ovariectomized, allowed to rest for 2 weeks, then treated with either oil or estradiol. (B) mRNA level of amphiregulin (AREG) was measured by qRT-PCR. Data are mean±SD. *, P< 0.05; **, P< 0.01.
### TABLE 3.1

Analysis of the body weights of pups used in box-plots of Fig. 3.4

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### Analysis of the body weights of pups used in box-plots of Fig. 3.5

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