University of Cincinnati

Date: 4/4/2011

I, Cheryl Minges , hereby submit this original work as part of the requirements for the degree of Master of Science in Pharmaceutical Sciences.

It is entitled:

Targeted Deletion of Estrogen Receptor Alpha in Mouse Pituitary Lactotrophs

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Targeted Deletion of Estrogen Receptor Alpha in Mouse Pituitary Lactotrophs

A thesis submitted to the
Graduate School
of the University of Cincinnati
in partial fulfillment of the
requirements for the degree of

Master of Science

In the Division of Pharmaceutical Sciences
Of the James L. Winkle College of Pharmacy
2011

by

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ABSTRACT

Prolactin (PRL) is a major anterior pituitary (AP) hormone that is necessary for mammary gland development, lactation, and female fertility. The synthesis and release of PRL from specialized pituitary cells (lactotrophs) is tightly regulated and primarily under tonic inhibition by dopamine (DA), produced by the tuberoinfundibular dopaminergic neurons (TIDA) of the hypothalamus. In turn, PRL regulates its own release by acting on the TIDA neurons in a short feedback loop to increase inhibition by DA and maintain homeostasis. Superimposed on this negative regulation are the stimulatory effects of estrogen. Estrogen (E_2) stimulates PRL synthesis, secretion, and lactotroph proliferation by direct action at the level of the AP. E_2 also stimulates PRL indirectly at the level of the hypothalamus by inhibiting TIDA activity. Finally, E_2 can also modify lactotroph response to DA and other PRL-regulatory factors. The normal growth and function of lactotrophs depends on an interactive balance between DA and E_2. However, the *relative* contribution of E_2 regulation directly on the pituitary lactotrophs *versus* hypothalamic DA, particularly during various physiological states in the female, is not well defined and current animal models are unable to distinguish this. Thus, there is a need for a new model with which to study the regulatory role of E_2 action at the level of the pituitary *versus* the hypothalamus. Through transgenic Cre/loxP technology and homologous recombination, we have generated a selective knockout mouse model in which the primary mediator of E_2 action in the lactotroph, estrogen receptor alpha (ERα), is deleted only in pituitary lactotrophs, while conserving E_2 action at the TIDA neurons. Herein is the generation of this animal model and its characterization. Results show the successful generation of mice with the ERα knockout targeted to lactotrophs but not in other ERα expressing tissues of these animals.

Estrogen responsiveness of the selective knockout mouse was evaluated. *In vivo* studies, utilizing a model of spontaneous prolactinoma formation, showed no significant differences in AP growth between mice with the selective deletion of ERα and those without. DA levels in the AP were low in both groups, suggesting that loss of dopaminergic inhibition contributes more to lactotroph proliferation than does a direct action of E_2 however further investigation is required to support this conclusion. *In vitro* studies demonstrated that lactotrophs with ERα deletion did not respond to E_2 or E_2 antagonist with regard to cell content of PRL, highlighting the direct action of E_2 on the lactotroph in modulating PRL synthesis.

Our mouse model, with selective loss of ERα in pituitary lactotrophs, and E_2 action at the level of the hypothalamus maintained, will provide an effective tool in elucidating the primary effects of estrogen, as well as other factors, on PRL regulation.

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Abbreviations

AC: Adenylyl Cyclase
AN: Acruate Nucleus
AP: Anterior Pituitary
BrdU: Bromodeoxyuridine
D_2R: D_2-like G-protein Coupled Receptor
DA: Dopamine
DAergic: Dopaminergic
E_2: Estrogen
ER: Estrogen Receptor
ERα: Estrogen Receptor alpha
ERβ: Estrogen Receptor beta
ERKO: Estrogen Receptor Knockout
ERαKO: Estrogen Receptor alpha Knockout
ERβKO: Estrogen Receptor beta Knockout
$ER\alpha^{fl/fl}$: Floxed Estrogen Receptor alpha
$ER\alpha^{fl/fl}$- pitPRL-Cre^{+}: Selective Estrogen Receptor Knockout
$ER\alpha^{fl/fl}$- pitPRL-$Cre^{0/0}$: Selective Estrogen Receptor Control
FACS: Fluorescence Activated Cell Sorting
GIRK: G-protein Inward Rectifying Potassium channel
GPR30: Membrane estrogen receptor
ICI: ICI 182780, estrogen receptor alpha antagonist
IL: Intermediate Lobe
LH: Luteinizing Hormone
MG: Mammary Gland
mGPHα: Mouse glycoprotein hormone alpha subunit
NL: Neural Lobe
Ov: Ovary
pitPRL-Cre^{+}: Pituitary prolactin promoter driving expression of Cre recombinase
PRL: Prolactin
PRL-R: Prolactin receptor
$PRL^{+/+}$: Prolactin wild type
$PRL^{+/-}$: Prolactin heterozygote
$PRL^{-/-}$: Prolactin knockout
RHPA: Reverse Hemolytic Plaque Assay
RIA: Radioimmunoassay
TH: Tyrosine Hydroxylase
THDA: Tuberohypophyseal Dopaminergic
PHDA: Periventricular Hypophyseal Dopaminergic
TIDA: Tuberoinfundibular Dopaminergic
Ut: Uterus
WAP: Whey acidic protein
WT: Wild Type

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Chapter 1

Introduction and Background

INTRODUCTION and BACKGROUND

Prolactin

Prolactin (PRL) is a polypeptide hormone found in all vertebrates that has many important biological functions including effects on osmoregulation, metabolism, reproduction, metamorphosis, and behavior (1). In mammals, PRL has highly specialized roles in female reproduction. It is required for mammary gland development and lactation, and appropriate secretion of PRL is required for female fertility (1, 2, 3). Hypersecretion of PRL in rats results in infertility in females and also causes infertility and impotence in males (4). On the other hand, female mice who are deficient in PRL are completely infertile, while male mice remain fertile (5). The same phenotype is observed in mice carrying a null mutation for the PRL receptor gene (6). Although rare, isolated PRL deficiency in humans has a similar phenotype – fertility problems in females, but not in males (7, 8, 9, 10).

PRL is synthesized and secreted from specialized endocrine cells of the anterior pituitary (AP) called lactotrophs. Lactotrophs are one of six different secretory cell types of the AP and comprise twenty to fifty percent of the entire cell population, depending on sex and physiological status (1, 2). These cells secrete PRL spontaneously in the absence of stimuli and are predominantly under tonic inhibitory control, however lactotrophs respond to physiological stimuli such as suckling, stress, increased ovarian sterioids, and other PRL-releasing factors (1, 2, 11, 12).

Regulation of Prolactin

There are many factors that regulate the synthesis and secretion of PRL, as well as the proliferation of lactotroph cells, in order to maintain homeostasis. The AP is located immediately below the brain, and is connected to the ventral surface of the brain by a portal vasculature, known as the long hypophyseal portal vessels. These blood vessels transport regulatory neurohormones and neurotransmitters from the hypothalamus to the AP. PRL is one of several different hormones secreted into the general circulation by the AP that acts on peripheral target tissues and is regulated by several factors from the hypothalamus. However, the principle hypothalamic influence on lactotrophs is inhibitory, through the neurotransmitter, dopamine (DA). DA has several direct actions on lactotrophs including inhibition of PRL synthesis, release, and lactotroph proliferation (13,14). Because lactotrophs are spontaneous secretors, this tonic inhibition requires a continuous input of DA and is required to maintain low circulating levels of PRL (1, 2, 11, 12, 14).

Dopamine

DA delivered to the AP comes from three distinct dopaminergic (DAergic) pathways in the hypothalamus: 1) the tuberoinfundibular dopaminergic (TIDA) neurons, whose cell bodies are located in the arcuate nucleus (AN) and whose axons project to the median eminence, terminating in the perivascular space around capillaries that drain into the long hypophyseal

portal vessels (15); 2) the tuberohypophyseal DAergic (THDA) neurons, whose cell bodies are also located in the AN but whose axons project to the neural lobe (NL) and intermediate lobe (IL) of the pituitary, which do not contain lactotrophs (16); and 3) the periventricular-hypophyseal DAergic (PHDA) neurons, whose cell bodies are located in the periventricular nucleus with axons terminating in the IL of the pituitary (17). DA released from THDA and PHDA neurons can reach the AP via short portal vessels connecting it with the NL and IL. However, the majority of DA comes from the TIDA neurons. This DA, as well as other hypothalamic regulatory factors, is delivered to the AP via the long portal vessels, which extend down the hypophyseal stalk, connecting the hypothalamus to the AP. These vessels are the primary vascular input to the AP (1, 2). The concentration of DA in the long hypophyseal portal vessels has been shown to be much greater than in the systemic circulation, and concentrations of DA in the hypophyseal stalk are inversely related to PRL levels (18, 19).

The D_2-like G protein-coupled receptor (D_2R) is the predominant isoform of the DA receptor in the AP and mediates DA's actions on the lactotroph. The D_2R is coupled to the pertussis toxin-sensitive $G_{i/o}$ family of heterotrimeric guanine nucleotide-binding proteins (G proteins) (20, 21). Studies have shown that the level of $G_{i/o}$ in lactotrophs is important in determining the secretory activity of these cells, and these proteins play an important role in the inhibitory actions of DA (20, 22, 23).

When DA binds to its receptor, the D_2R is activated, leading to inhibition of adenylyl cyclase (AC) and reduction of cAMP in the lactotroph. This leads to a decrease in PRL synthesis. However, the inhibiton of cAMP as a mediator of DA inhibition of acute PRL release is

controversial as studies have shown inhibition of PRL release by DA with elevated cAMP. It has also been shown that extracellular potassium can stimulate PRL release while DA maintains inhibition of cAMP (11, 24, 25, 26). Therefore, while DA suppression of cAMP is sufficient to decrease PRL synthesis, it is not sufficient or necessary for PRL release.

What is clear is DA inhibition of PRL release is dependent on extracellular calcium (Ca^{2+}) influx through voltage gated ion channels. Active lactotrophs exhibit spontaneous membrane depolarization due to influx of extracellular Ca^{2+}. The elevations in cytosolic Ca^{2+} in active lactotrophs results in a high rate of PRL release, and DA inhibits this depolarization and lowers cytosolic Ca^{2+} (27, 28).

Potassium (K^+) channels also play a role in DA inhibition of PRL release, where increases in K^+ permeability in response to DA cause membrane hyperpolarization and inactivation of voltage gated Ca^{2+} channels. Evidence supports that the K^+ channel in the lactotroph activated by DA is a member of the G protein activated inward rectifying potassium (GIRK) channels. Evidence supports D_2R coupling to GIRK, and studies have shown using primary pituitary cells from proestrous rats expressing a dominant negative mutant of GIRK1, that DA inhibition of PRL release is reversed in the absence of a functional GIRK. During proestrous, 90% of lactotrophs express functional DA activated GIRKs and regulate PRL release, however these channels do not appear to be active on any other day of the cycle (27, 29).

DA action through the D_2R is also important in the regulation of lactotroph proliferation, and studies have demonstrated the anti-proliferative effects of DA on these cells. Mice lacking the

D_2R display chronic lactotroph hyperplasia, formation of prolactinomas, and hyperprolactinemia (30). When these mice are treated with D_2R agonists, proliferation of the lactotrophs is reduced, as evidenced by tumor regression *in vivo* and decreased BrdU incorporation *in vitro* (12, 14). Also, changes in cell morphology occur and DA inhibition of lactotroph proliferation has been shown to be closely related to actin organization and cell shape (31).

Feedback by Prolactin

PRL receptors are present in the AN of the hypothalamus where the TIDA neurons originate, and they are also found in the median eminence (1, 32, 33). As serum PRL levels rise, PRL regulates its own release by acting on the TIDA neurons in a short feedback loop to increase their activity and DA synthesis in order to maintain homeostasis (2, 12, 33). Studies have demonstrated an increase in DA in the hypophyseal portal blood upon stimulation by PRL *in vivo* (34). Tyrosine hydroxylase (TH) is the rate-limiting enyme in DA synthesis and is constitutively active in the hypothalamo-pituitary system (1, 12). When PRL binds to its receptor, TH activity increases in order to increase DA synthesis and release. This has been evidenced, for instance, by the increase in enzyme activity in cultured fetal hypothalamic neurons treated with PRL *in vitro* (1, 33).

Estrogen

As mentioned, lactotrophs can comprise anywhere from 20 to 50 percent of cells in the AP in mammals. However, basal serum PRL levels and pituitary PRL content are lower in males than

in females (11, 12). Consistent with sex differences in PRL, basal TIDA activity is higher in females than in males (2). These differences are due to estrogens.

Estrogen (E_2), a steroid hormone primarily from the ovary, is important in the regulation of cell proliferation, cell survival, and differentiation in a variety of organ systems and tissues including the AP. This is evidenced by changes in secretion of hormones due to changes in ovarian status associated with puberty, cyclicity, pregnancy, lactation and disease (35, 36).

Superimposed on the negative regulation of lactotrophs by DA are the stimulatory effects of E_2. E_2 increases PRL gene transcription and PRL synthesis (37, 38). E_2 does not stimulate acute secretion of PRL, but it increases the storage pool of PRL that can be readily released upon stimulation by releasing factors or removal of inhibitory factors. It also increases proliferation and cell growth (39, 40), and plays a role in lactotroph genesis and survival in PRL-secreting tumors (36, 41, 42).

E_2 regulation of PRL has been shown *in vivo* and *in vitro*. Treatment of cultured pituitary cells with E_2 results in an increase in PRL mRNA and hormone synthesis (43, 44). Chronic exposure to E_2 results in the development of hyperprolactinemia and hyperplasia of lactotrophs *in vivo* (45, 46). The lactotrophs are clearly responsive to changes in endogenous E_2 levels as seen with their growth in number during pregnancy and lactation, and decrease following the end of lactation (36). A rise in circulating E_2 that occurs during the estrous cycle results in a surge in PRL release and increased lactotroph proliferation. It has been shown the proliferative activity in

female rats is high in estrus and during parturition due to the sensitizing action of E_2 (47, 48, 49).

E_2 action on PRL regulation is mediated by the estrogen receptor (ER) in the lactotroph. The ER is a member of the nuclear receptor family. The receptor is activated upon ligand binding and acts as a transcription factor when it dimerizes and binds to E_2 response elements (EREs) in the regulatory regions of target genes (12, 42). There are at least two isoforms of this receptor, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). The two isoforms are products of different genes, however they share significant homology except in the transcriptional activation domain. ERα and ERβ have equal binding affinities for E_2, but there are some differences between their transcriptional properties (12, 42, 50).

E_2 action in the pituitary is mediated primarily by ERα (12, 51, 52, 53). The lactotrophs, as well as the gonadotrophs and corticotrophs express ERα with highest expression detected in the lactotroph cells (36, 54). Many studies using the global ERα knockout mouse (ERαKO), where the receptor is genetically deleted in all tissues and cell types, have shown the requirement for ERα in regulation of PRL. Gene disruption of ERα leads to a 10 to 20 fold decrease in PRL mRNA levels in the anterior pituitary, and there is also a slight reduction in lactotroph cell number (41, 42, 55). This supports ERα as a primary mediator of E_2 action in the pituitary lactotrophs.

ERβ transcript has been detected in the pituitaries of rats and humans, whereas levels in mice are negligible (42). Studies show no immunoreactivity for ERβ in pituitary glands of wild type

(WT), ERαKO, or ERβKO mice, and no change was found in PRL immunoreactivity in ERβKO mice (56). This suggests that ERβ is not involved in the regulation of PRL in mice. Studies have shown that ERβ is detectable in the ovary, uterus, prostate, lung, and brain, and it may be the primary mediator of E_2 action in FSH cells and in the regulation of folliculogenesis (12, 57, 50).

A seven transmembrane G-protein coupled receptor, known as GPR30, has been studied and proposed as an ER. It is believed to mediate rapid and transcriptional responses to E_2 by transactivation of EGFR and activation of the ERK1/2 pathway (58, 59). Immunohistochemical studies have shown the expression of GPR30 in the AP, with approximately 50 percent of cells in the anterior lobe intensely stained for GPR30, with highest expression found in the IL. Abundant staining was also seen in the AN of the hypothalamus where the classical ERα is also highly expressed. However, GPR30 expression was most prominent in the stomach, followed by the adrenal gland (59, 60).

Recent studies have evaluated E_2 responsiveness of GPR30. It has been shown in MDA-MB-231 cells expressing only ERβ, transfection of GPR30 into these cells restored the activation of ERK1/2 in response to E_2, demonstrating a role for the membrane receptor in E_2 responsiveness. E_2 binding to GPR30 has been shown to stimulate cAMP production, calcium mobilization, regulation of growth factor signaling pathways, insulin release in females, reduced blood pressure, and has been implicated in bone development (60, 61).

GPR30 has also demonstrated a role in E_2-mediated cell proliferation (58). Additional studies have demonstrated E_2 effects on the secretory and proliferative activity of lactotrophs through interaction with the membrane receptor and growth factors such as insulin. These results confirmed this response to be mediated by a membrane ER following treatment with an E_2-BSA conjugate that is unable to cross the plasma membrane to bind to nuclear receptors (62, 63). While E_2 responsiveness of GPR30 has been demonstrated, its existence as an ER is still controversial, and a significant role in E_2 regulation of PRL is questionable.

E_2 also alters responsiveness of the lactotrophs to DA. E_2 down regulates the D_2R, uncouples D_2R from its effector systems, and has been shown to decrease the expression of $G_{i/o}$, further decreasing the inhibitory tone by DA on the lactotroph cells (14, 23). Similarly, D_2R deficient mice illustrate pituitary growth is dependent on stimulation by ovarian E_2 as these mice develop hyperprolactinemia and hyperplasia in the absence of DA inhibitory tone, and this phenotype is more severe in females than in males (30).

In addition to direct actions on the AP, E_2 also stimulates PRL synthesis, secretion, and lactotroph proliferation indirectly at the level of the hypothalamus. E_2 can alter the secretion of hypothalamic regulatory factors in addition to the responsiveness of the AP to these factors. For instance, it alters the activity of the TIDA neurons and decreases DA by inhibiting transcription, phosphorylation, and activity of TH (2, 36). *In vitro* analyses using hypothalamic neuronal cultures show suppression of TH activity and reduced TH mRNA following treatment with E_2 (12, 64). It is also evident that TIDA neurons respond to changing levels of endogenous E_2, as

immunoreactivity to TH and DA levels decreases during late pregnancy and lactation when E_2 levels increase (65).

There is evidence E_2 mediates its actions through ERα within the hypothalamus. Expression of ERα has been found to be greater than expression of ERβ in the arcuate nucleus (65, 66). Because studies using ERα knockout animal models show significant decreases in PRL levels, it is likely E_2 action at the hypothalamus is mediated by ERα, however this receptor is the primary mediator of E_2 action in the mouse lactotroph leaving regulation of PRL by E_2 to question.

Overview

It is evident that there is an interactive balance between DA and E_2 that underlies normal lactotroph growth and function. This is clear when observing the dynamic changes of PRL secretion that occur during the reproductive cycle of a female mouse. PRL remains relatively low and stable throughout most of the estrous cycle, from the morning of estrus through the afternoon of proestrus. On the afternoon of proestrus there is a periovulatory surge of PRL, coincident with the surge of luteinizing hormone (LH) that is necessary for ovulation. The amount of DA being released remains steady throughout the cycle, maintaining its tonic inhibition on the lactotrophs; however there is a precipitous drop in DA prior to the surge of PRL. DA levels, activity of TH, turnover of DA to median eminence, and levels in the anterior pituitary have been reported to decrease prior to the PRL surge (2, 11, 67).

The surge in PRL, surge in LH, and drop in DA are all dependent on an increase in circulating E_2

that begins in diestrus II, twenty-four to thirty-two hours prior to the surge. Indeed, treatment of ovariectomized rats with E_2 that produces circulating levels comparable to late diestrus II or early proestrus, results in daily surges of both PRL and LH (49, 68). Treatment with antiserum to E_2 on diestrus II blocks the PRL surge on the afternoon of proestrus (11, 49).

It is clear that both DA and E_2 are essential in the regulation of PRL throughout the reproductive cycle, however, the *specific* contribution of E_2 regulation directly on the lactotrophs and regulation on hypothalamic DA is not well defined. Many studies have used global ERα KO mouse models, in order to study the function and mechanism of E_2 action on PRL regulation. However, the relative contribution of E_2 action at the level of the pituitary *versus* the level of the hypothalamus in the regulation of PRL synthesis, secretion, and lactotroph proliferation cannot be distinguished with these models. Thus, there is a need for a new model with which to study the regulatory role of E_2action at the level of the pituitary *versus* the hypothalamus.

Specific Aims

Aim 1: Generate a selective deletion of ERα in pituitary lactotrophs in mice using transgenic and Cre/loxP techniques

Aim 2: Verify that ERα is deleted only in pituitary lactotrophs using various methods including single cell RT-PCR

Aim 3: Evaluate the effects of the selective deletion of ERα in pituitary lactotrophs on responsiveness to estrogen, in terms of prolactin synthesis and lactotroph proliferation.

Chapter 2

Generation and Validation of

Targeted Deletion of the Estrogen Receptor-alpha in Pituitary Lactotrophs

of the Mouse

Chapter 2

INTRODUCTION

ERKO Models

A variety of techniques have been used to study the action of E_2, but many of these have had limitations. For example, studies using aromatase inhibitors to block E_2 synthesis, and E_2 antagonists to block its receptor were limited by compound efficiency, tissue and cell specificity, and bioavailability of the compounds. *In vitro* approaches using cell culture were limited in determining E_2 action as it would occur *in vivo* due to the removal of tissues or cells from whole animal systems. Techniques using castration approaches, such as ovariectomy, to remove endogenous E_2 followed by replacement with exogenous treatment removed multiple gonadal hormones in addition to E_2 from the animal. Also, these methods cannot distinguish whether responses to steroid treatment is mediated by the receptor or alternative mechanisms such as responses initiated by growth factor-regulated signal transduction pathways (42, 57, 69, 70).

Global estrogen receptor knockout (ERKO) mouse models, including ERαKO, ERβKO, and the double knockout, ERαβKO, have been generated to provide a better tool to study the function and mechanism of E_2 action. These models are useful in studying the role of the ER in an animal as a whole, and in development and normal physiology of all systems during an entire life span. Deletion of ERα as well as ERβ, is not lethal to the animal, and while the ablation of the ERs results in many abnormalities including infertility in ERαKO and ERαβKO males and females, the animals develop normally and live effectively as long as their wild type counterparts (55, 71). These models retain the ability to synthesize the natural ligand, E_2, as well as other gonadal

hormones *in vivo*. This allows for the physiology of E_2 to be studied in the presence of intact systems, and an understanding of the role of its receptor to be gained (42).

Although global ERKO models have proven to be useful in studying the effects of E_2 in many tissues, they are unable to distinguish among multiple actions of E_2 and its regulation on a specific system *in vivo*. It has been well established using ERαKO, ERβKO and ERαβKO mouse models that ERα is the primary mediator of estrogen action in the pituitary (41, 50, 53, 57). However *in vivo* studies cannot distinguish between the role of this receptor in the pituitary versus the hypothalamus in the regulation of ER-expressing cells.

The lactotrophs of the anterior pituitary synthesize and secrete PRL, and this synthesis and release of PRL, as well as lactotroph proliferation is under tonic inhibition by hypothalamic DA. Superimposed on the inhibitory effects of DA is stimulation by E_2 primarily on PRL synthesis and cell proliferation. The stimulatory actions of E_2 are mediated through ERα at the level of the anterior pituitary and the TIDA neurons of the hypothalamus. Because ERα is expressed in both lactotrophs and TIDA neurons, the relative contribution of E_2 action at the pituitary (direct) from that at the hypothalamus (indirect) in regulating lactotrophs is unclear (2, 12, 13, 14). Global ERαKO models cannot be used to determine this because ERα is removed from both the pituitary and the hypothalamus. To overcome some of these limitations, we have generated mouse lines that have targeted deletion of a functional ERα only in pituitary lactotrophs while conserving ERα and E_2 action at the level of the hypothalamus.

Selective Deletion: Cre/loxP System

In this chapter, I describe the generation of a lactotroph-specific deletion of ERα using homologous recombination with the Cre/loxP system. This technique has been successfully used to delete ERα in pituitary gonadotrophs in order to elucidate the direct actions of estrogen on gonadotrophs and the regulation of LH secretion and, consequently, reproductive function (41, 53, 72). This technique was also used by Feng *et al.*(74) to selectively delete ERα in epithelial cells of the mouse mammary gland, while conserving the receptor in the stroma, in order to study the role of ERα in the epithelium during different stages of mammary gland development.

The Cre/*loxP* method consists of flanking the gene of interest with two loxP sequences ("floxed" ERα). This is a global modification, floxing the gene sequence in all cell types and tissues. *LoxP* is a Cre-specific recognition sequence and Cre recombinase, a cyclization recombination gene of bateriophage P1, caused the two *loxP* sequences to recombine into one ("homologous recombination"), excising the intervening sequences (73). Tissue- or cell-specific deletion is accomplished by targeting the expression of Cre recombinase to the cell of interest using transgenic technology. In our model, the third exon of the ERα gene was floxed (74) and the rat pituitary PRL promoter was used to target expression of Cre recombinase to pituitary lactotrophs. This resulted in the "knock-out" of a functional ERα in the pituitary lactotrophs of our mice. Following the generation of these genetically-engineered mice, the next crucial step was to verify that ERα was deleted only in the pituitary lactotrophs and conserved in other ER-expressing cell types as well as other tissues. Herein I describe this verification and characterization of our model.

Chapter 2

MATERIALS AND METHODS

Transgene Construction and Generation of Animals

To generate mice positive for the expression of Cre recombinase in pituitary lactotrophs, a construct with the full-length pituitary prolactin promoter to drive the expression of Cre recombinase was created. Briefly, the Cre recombinase cDNA of bacteriophage P1 (Accession No. NC_005856.1) was cloned behind the full-length, ~3 kb (3,172 bp) rat PRL promoter (Accession No. X70518.1) to create a transgene with the PRL promoter, Cre recombinase, and SV40 polyadenylation sequences. This pitPRL-Cre transgene was purified, sequenced, and then delivered to the University of Cincinnati Transgenic and Mouse Knock-Out Core where it was injected into the pronucleus of fertilized murine oocytes. Injected oocytes were then implanted into pseudopregnant female mice. Viable offspring were genotyped using PCR of a sequence encoding a unique site in the Cre-recombinase sequence, from tail DNA. Positive offspring were mated to wild type mice to produce F1 generation transgenic mice, which were then inbred to produce lines of lactotroph-specific Cre-recombinase transgenic animals (pitPRL-Cre^{+}).

Mice with Exon 3 of Estrogen Receptor alpha flanked by two *loxP* sites in all cells ("floxed" ERα), were generated in the laboratory of Dr. Sohaib Khan from the University of Cincinnati (74). Dr. Khan generously donated a male mouse to the Gregerson Laboratory and transgenic mice positive for the expression of Cre Recombinase (pitPRL-Cre^{+}) were bred onto the floxed ERα background. Mice homozygous for floxed ERα and positive for Cre ($ER\alpha^{fl/fl}$- pitPRL-Cre^{+}) should have a functional ERα selectively deleted from the pituitary lactotrophs.

DNA Extraction and Genotyping

Genomic DNA was extracted from tail clippings of weanling mice. Approximately 2 mm clippings were digested with 300 µL digestion buffer [5 mM EDTA, pH 8.0, 200 mM NaCl, 100 mM Tris, pH 8.0, 0.2% SDS] with 0.4 mg proteinase K per 1 mL digestion buffer at 55^{o}C overnight. Tail digests were washed by adding 1 mL of 100% ethanol, centrifuged at 16,000 x g for 30 min. Ethanol was poured off, and the digests washed again by adding 1 mL of 70% ethanol followed by centrifugation at 16,000 x g for 20 min. Ethanol was poured off, and the digests incubated at 55^{o}C for 2 hours with lids open in 300 µL TE buffer [10 mM Tris-HCl, 0.2 mM Na_2EDTA, pH 7.5] (75).

Cre Recombinase

pitPRL-Cre+ mice were identified through PCR analysis of tail genomic DNA using a Cre-specific primer pair, corresponding to base pair 63-86 (NC_005856.1; sense 5'-TGAGGTTCGCAAGAACCTGATGGA-3'; Tm 60.3 ^{o}C) and base pair 331-355 (antisense 5'-GCCGCATAACCAGTGAAACAG-3'; Tm 60.3 ^{o}C). Each PCR reaction was carried out in 25 µL containing 1 µL (not quantified, estimated approximately 10 ng) of genomic DNA, 600 nM of the 5' and 3' primers, and reagents from the FastStart Taq DNA Polymerase Kit- PCR Reaction system (Roche) including 2 units of *FastStart Taq* DNA polymerase. The reactions were initiated at 95^{o}C for 3 minutes and then sequentially cycled 28 times at 93^{o}C for 30 seconds [*denaturing*], 61^{o}C for 30 seconds [*annealing*], and 72^{o}C for 45 seconds [*extension*], followed by

a final extension at 72°C for 10 minutes. PCR products were separated by electrophoresis on a 1.2% agarose gel to identify the presence or absence of the 293 bp transgene product.

Floxed Estrogen Receptor α

Animals homozygous for *loxP* sites flanking Exon 3 of ERα were identified by PCR using primers designed to align with sequences of the ERα gene that were directly outside the inserted *loxP* sites (see Figure 3) (sense 5'-TGGGTTGCCCGATAACAATAAC-3', Tm 55.5 °C; antisense primer 5'-AAGAGATGTAGGGCGGGAAAAG-3', Tm 56.8 °C; (74)). Each PCR reaction was carried out in 25 μL containing 1 μL (not quantified, estimated approximately 10 ng) of genomic DNA, 3.3 ng/μL of the 5' and 3' primers, and reagents from the FastStart Taq DNA Polymerase Kit- PCR Reaction system (Roche) including 2 units of *FastStart Taq* DNA polymerase. The reactions were initiated at 94°C for 3 minutes and then sequentially cycled 31 times at 94°C for 45 seconds [*denaturing*], 58°C for 45 seconds [*annealing*], and 72°C for 2 minutes and 20 seconds [*extension*], followed by a final extension at 72°C for 5 minutes. PCR products were separated by electrophoresis on a 1.2% agarose gel to identify the presence or absence of 1280 bp $ER\alpha^{fl/fl}$ (homozygous) product, 1200 bp $ER\alpha^{o/o}$ (wild type) product, and two products at 1280 and 1200 bp for $ER\alpha^{fl/o}$ (heterozygous).

RNA Extraction and RT-PCR

Animals were sacrificed by rapid decapitation, and a full necropsy performed. Tissues were stored at -80°C until RNA was extracted.

RNA Extraction

Tissue samples were homogenized in 500 µL (small tissues, i.e. anterior pituitary) to 1 mL (larger tissues, i.e. mammary gland) of TRIzol Reagent. Following a 5-minute at room temperature, 0.2 mL of chloroform per 1 mL of TRI reagent was added, tubes shaken for 15 seconds, then incubated at room temperature for 2 to 3 minutes. Samples were then centrifuged at 12,000 x g for 15 minutes at 4^{o}C. The aqueous phase, containing the RNA, was then transferred to a new, clean tube. RNA was precipitated by adding 0.5 mL of isopropyl alcohol per 1 mL of TRI reagent used for initial homogenization. Samples were incubated at room temperature for 10 minutes then centrifuged at 12,000 x g for 10 minutes at 4^{o}C. The RNA precipitates as a gel-like pellet at the bottom of the tube. The supernatant was removed and the pellet washed with 75% ethanol. The pellet was then briefly dried, removing excess ethanol with a sterile cotton applicator without disturbing the RNA pellet. The RNA was dissolved in RNase-free water and incubated for 10 minutes at 55^{o}C. RNA samples were stored at -80^{o}C until used for reverse transcription.

Reverse Transcription

Samples were quantitated on a spectrophotometer, and 1 µg of sample RNA was reverse transcribed to produce complementary DNA (cDNA) using the QuantiTect Reverse Transcription Kit (Qiagen). To the volume equivalent to 1µg of RNA, 2 µL of *gDNA wipeout* buffer was added to eliminate genomic DNA contamination. A volume of RNase-free water was

added to the sample to bring the total volume to 14 μL. Samples were incubated at 42°C for 2 minutes and then immediately placed on ice for 2 additional minutes. To each reaction, 4 μL of *Quantiscript* RT buffer, 1 μL of RT *dNTP mix*, and 1 μL of *Quantiscript reverse transcriptase* were added for a final volume of 20 μL. Samples were incubated at 42°C for 20 minutes, 95°C for 3 minutes, then stored at 4°C until used for PCR. Negative control reactions (RT-) were handled in an identical manner except that distilled water was substituted for the reverse transcriptase.

Cre-recombinase transcription was determined by PCR on the cDNA from tissues using the same primers and thermocycler conditions designed for genotyping [see Genotyping: Cre Recombinase].

DNA Extraction from Animal Tissues

Animals were sacrificed by rapid decapitation, tissues dissected free, and stored at -80°C. DNA was extracted using the High Pure PCR Template Preparation Kit and protocol for "Isolation of Nucleic Acids from Mammalian Tissue" (Roche, Indianapolis IN).

Assessment of homologous recombination and deletion of ERα was performed by PCR on DNA extractions from various tissues, using the same primers and thermocycler conditions designed for genotyping for the floxed ERα (see above). PCR products were separated by electrophoresis on a 1.2% agarose gel. In the absence of homologous recombination, only a 1280 bp product

was generated from tissues of ERα$^{fl/fl}$ mice. If homologous recombination occurred and Exon 3 of ERα spliced out, a 600 bp product was generated.

Primary Cell Dissociation

Wild type C57/Bl6 mice and ERα$^{fl/fl}$- pitPRL-Cre^{+} mice were sacrificed by rapid decapitation and the anterior pituitaries dissected and immediately placed in a sterile 35mm Petri dish containing 2 to 3 mL of sterile Hank's Balanced Salt Solution [HBSS; 1 L solution containing 9.52 g of HBSS (Sigma 6136), 10 mg/mL Penicillin-Streptomycin, 0.35 g $NaHCO_3$ (Sigma S-8875), 5.96 g Hepes-HCl (Sigma H-3375), pH 7.2, Osmolarity 295-305 mOs/L). The tissue was then transferred to a second Petri dish containing fresh HBSS. Using sterile forceps and a sterile scalpel, the glands were minced into small pieces (≤1mm^3). Fragments were transferred to a 15 mL centrifuge tube using a sterile, fire-polished, siliconized pipet. Fragments were settled by centrifuging at 150 x g for 4 minutes. Medium was manually aspirated off without disturbing the tissue fragments. Fragments were washed with 5 mL of fresh HBSS, two more times. Trypsin (Sigma T-1426) was weighed out and dissolved in 5 mL of HBSS for a final 0.2% concentration. The trypsin solution was passed through a sterile 0.22 μm pore filter unit into the sterile tube containing the washed tissue fragments. The tube was then sealed and submerged in a 37^{o}C water bath and shaken gently for 20 to 25 minutes. After incubation, 5 mL of sterile Hanks Calcium, Magnesium Free solution [Hank's-CMF; 1 L solution containing 9.52 g HBSS without calcium and magnesium (Sigma H-2387), 10 mg/mL Penicillin- Streptomycin, 0.35 g $NaHCO_3$, 5.96 g Hepes-HCl, pH 7.2, Osmolarity 295-305 mOs/L] was added to the tube and the tube was centrifuged at 150 x g for 4 minutes. The medium was manually aspirated off and replaced by

10 mL of fresh Hank's-CMF, repeating two more times for additional washing. Fragments were then resuspended in 2 mL Hank's-CMF with 75 µL of DNase and trypsin inhibitor. Gentle trituration using a sterile, fire-polished and siliconized Pasteur pipet was performed for 2 to 3 minutes to disperse the cells. Eight mL of Hank's-CMF was then added and the tube centrifuged at 150 x g for 4 minutes to pellet the cells. The medium was removed and the cells were resuspended in 1 to 2 mL of Hank's-CMF. Cells were separated from any remaining tissue fragments by passing the suspension through a sterile 20-µm pore nylon mesh. Cells were counted and viability determined. Normal cell yield ranged between 100,000 and 300,000 cells per pituitary, and typical viability achieved was 95% or greater.

Fluorescence Activated Cell Sorting (FACS)

Anterior pituitaries were dissociated as described above. Media was prepared without phenol red. Cells, filtered and suspended in 2 mL of Hank's-CMF, were added to 2 mL of 10% DMES culture media (500 mL solution containing 4.37 grams DMEM phenol red free (Sigma), 1.85 grams NaHCO3, 2.98 grams Hepes-HCl; pH 7.35-7.42; osmolarity 295-305 mOs; 10% horse sera; 4mg/mL gentamycin) without phenol red in a culture dish, and incubated at $37^{o}C/CO_2$ for two hours. After incubation, 1 mL of Buffer B (Earle's balanced salt solution with 25 mmol Hepes/L; pH 7.2, 4 mg BSA/ml) was added to the cells. The solution was then transferred to a sterile culture tube. The culture dish was then washed with 1-2 mL of Buffer B and added to the culture tube in order to collect any residual cells. Cells were centrifuged at 150 x g for 4 minutes. The supernatant was removed and the cells were then washed twice with 7 mL Buffer

B. After the final wash supernatant was removed, anti-mouse prolactin antibody (α-rat PRL, rabbit IgG; AFP-131078; National Hormone and Peptide Program; 1:500 in Buffer B) was added. The cells were transferred to a culture dish and incubated for 1 hour and 15 minutes at $37^oC/CO_2$. After incubation, 1 mL of 10% DMES culture media was added to the dish and the solution, with cells, was transferred to a sterile culture tube. The culture dish was washed with an additional 1-2 mL of DMES and added to the cell suspension. This suspension was then centrifuged at 150 x g for 4 minutes and the supernatant removed. Culture media was added again and the cells centrifuged two additional times for washing. After the final wash, the supernatant was removed and the secondary antibody (goat anti-rabbit FITC; Sigma F0382; 1:25) was added and the cells incubated for 1 hour and 15 minutes at $37^oC/CO_2$. After incubation, they were washed twice with Hank's-CMF and resuspended in a final volume of 1 mL Hank's-CMF. The solution was then taken to the Flow Cytometry Core at the Shriners Hospital where the cells were analyzed.

Reverse Hemolytic Plaque Assay (RHPA)

Preparation of RBCs

For the RHPA, ovine erythrocytes (oRBCs; Colorado Serum Company) were coated with Staphylococcus protein A. Briefly, one mL of packed oRBCs was washed three times with saline. After a final centrifugation (800 x *g*; 10 min), the supernatant was removed and the RBCs resuspended in 5 mL sterile saline in a 15-ml conical centrifuge tube.

The centrifuge tube was then wrapped in foil in order to protect the mixture from light exposure. To the 5 mL oRBC suspension, 5 mL of aged (> 2 months) chromium chloride solution (0.2 mg/mL) and 1 mL staph protein A solution (0.5 mg/mL) was added and mixed well. The centrifuge was sealed and then submerged in a 30 ^{o}C shaking water bath for one hour. After incubation, the mixture was spun at 800 x g for 10 minutes and the supernatant discarded. The oRBCs were washed twice in 10 mL of sterile saline. After the second wash, the oRBCs were resuspended in 10 mL of sterile DMEM/BSA [500 mL solution containing 4.14 g DME modified (Sigma D-2902), 1.85 g NaHCO3 (Sigma S-8875), 0.5 g BSA Fraction V (Sigma A-8022), 2.98 g Hepes-HCl (Sigma H-3375); pH 7.3, 295-305 mOsm/L]. The suspension was spun at 800 x g for 10 minutes, and this was repeated for a total two washes with DMEM/BSA. The oRBCs were then resuspended in 50 mL of DMEM/BSA and stored in a foil wrapped, sterilized, siliconized bottle as a 2% (vol/vol) suspension at 4 ^{o}C for no longer than one week before use.

Preparation of Chambers

Cleaned, small round coverslips (13 mm) were incubated in 0.1N HCl overnight. Prior to the plaque assay, the coverslips were rinsed briefly in EtOH, then soaked in poly-L-lysine (Sigma P-9155; 0.25 mg/ml) for 5 minutes, then rinsed with water and set to dry. While drying, chambers were constructed on clean microscope slides. Strips of double-sided adhesive tape were positioned across a microscope slide, parallel to each other and separated by 10mm. When the coverslips were dry, they and the microscope slides were UV sterilized. The coverslips were then placed onto the chambers, such that each side of the coverslip slightly overlapped and held

to the adhesive tape. The completed chambers were then incubated under UV light for further sterilization.

Plaque Assay

Anterior pituitaries were collected and dissociated as described above. Equal volumes of pituitary cell suspension (150,000 cell/mL) and coated oRBCs (6% vol/vol in DMEM/BSA) were mixed. The mixture was then infused into chambers, which were immediately inverted and incubated for 30 minutes at 37°C/CO_2 to allow cells to attach to the coverslip. Unattached pituitary cells and oRBCs were rinsed from the chambers with DMEM/BSA and then the chambers were infused with primary rabbit anti-rat PRL antibody (αrPRL-86, final concentration 1:200; *see Ho,M.Y. et al.*), inverted, and incubated for 2 hours at 37°C/CO_2. Chambers were again washed with DMEM/BSA to remove unbound antibody. Guinea pig compliment at a dilution of 1:40 with DMEM/BSA was infused, chambers inverted, and incubated at 37°C/CO_2 until clear plaques began to form (~30 minutes). Chambers were then disassembled and coverslips were transferred to a 12-well plate and cultured in 10% DMES at 37°C/CO_2.

Single Cell RT-PCR

Single Cell Collection

Lactotroph cells identified by the RHPA as described above, were collected in heat sterilized (400°F) glass microelectrodes operated with a micromanipulator. Microelectrodes were

fabricated on a Sutter puller (pulled at temperature: 495-500, pressure: 700, velocity: 30). Coverslips containing the plaques were submerged in 1x PBS in a chamber on the microscope. Once plaques were identified, pipette tips were backfilled with 1x PBS and the cells were pulled up into the tip containing the solution. The tips were then broken into a sterile PCR tube in order to ensure collection of the cell. The cell was then suspended in 6 μL of RNase-free water and samples were stored at -80^{o}C until RT-PCR analysis.

RT-PCR

Cell samples were reverse transcribed as described above, using half reaction volumes (6 μL RNA in water, 1 μL gDNA wipeout buffer, 2 μL RT buffer, 0.5 μL primer mix, 0.5 μL reverse transcriptase).

The presence or absence of transcripts in each single cell sample was assessed with PCR as described previously, using nested reactions. Initiation for each occurred at 94^{o}C for 2 minutes, then sequential cycles a set amount of times (as listed) at 94^{o}C for 45 seconds [*denaturing*], an optimal temperature as listed [*annealing*] for 45 seconds, and 72^{o}C for 45 seconds [*extension*] followed by a final extension at 72^{o}C for 5 minutes. The accession number for the gene mRNA sequence, corresponding base pairs, optimal annealing temperatures, and cycle numbers for each primer set were as follows: mouse PRL outside (NM_011164.2; base pair 29-47; sense 5'-GGAGAAGTGTGTTCCCAGC-3', Tm 55.8 ^{o}C; base pair 645-662 antisense 5'-CAGCGAATGGTGTTGCGC-3', Tm 58^{o}C; 57^{o}C annealing temperature; 26 cycles) followed by mouse PRL inside (NM_011164.2; base pair 64-84; sense 5'-AACAGCCAGGGGTCAGCCCA-

3', Tm 64.3°C; base pair 389-408 antisense 5'-CTGCACCAAACTGAGGATCA-3', Tm 55 °C; 60°C annealing temperature; 30 cycles); mouse ERα outside (NM_007956.4; base pair 181-200 sense 5'-CATGACCATGACCCTTCACA-3', Tm 54.8 °C; base pair 803-822 antisense 5'-TTAAAGAAAGCCTTGCAGCC-3', Tm 53.8 °C; 54°C annealing temperature; 40 cycles) followed by mouse ERα inside (NM_007956.4; base pair 625-644 sense 5'-CGGCCCTCCCGCCTTCTACA-3', Tm 64.2 °C; base pair 739-758 antisense 5'-TCGACACGGCACAGTAGCGA-3', Tm 61.9 °C; 63°C annealing temperature; 45 cycles); mouse glycoprotein α subunit outside (NM_009889.2; base pair 130-151 sense 5'-TCTGGTCATGCTGTCCATGT-3', Tm 56.3 °C; base pair 395-414 antisense 5'-TTCTCCACTCTGGCATTTCC-3', Tm 54.8 °C; 55°C annealing temperature; 26 cycles) followed by mouse glycoprotein α subunit inside (base pair 274-293 sense 5'-TTGCTTCTCCAGGGCATATC-3', Tm 54.7 °C; antisense is that used for the outside reaction; 55°C annealing temperature; 30 cycles).

Chapter 2

RESULTS

Generation of ERα KO Mice

From the fertilized eggs injected with the pitPRL-Cre construct and implanted into pseudopregnant females, two of the pups delivered were positive for the transgene in their genomic DNA. These two founders were bred with wild type C57-Bl6 mice and did pass the transgene to their offspring, thereby producing two independent lines of pitPRL-Cre$^+$ mice. As shown in Figure 1A, animals carrying the transgene (Tg1, Tg2) had a PCR product of 259 bp, while PCR of DNA obtained from a wild type (WT) animal generated no product.

To verify transcription of the transgene, RNA was extracted from various tissues of pitPRL-Cre$^+$ animals. The RNA was reverse transcribed and the resulting cDNA subjected to PCR analysis using the Cre specific primers. Only cDNA from APs of pitPRL-Cre$^+$ mice generated a PCR product of the correct size (259 bp) (Figure 1B). This product was much more abundant in the female AP than in the male AP, which is most likely due to the greater amounts of E_2 in females driving the pituitary PRL promoter. Other tissues from these mice were negative for Cre recombinase (products for GAPDH were generated demonstrating the integrity of the RNA preparations), demonstrating that expression of the transgene was restricted to the AP.

In order to obtain mice with a selective deletion of ERα, it was necessary to cross pitPRL-Cre$^+$ mice with mice in which the ERα gene was floxed. A line of mice in which exon 3 of the ERα gene was flanked by loxP sites (floxed ERα), had been generated in the laboratory of Dr. Sohaib

Khan (University of Cincinnati) and a homozygous floxed ERα animal was obtained from Dr. Khan. Genotyping this line of mice used PCR primers complementary to ERα gene sequences outside of the loxP sites (*see* Figure 2A), designed so that a WT ERα gene, with no loxP sequences inserted, would generate a PCR product of 1200 bp. A floxed ERα gene, with the addition of the two loxP sequences, would generate a larger product of 1280 bp (Figure 2A). Depending upon the genotype of the animals, three different patterns of PCR products could be observed (Figure 2B). Genomic DNA from homozygous WT (0/0) mice had a single 1200 bp PCR product, while that from homozygous floxed ERα (fl/fl) mice had a single 1280 bp product. A heterozygous mouse in which one allele for ERα was floxed and the other allele was not (fl/0), had PCR products of both sizes.

Verification of targeted ERα deletion

The floxed ERα mouse from the Khan lab was mated with pitPRL-Cre^{+} mice to generate offspring with deletion of ERα targeted to pituitary lactotrophs (ERα$^{fl/fl}$- pitPRL-Cre^{+}). Cre recombinase expressed in any cell catalyzes the recombination of the two loxP sites, splicing out Exon 3 of the ERα gene, thereby shortening the DNA sequence between the two primer sites and resulting in a 600 bp PCR product (*see* Figure 2A). The presence of this 600 bp product in PCR analysis of DNA extracted from the AP of mice genotyped as ERα$^{fl/fl}$- pitPRL-Cre^{+}, demonstrated that recombination and excision of Exon 3 had occurred (Figure 2C, *lane 1*). APs from mice that had floxed ERα without Cre (ERα$^{fl/fl}$- pitPRL-Cre$^{0/0}$;Figure 2C, *lane 2*) or Cre expression without ERα being floxed (ERα$^{0/0}$- pitPRL-Cre^{+};Figure 2C, *lane 3*) did not have recombination as demonstrated by the absence of the 600 bp product.

Although recombination had occurred in the AP of the ERα$^{fl/fl}$- pitPRL-Cre^{+} mouse, two other PCR products were observed: a 1280 bp product, representing floxed ERα genes that did not undergo homologous recombination, and an intermediate product of approximately 940 bp. Both of these products were also generated in PCR of mammary gland (MG) DNA obtained from a ERα$^{fl/fl}$- WAP-Cre^{+} mouse from Dr. Khan's lab (Figure 2C, *lane 4*). This animal had Cre recombinase expression targeted to MG epithelial cells using the whey acidic protein (WAP) promoter (74).

Unsure of what the 940 bp product could be, this band was extracted from the gel for sequencing. The amount of DNA recovered was not enough for sequencing so it was used as the template for an additional PCR amplification. However, this only produced the 600 bp product, so we concluded that the intermediate product was a hybrid of the 600 and 1280 bp products that were generated during the first PCR amplification.

Due to the large amount of background (i.e., 1280 bp product) from the floxed ERα product present in other pituitary cell types, ERα$^{fl/fl}$- pitPRL-Cre^{+} mice were crossed onto a mouse PRL knockout (PRL$^{-/-}$) background. PRL$^{-/-}$ mice do not synthesize biologically active PRL, which removes the feedback stimulus to the TIDA neurons (76). Subsequently, TIDA synthesis and secretion are dramatically lowered. With the loss of DAergic inhibitory tone, lactotrophs begin to proliferate and eventually form large pituitary tumors (6, 30, 77). With the increased ratio of lactotrophs to other ERα-positive cell types, DNA from APs of the ERα$^{fl/fl}$- pitPRL-Cre^{+} on the PRL$^{-/-}$ background were analyzed in order to obtain a more intense recombinant signal. As shown in Figure 3 (*lane 9*), a band at 600 bp was generated from these APs that was much more

intense relative to the 1280 bp product than had been generated from APs on the WT PRL background (see Figure 2C, *lane 1*). In fact, using DNA from glands with much greater numbers of lactotrophs also eliminated the generation of the intermediate PCR products of ~940 bp. No evidence of recombination was found in APs or other tissues from floxed ERα mice that did not express the transgene for Cre ($ER\alpha^{fl/fl}$- pitPRL-$Cre^{0/0}$; Figure 3, *lanes 2-5*). Moreover, the ERα deletion only occurred in the AP of $ER\alpha^{fl/fl}$- pitPRL-Cre^{+} mice and not in other ERα-expressing tissues such as the mammary gland (MG), uterus (Ut) or ovary (Ov) (Figure 3, *lanes 6, 7 and 8*, respectively).

Fluorescence activated cell sorting

Homologous recombination was demonstrated to occur only in the APs of $ER\alpha^{fl/fl}$- pitPRL-Cre^{+} mice. However, it was necessary to verify that this was occurring only in the pituitary lactotrophs and not other cell types such as the gonadotrophs and corticotrophs, which also express ERα. To accomplish this, a purified preparation of lactotrophs was required for PCR analysis. Fluorescence activated cell sorting (FACS) was performed on dissociated AP cells of WT mice to determine if this technique could produce the required purification. Dissociated AP cells were labeled by incubation with a PRL-specific antibody followed by a FITC-conjugated secondary antibody, and then were sorted. In this initial sorting, two peaks of signal were detected, representing the unlabeled (non-lactotrophs) and labeled cells (lactotrophs). However, there was not a clean separation of these two populations, but rather a considerable overlap of the unlabeled and labeled cells (Figure 4B). After isolating and discarding the cells in the

ambiguous region of overlap, each population was resorted. While the second sorting of the unlabeled cells demonstrated little or no contamination by labeled cells (Figure 4C), the same was not true for the labeled, and presumed lactotroph, population (Figure 4D). The presence of a small, but significant, population of cells falling closely to the "labeled" region indicated that there was measurable contamination of other cell types in the lactotroph population. Therefore, this method only enriched and did not completely purify the lactotroph cells. Since even small amounts of contaminating DNA would amplify exponentially in the PCR analysis, this enrichment was deemed unsuitable for the definitive demonstration that ERα was knocked out only in lactotrophs.

Reverse Hemolytic Plaque Assay and Single Cell RT-PCR

In order to obtain pure lactotroph cell samples to analyze for verification of the selective deletion of ERα, single cell RT-PCR was combined with the reverse hemolytic plaque assay (RHPA). Plaque-identified lactotrophs from dissociated APs of WT mice, control $ER\alpha^{fl/fl}$- pitPRL-$Cre^{0/0}$ mice, and experimental $ER\alpha^{fl/fl}$- pitPRL-Cre^{+} mice, were collected as single, individual cell samples using a glass micropipette (Figure 5*A*). RNA from each cell was reverse transcribed and analyzed with PCR using primers designed against unique sequences in the prolactin (PRL) gene (to specifically identify lactotrophs); the ERα gene (to determine the presence or absence of the receptor in these cells); and the α-glycoprotein hormone (mGPHα) subunit, specific to gonadotrophs and thyrotrophs, as a negative control. Results showed this technique to prove sufficient, and a signal was able to be seen using single cell collections (Figure 5B-D). Also,

plaque identified lactotrophs showed a positive signal for PRL and no signal for mGPHα (Figure 5C-5D), showing this technique was efficient in obtaining purified lactotroph samples.

Table 1. Percent of PRL-positive lactotrophs with detectable transcript for ERα in single-cell RT-PCR.

Genotype	**% Lactotrophs expressing ERα transcript**
$ER\alpha^{fl/fl}$-pitPRL-$Cre^{0/0}$	37
$ER\alpha^{fl/fl}$-pitPRL-Cre+	6

Table 1 shows percent expression of ERα in PRL-positive lactotroph samples. When beginning this analysis, multiple lactotrophs were pooled together in order to increase the amount of RNA to analyze by RT-PCR. Since there is little known about the expression level of ERα in the lactotroph population in mice and a good possibility of cell-to-cell variability, single one cell samples were collected and individually analyzed for PRL and ERα. Table 1 is an overall analysis of the pooled and single cell samples that were positive for PRL expression (lactotrophs) and ERα. Expression in $ER\alpha^{fl/fl}$- pitPRL-$Cre^{0/0}$ control mice was not as high as expected, but again there is little known about the expression levels of ERα in the lactotroph population in mice. There was also a low level of transcript found in the $ER\alpha^{fl/fl}$- pitPRL-Cre^{+} mice, contrary to what would be expected if ERα was genetically deleted in the lactotrophs of these animals. Non-plaque forming cells showed no PRL product, with some expression of mGPHα and ERα (data not shown).

Chapter 2

DISCUSSION

Mice containing the deletion of ERα targeted to pituitary lactotrophs were successfully generated. A transgene, using the full-length (3,172 bp) rat PRL promoter to drive expression of Cre recombinase in lactotroph cells, was successfully inserted into the mouse genome, resulting in a working transgenic line of mice, either Cre^{+} or $Cre^{0/0}$, as shown through PCR analysis of genomic DNA. Expression of the transgene was also demonstrated in RT-PCR analysis of isolated RNA from mouse APs. It has been demonstrated, both in cell lines and in transgenic mice that the full-length PRL promoter targets transgene expression exclusively to pituitary lactotrophs, with no break-through expression in somatotrophs or thyrotrophs, which arise from the same cell lineage (78). The line of Cre^{+} mice was then bred onto the floxed ERα background, and healthy offspring were generated.

Numerous studies have shown that ERα is the primary mediator of E_2 action on PRL regulation, at both the level of the lactotroph and the level of the TIDA neuron (41, 55). ERβ has not been found in lactotrophs of the mouse (although it has been demonstrated in rat lactotrophs), and ERβ expression is low in the hypothalamus and has not been demonstrated in TIDA neurons (51, 66). Therefore, we targeted ERα for deletion in our efforts to create an experimental model in which to study the direct and indirect actions of E_2 in the regulation of PRL. The $ERα^{fl/fl}$-pitPRL-Cre^{+} mice, identified through genotyping, represented the needed model with the selective deletion of ERα in the pituitary lactotrophs.

The Cre/loxP system has been shown to effectively result in homologous recombination and gene deletion in target tissues and cell types, and this is characterized by a smaller recombinant product when analyzing the DNA from these tissues and cells (53, 72, 74). Using the floxed ERα genotyping primers on DNA extracted from various tissues from these animals, homologous recombination was shown to occur only in the AP and not in any other reproductive tissues, as confirmed by the presence or absence of a 600 base pair PCR product. This product was also generated from MGs of floxed ERα mice expressing the Wap-Cre transgene, generated in the Khan lab, indicating that homologous recombination was successfully occurring in the tissue of interest (74).

The AP is composed of a heterogeneous mixture of different cell types, several of which (lactotrophs, somatotrophs, gonadotrophs and corticotrophs), express ERα (36). As a result, if the ERα deletion was correctly targeted to only the lactotrophs, then there would be considerable "background" of the undeleted floxed ERα gene. This is evident in the PCR analysis of AP DNA shown in Figure 2 (*lane 1*). The 1280 base pair band generated from intact floxed ERα is much more intense than the 600 base pair band representing the recombinant product, and should be due to the background signal from other ERα-expressing pituitary cell types. The 1280 bp product is also evident in PCR of MG DNA from $ER\alpha^{fl/fl}$- Wap-Cre^{+} mice (Figure 2, *lane 4*), although it is less intense than the 600 bp recombinant band. This can be explained by the fact that while some interstitial cells of the MG express ERα, the majority of the cells expressing ERα are the epithelial cells, which are the cells of Wap expression. Thus, in that model (MG), recombination occurs in the majority of ERα-expressing cells; while in our model (AP),

recombination occurs in a minority of ERα-expressing cells. This interpretation is supported by the DNA analysis of $ER\alpha^{fl/fl}$- pitPRL-Cre^{+} mice bred onto the mouse PRL-knock-out ($PRL^{-/-}$) background. The lactotrophs of $PRL^{-/-}$ mice proliferate to eventually form large tumors (77). This proliferation is a result of decreased DAergic inhibition of lactotrophs that occurs in the absence of PRL stimulation of TIDA neurons (79). The relative increase in lactotroph cell number, without a concomitant increase in the numbers of other pituitary cell types, resulted in a much greater recombinant (600 bp) product relative to the 1280 bp product (Figure 3, *lane 9*).

Analysis of various tissues demonstrated that in the $ER\alpha^{fl/fl}$- pitPRL-Cre^{+} mice recombination occurred only in the AP of these animals (Figure 3) and the effect of the $PRL^{-/-}$ background on the relative levels of recombination in the AP was consistent with targeted deletion in the lactotrophs. However, this was not a direct demonstration and it remained to be shown that ERα deletion occurred only in the lactotrophs of the $ER\alpha^{fl/fl}$- pitPRL-Cre^{+} mice. In order to do this, it was necessary to purify the lactotroph population or separate it from the other cell types of the AP.

Fluorescence-activated cell sorting (FACS) has been used previously to isolate a homogeneous cell population and sort dispersed cells based on size, granularity, and cell surface fluorescence (80). In this study, FACS was used on dispersed anterior pituitary cells from WT animals to evaluate whether this method would provide a pure population of lactotrophs to analyze for the presence or absence of ERα in the experimental animals. Results showed only enrichment of the lactotroph population with a small amount of contamination of other cell types. Other studies have shown this method to nearly purify, but not to 100% (81). Because other cell types in the

AP express ERα, any amount of contamination would produce equivocal findings when characterizing the selective ERα knockout mice.

Many studies have used the reverse hemolytic plaque assay (RHPA) to determine PRL gene expression in individual lactotrophs and their secretion, as well as electrophysiological characterization of lactotrophs (82, 83, 84). This method identifies cells by their secretory product. Thus, using a PRL-specific antibody, only the PRL-secreting cells formed plaques (85). This unequivocal identification of single lactotrophs enabled collection of pure, single cell samples for RT-PCR analysis. This method of cell collection eliminated contamination seen with FACS, and it also allowed for individual cells to be screened for a variety of different genes, most importantly ERα. A minority of lactotrophs from WT and control $ERα^{fl/fl}$- pitPRL-$Cre^{0/0}$ animals have shown expression for ERα. To this respect, there is little known regarding the percent expression of ERα in the AP cell population of the mouse. A study by Mitchner *et. al.*(100) used immunocytochemistry and *in situ* hybridization methods to compare expression of ERα and ERβ by pituitary cells in the rat, showing 47% expression of ERα in lactotrophs. However, expression in the mouse pituitary may be very different, especially with ERβ negligible in this species.

There was a low level of ERα transcript found in the $ERα^{fl/fl}$- pitPRL-Cre^{+} mice, contrary to what would be expected if ERα was genetically deleted in the lactotrophs of these animals. However, lactotrophs expressing the receptor were obtained from young mice approximately 3 months of age. The PRL promoter driving the expression of the Cre transgene is regulated by circulating E_2 (Chapter 2, Figure 6), and because these mice were sacrificed at a young age, Cre efficiency

was most likely not optimal in order to completely delete ERα in the entire lactotroph population. This is discussed further in Chapter 3.

To summarize, mice carrying the selective deletion of ERα in pituitary lactotrophs were successfully generated providing a new selective knockout model in which to study the regulation of PRL by E_2. However, further investigation is needed to determine the conditions required for complete deletion in all lactotrophs. Also, investigation is needed to determine any changes in E_2 responsiveness of these animals, as will be determined by *in vitro* as well as *in vivo* studies.

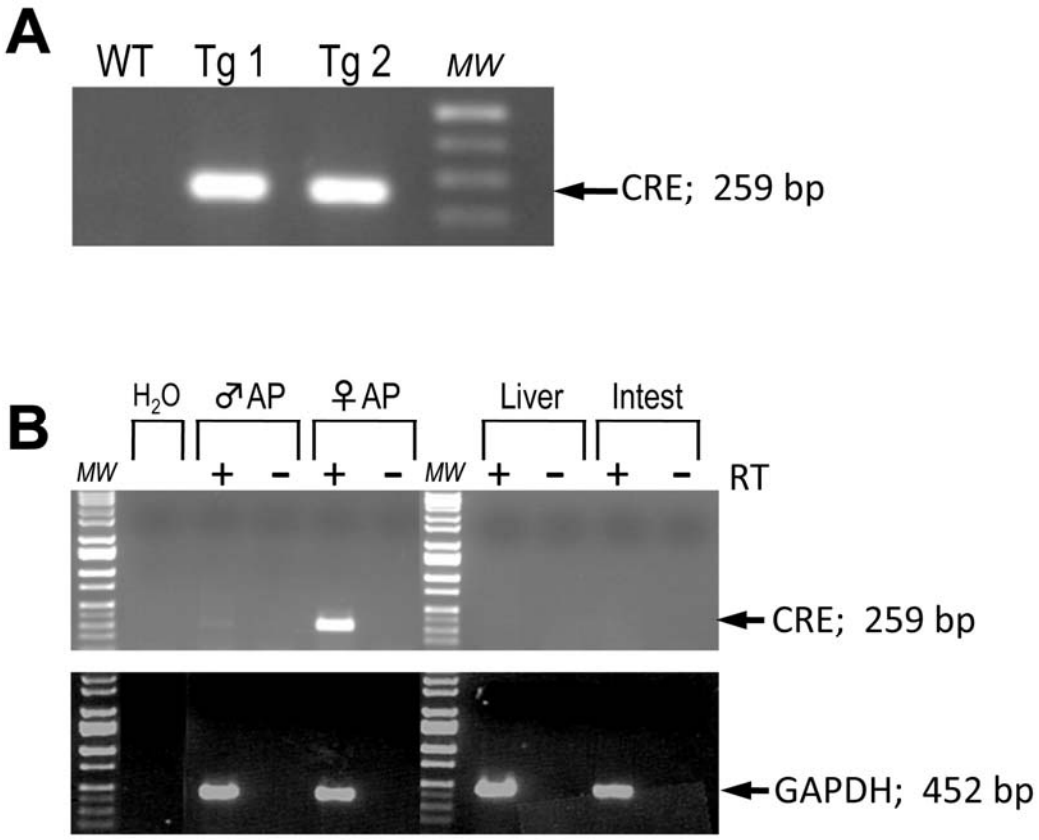

Figure 1. Genotyping and tissue expression of Cre recombnase.

A. Genotyping: PCR analysis and gel electrophoresis of genomic DNA extracted from mouse tail clippings. Mice carrying the Cre recombinase transgene (Tg 1 and Tg 2) produced a band at the primer-specific size of 259 bp. Wild type (WT) mice had no product. ***B.*** Tissue expression of Cre recombinase transgene: RNA extracted from various tissues of Cre positive mice was reverse transcribed (+) and the cDNA subjected to PCR analysis using the same primers as used for genotyping. Negative controls, with the reverse transcriptase (RT) omitted (-) were run to verify that samples were not contaminated with genomic DNA. Only anterior pituitary (AP) cDNA, from either male or female mice, generated the 259 bp PCR product, while other tissues (liver and intestine shown here) did not. A PCR product (452 bp) using mGAPDH-specific primers could be amplified in all samples, demonstrating the integrity of the RNA preparation.

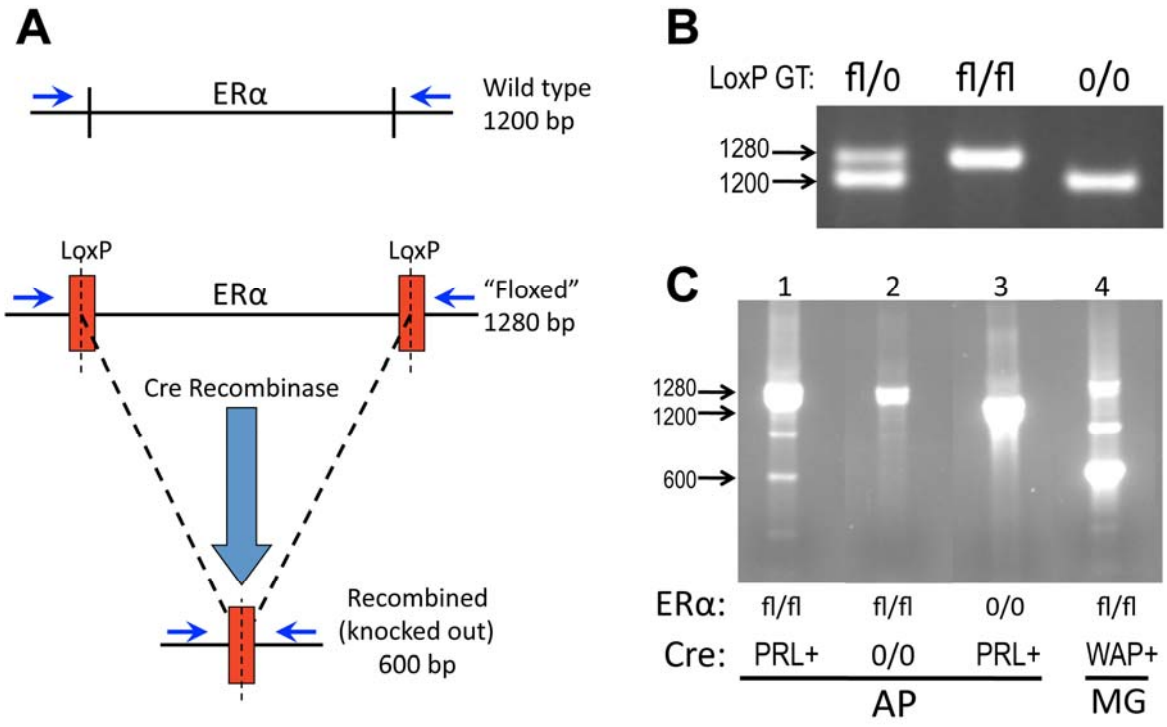

Figure 2. Generation of mice with targeted deletion of ERα in lactotrophs.

A*.** Scheme for targeted disruption of the ERα gene: Exon 3 of the ERα gene was flanked by loxP sites in all tissues and cell types in one line of mice. When Cre recombinase is expressed, homologous recombination occurs, splicing out Exon 3. The horizontal arrows indicate the primer annealing sites. ***B*.** Genotyping of floxed ERα mice: Genomic DNA extracted from tail clips were analyzed by PCR using the primers illustrated in ***A. Wild type (0/0) DNA generated one product of 1200 bp. Homozygous floxed (fl/fl) ER-alpha DNA generated a single product of 1280 bp. Heterozygous DNA (fl/0) produced two PCR bands – 1200 and 1280 bp. ***C*.** Demonstration of recombination: DNA was extracted from tissues and subjected to PCR using the primers as illustrated in ***A***. *Lane 1:* AP DNA of a mouse homozygous for floxed ERα and positive for the Cre transgene driven by the PRL promoter had a 600 bp product, demonstrating homologous recombination of the ERα gene (see diagram in A); *Lane 2:* a single 1280 bp product was generated from the AP of a mouse homozygous for floxed ERα and negative for Cre recombinase; *Lane 3:* a single 1200 bp product was generated from WT AP DNA; *Lane 4:* mammary gland DNA from a mouse homozygous for floxed ERα and positive for Cre recombinase driven by the WAP promoter also had a 600 bp product.

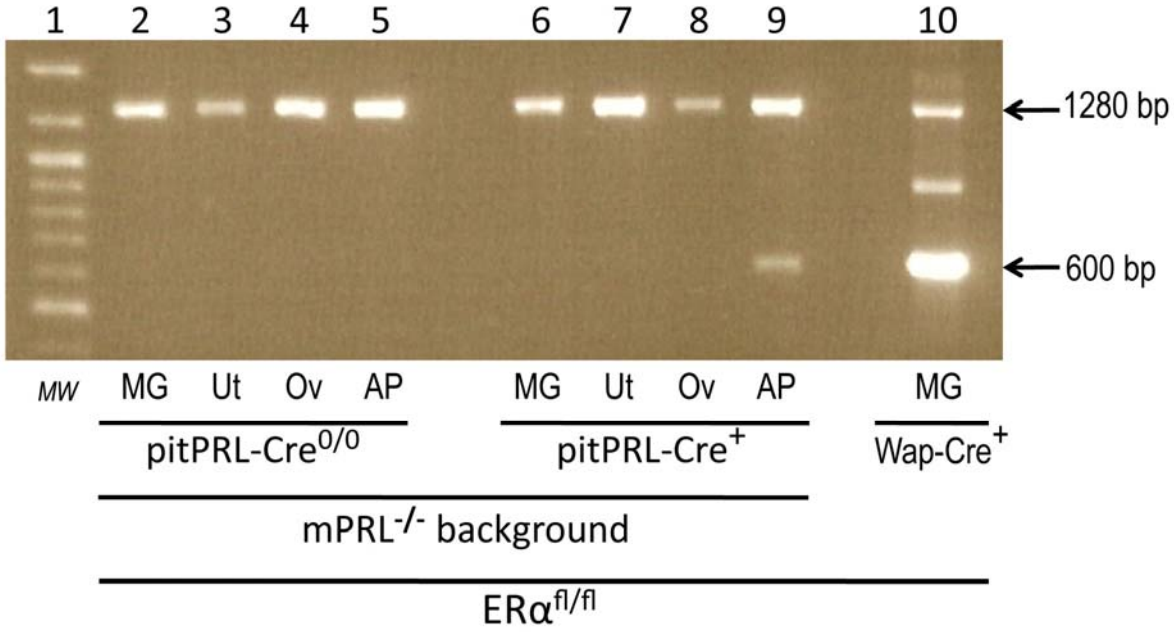

Figure 3. Homologous recombination occurs only in the AP of ERα$^{fl/fl}$- pitPRL-Cre^{+} mice.

DNA was extracted from various tissues of mice that were negative (pitPRL-Cre$^{0/0}$) or positive (pitPRL-Cre^{+} or Wap-Cre^{+}) for Cre recombinase transgene. All animals were homozygous for floxed ERα and all animals, except the Wap-Cre^{+} mouse, were on the mPRL knock-out background. The 600 bp product is present only in the AP of pitPRL-Cre^{+} mice (*lane 9*) and the mammary gland (MG) of Wap-Cre^{+} mice (*lane 10*). In the pitPRL-Cre^{+} mice, homologous recombination does not occur in other ERα expressing tissues such as MG, uterus (Ut) and ovary (Ov) (*lanes 6-8*).

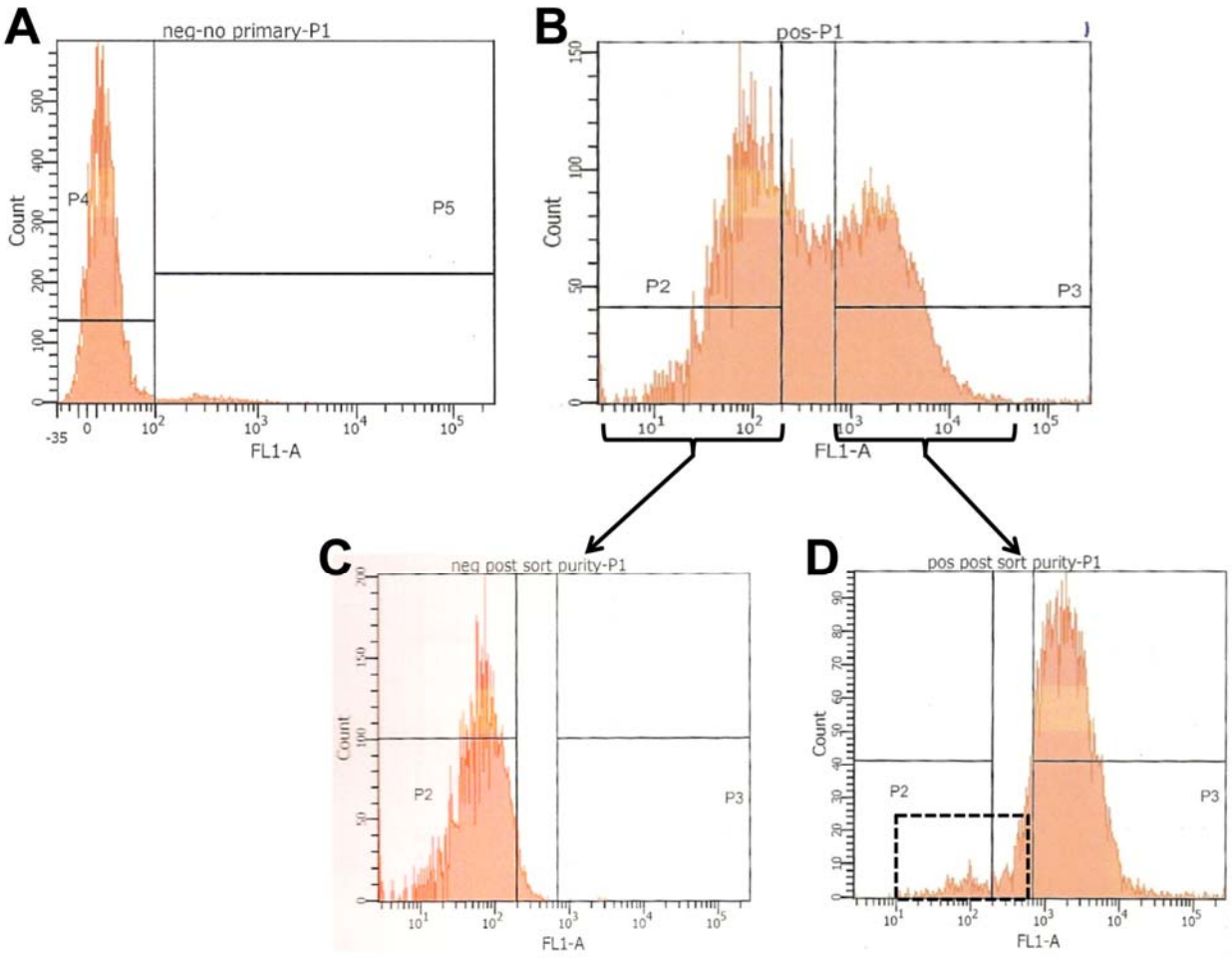

Figure 4. Fluorescence-activated cell sorting of dissociated mouse AP cells for purification of lactotrophs.

A. FACS profile of non-labeled (primary antibody omitted) cells. The peak to the left of the vertical line represents the background signal from the unlabeled cells. ***B.*** FACS profile of AP cells labeled with a PRL-specific primary antibody and FITC-labeled secondary antibody. The peak to the left represents the negative population and the group on the right represents the anti-PRL labeled population. Cells sorted in the left ("P2") and cells sorted in the right ("P3") peaks were saved for resorting (***C*** and ***D***). The cells in the center group between the two vertical lines were discarded. ***C.*** Re-sorting of the "negative" population of cells obtained in ***B***. ***D.*** Resorting of the "positive" population of cells obtained in ***B***, with contaminating or ambiguous cells highlighted by the dash-lined box.

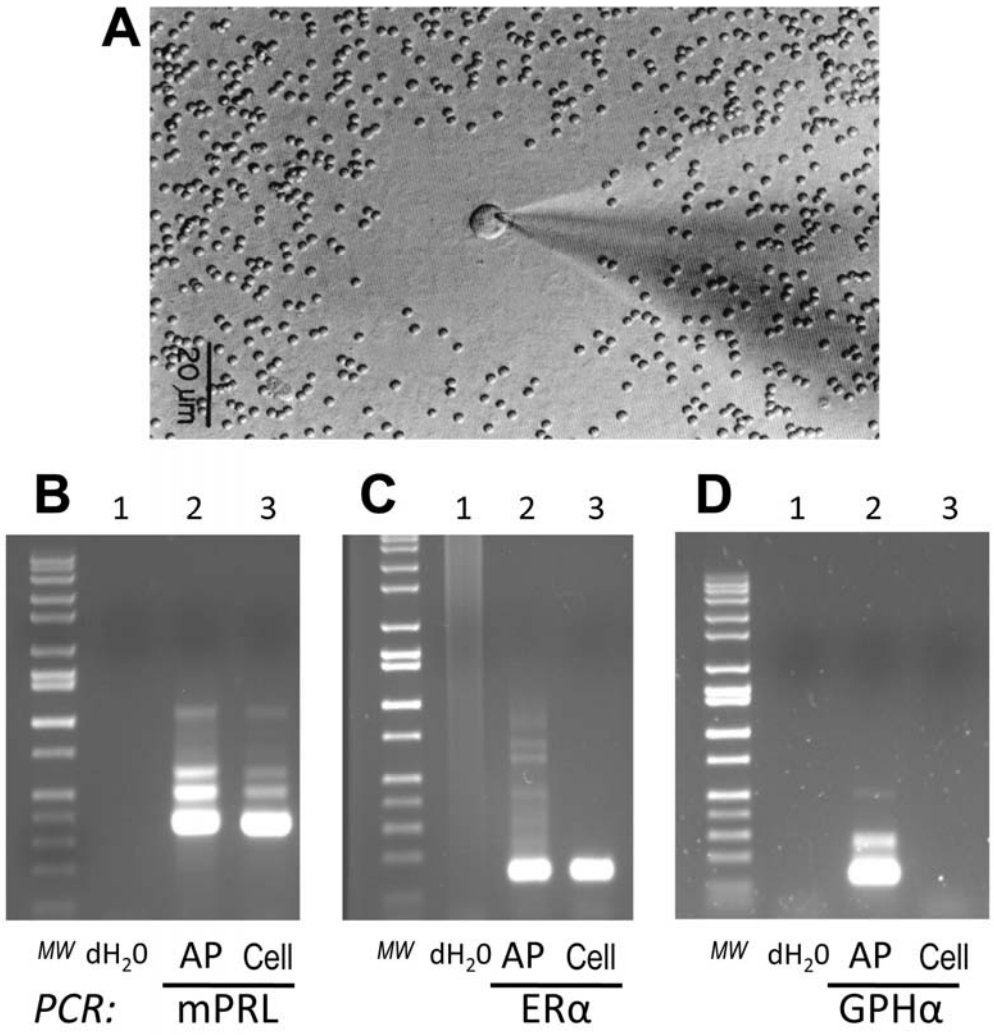

Figure 5. Single cell RT-PCR on plaque-identified lactotrophs.

A. Photomicrograph of a glass pipette touching the surface of a plaque-forming cell (lactotroph). A single lactotroph was drawn into a glass pipette and expelled into a sterile microcentrifuge tube. RNA was extracted from the cell and cDNA transcribed. The cDNA was then subjected to PCR analysis for mouse PRL (mPRL; ***B***), ER-alpha (***C***), or GPH-alpha (***D***). In each gel, beginning with the molecular weight marker (*MW*), *lane 1* is a negative control (dH_20), *lane 2* is a positive control RT-PCR from whole AP, and *lane 3* is the PCR product from a single plaque-forming cell. Note that transcripts for both mPRL and ER-alpha are detectable in the plaque-forming cell, while there is no detectable transcript for GPH-alpha.

Chapter 3

Experiments Testing the Role of Estrogen Receptor-alpha in Lactotroph Proliferation and Prolactin Synthesis Using Mice with Targeted Deletion of Estrogen Receptor-alpha in Lactotrophs

Chapter 3

INTRODUCTION

Hyperprolactinemia and pituitary PRL-secreting adenomas are the most common pituitary disorders. Lactotroph hyperplasia leads to the formation of adenomas, referred to as prolactinomas, and this has been estimated to occur in more than 20% of individuals (11, 86). Prolactinomas can lead to visual field defects and hyperprolactinemia can cause menstrual disturbances, anovulation, galactorrhea, amenorrhea, and infertility (1, 12).

Lactotroph cells of the AP are E_2 responsive cells. E_2 action through its receptor, ERα, stimulates PRL synthesis and lactotroph proliferation (37, 43, 44, 45, 46). In addition, E_2 enhances cell survival. Therefore, it is not surprising that E_2 has been found to contribute to pituitary tumorigenesis and the formation of prolactinomas. These tumors are E_2 sensitive, which correlates with a higher incidence in females. Studies have shown the growth of prolactinomas to be reduced or blocked following ovariectomy in female mice (14). It has also been shown that prolactinomas express ERα which exerts the effects of E_2 on lactotroph hyperplasia and tumor pathogenesis (87).

A useful model of prolactinoma formation is the mouse prolactin knockout ($PRL^{-/-}$) model, where mice possess a targeted disruption of the PRL gene. In this model, the absence of biologically active PRL eliminates the stimulatory feedback on the TIDA neurons (79). The resulting decrease in endogenous DAergic input to the AP leads to proliferation of the

lactotrophs and a coincident increase in pituitary weight, which can be inhibited by treatment of these mice with the D_2 agonist, bromocriptine (77). Thus, over time, large pituitary tumors spontaneously develop in $PRL^{-/-}$ mice, with females developing enlarged pituitaries as early as six weeks (77). Similar prolactinomas develop spontaneously in mice carrying the genetic deletion of the PRL receptor (PRL-R), again due to loss of feedback at the TIDA neurons leading to greatly reduced DA input to the AP (88).

Prolactinoma sensitivity to E_2 and ERα-mediated action is emphasized with studies utilizing ICI-182,780 (ICI), a pure estrogen receptor antagonist, with no agonist activity. *In vitro*, it has been demonstrated that E_2 stimulates PRL synthesis (52, 89). In pituitary cell lines it has been shown that chronic exposure to E_2 results in hyperprolactinemia and lactotroph hyperplasia, and treatment with ICI has been shown to inhibit cell proliferation by degrading ERα protein. ICI can also inhibit PRL synthesis by occupying ERα (46, 90). *In vivo*, the growth and transformation of the spontaneous lactotroph tumors in $PRL^{-/-}$ mice, are accelerated in female mice as compared to males, consistent with a role of E_2 in the development of these tumors (77). However, an interesting finding is that $PRL^{-/-}$ female mice have only about one-half the levels of circulating E_2 as wild type or heterozygous controls, which do not develop prolactinomas. $PRL\text{-}R^{-/-}$ female mice have similar low levels of circulating E_2 (91). Thus, pituitary tumorigenesis in these models cannot be explained by exposure to excess estrogens, but may reflect an increased sensitivity to estrogens in the absence of DAergic tone. However, elimination of E_2 action at the level of the lactotroph would be needed to test this hypothesis.

The goal of this investigation was to determine E_2 responsiveness of $ER\alpha^{fl/fl}$-pitPRL-Cre^{+} mice . An *in vivo* approach was taken to determine lactotroph proliferation by measuring pituitary wet weight of $ER\alpha^{fl/fl}$-pitPRL-Cre^{+} mice on a $PRL^{-/-}$ background. DA content was also measured as an indirect marker of hypothalamic response. Because these animals do not make PRL that is either biologically active, or that can be detected in RIA, we examined PRL synthesis *in vitro* in response to E_2 and ICI in pituitary cell cultures from $ER\alpha^{fl/fl}$ -pitPRL-Cre^{+} and $ER\alpha^{fl/fl}$ -pitPRL-$Cre^{0/0}$ mice. Our results show no effect of the selective deletion of ERα on prolactinoma formation, while DA levels reaching the pituitary were significantly lowered with receptor deletion. E_2 responsiveness directly at the pituitary was disrupted and PRL synthesis decreased, however, there is evidence this is dependent upon induction of the Cre tansgene promoter by previous E_2 exposure.

Chapter 3

MATERIALS AND METHODS

Anterior Pituitary Weights

The selective knockout mice were crossed onto a mouse prolactin knockout ($PRL^{-/-}$) background. $ER\alpha^{fl/fl}$ -pitPRL-Cre^{+} and $ER\alpha^{fl/fl}$ -pitPRL-$Cre^{0/0}$ mice were mated with either male $PRL^{-/-}$ or heterozygous female ($PRL^{(+/-)}$) in order to produce offspring that could then be crossed to produce the following genotypes: selective knockouts with mouse prolactin ($ER\alpha^{fl/fl}$ -pitPRL-Cre^{+} -$mPRL^{+/-}$); selective knockouts without mouse prolactin ($ER\alpha^{fl/fl}$ -pitPRL-Cre^{+} -$PRL^{-/-}$); control mice with ERα and mouse prolactin ($ER\alpha^{fl/fl}$ -pitPRL-$Cre^{0/0}$ -$PRL^{+/-}$); control mice with ERα and without mouse prolactin ($ER\alpha^{fl/fl}$ -pitPRL-$Cre^{0/0}$ -$PRL^{-/-}$). Healthy offspring were successfully produced. All animal handling and procedures were carried out in a facility accredited by the American Association for the Accreditation of Laboratory Animal Care, and were approved by the University of Cincinnati's Institutional Animal Care and Use Committee.

At six months of age, mice were anesthetized (0.1cc-0.3cc ketamine/xylazine solution) and intracardial perfusion performed. Blood was first cleared with 0.9% saline, followed by perfusion with 4% paraformaldehyde. When spontaneous movement ceased and limbs became stiff, and clearing of the liver was seen, the perfusion was stopped. The anterior pituitary (AP) was excised and a wet weight taken using a semi-analytical electrobalance. The tissue was then

post-fixed in 4% paraformaldehyde then stored in a 30% sucrose solution at 4^{o}C for later immunocytochemistry.

Primary Cell Dissociation

Animals were sacrificed by rapid decapitation and APs dissected and immediately placed in a sterile 35mm Petri dish containing 2 to 3 mL of sterile Hank's Balanced Salt Solution [HBSS; 1 L containing 9.52 g of HBSS (Sigma 6136), 10 mg/mL Penicillin-Streptomycin, 0.35 g $NaHCO_3$ (Sigma S-8875), 5.96 g Hepes-HCl (Sigma H-3375), pH 7.2, osmolarity 295-305 mOs/L). The tissue was then transferred to a second Petri dish containing 2 to 3 mL of fresh HBSS. Using sterile forceps and a sterile scalpel, the glands were minced into small pieces (<1mm^3). Fragments were transferred to a 15 mL centrifuge tube using a sterile, fire-polished, siliconized glass pipet. Fragments were settled by centrifuging at 150 x g for 4 minutes. Medium was manually aspirated off without disturbing the tissue fragments. Fragments were washed twice in 5 mL of fresh HBSS. Trypsin (Sigma T-1426) enzyme was weighed out and dissolved in 5 mL of HBSS for a final 0.2% concentration. The trypsin solution was passed through a sterile 0.22 µm pore filter unit into the tube containing the washed tissue fragments. The tube was then submerged in a 37^{o}C water bath and shaken gently for 20 to 25 minutes. After incubation, 5 mL of sterile Hanks Calcium, Magnesium Free solution [Hank's-CMF; 1 L containing 9.52 g HBSS without calcium and magnesium (Sigma H-2387), 10 mg/mL Penicillin- Streptomycin, 0.35 g $NaHCO_3$, 5.96 g Hepes-HCl, pH 7.2, Osmolarity 295-305 mOs/L] was added to the tube, and the tube was centrifuged at 150 x g for 4 minutes. The medium was manually aspirated off and replaced by 10 mL of fresh CMF, repeating two more times for additional washing. Fragments

were then resuspended in 2 mL Hank's-CMF with 75 μL of DNase and trypsin inhibitor. Gentle trituration with a sterile, fire-polished, siliconized glass pipet was performed for 2 to 3 minutes to mechanically disperse the cells. Eight mL of Hank's-CMF was added to the cell dispersal and centrifuged at 150 x g for 4 minutes. The medium was removed and resuspended in 1 to 2 mL of Hank's-CMF. Cells were filtered through a sterile Swinnex unit containing 20 μm-pore nylon mesh and the cells collected into a sterile culture tube. Cells were counted using a haemocytometer and viability determined based on Trypan blue exclusion.

Cell Culture

Primary cells were plated onto coverslips, treated with poly-L-lysine, in a 12-well plate. Cells were cultured in 1 mL of phenol-red free media (DME modified Sigma D-2902; 25mM Hepes; 10 μg/mL Insulin; 15% Horse Serum and 2.5% Fetal Bovine Serum—charcoal stripped and heat inactivated; gentamycin). Wells were divided into four treatment groups, each in triplicate: 0 (vehicle; 0.003% ethanol), 100 pM 17-β estradiol, 10 nM ICI 182780 (Tocris Bioscience), and 17-β estradiol with ICI. Cells were cultured for a total of 6 days, with a media change on the third day (3 day media stored at -20^{o}C). On the sixth day, media was removed and cells collected. For quantitative RT-PCR analysis, RNA was extracted from the cells as described (200 μL TRIzol) and stored at -80^{o}C until ready for use. In studies measuring PRL content, cells were collected in 200μl distilled water and stored at -20°C until assayed.

Reverse Transcription

RNA was quantitated on a spectrophotometer, reading at a 260/280 nm ratio. To determine the concentration of micrograms of RNA per microliter, the value from the 260 nm reading was multiplied by the dilution factor used to quantitate and a correction factor of 40. The volume equivalent to 1 μg of RNA was used in the reverse transcription reaction.

The reverse transcription reaction was done using the QuantiTect Reverse Transcription Kit (Qiagen). To the necessary volume of RNA, 2 μL of gDNA wipeout buffer was added in order to eliminate genomic DNA contamination. A volume of RNase-free water was added to the sample to make a total volume of 14 μL. Samples were incubated at 42^{o}C for 2 minutes and then immediately placed on ice for 2 additional minutes. To each reaction, 4 μL of Quantiscript RT Buffer, 1 μL of RT Primer Mix, and 1 μL of Quantiscript Reverse Transcriptase was added for a final volume of 20 μL [also included was a negative control for each sample, where each step was completed as described, but 1 μL of water was added in place of the reverse transcriptase enzyme]. Samples were incubated at 42^{o}C for 20 minutes, 95^{o}C for 3 minutes, then stored at 4^{o}C until ready for PCR.

To verify reverse transcription and absence of genomic contamination, PCR was run using primers designed against mGAPDH on the reverse transcriptase positive reactions (RT(+)) and negative reactions (RT(-)). Reactions were run as described (forward primer 5'-ACCACAGTCCATGCCATCAC- 3'; reverse primer 5'- TCCACCACCCTGTTGCTGTA- 3'). Denaturation occurred at 94^{o}C and 94^{o}C for 2 minutes and 45 seconds respectively, annealing at 62^{o}C for 45 seconds, and extension at 72^{o}C for 45 seconds for 26 cycles, with a final extension at

72°C for 5 minutes. Gel electophoresis was ran using a 1.2% gel. If signal was present in the RT(-) indicative of genomic contamination, the RNA was cleaned using a the Turbo DNA-free Kit (Ambion).

Quantitative/Real-time PCR

Real-time PCR was run on the clean cDNA samples using Fast SYBR Green Reagent and protocol (Applied Biosystems 4385612). Using a 48-well Micro-Amp reaction plate, 2 μL of each sample was dispensed into appropriate wells. A 1x SYBR Green master mix was made using primers designed against specific genes for real-time analysis: GAPDH (forward primer 5'-TGTTCCTACCCCCAATGTGT-3'; reverse primer 5'-GGTCCTCAGTGTAGCCCAAC-3'; annealing temperature); PRL (forward primer 5'- AGCCAGGCCTATCCTGAAGCCA- 3'; reverse primer 5'- ACCATTCGCTGCCTGCGCAG- 3'). Reactions were run on a real-time quantitative PCR instrument at an annealing temperature of 58.5°C.

Data Analysis for Real-time

Real-time data was analyzed using the Pfaffl method, which corrects the values to primer efficiency and reference genes (see *Pfaffl et al.*(101))

$$R = \frac{(E_{target})^{\Delta C_P \text{ target (MEAN control – MEAN sample)}}}{(E_{Ref\ index})^{\Delta C_P \text{ Ref index (MEAN control – MEAN sample)}}}$$

In this case, the reference used was mGAPDH, the target mPRL. The efficiency of mGAPDH primers was 0.93. The efficiency of the PRL primers was 0.97. Data for each sample was averaged and entered into the above equation for relative analysis.

Iodination for Radioimmunoassay

A sephadex column was prepared for iodination of mouse prolactin (mPRL). One millicurie (mCi) of iodine-125 (^{125}I) was used per 5 μg of mPRL. Hormone stock (AFP-10777D; 5 μg PRL/50 μL 0.01M $NaHCO_3$). To the vial containing 1 mCi of carrier-free ^{125}I, 50 μL of mPRL stock was added and mixed. Chloramine-T solution (10 μL; 2 mg C-T/2 mL 0.05 NaH_2PO_4) was added and mixed for 40 seconds. Sodium metabisulfite solution (6 mg $Na_2S_2O_5$/2.5 mL 0.05M NaH_2PO_4) was then immediately added to the solution and mixed to stop the reaction. The reaction mixture was layered on the top of a prepared chromatography column containing ~10 cc of G-200 preswollen Sephadex that had been treated with 3% BSA-PBS. When the reaction mixture had entered the column, 1 mL of 0.05M PBS buffer was carefully layered on top and continued to be added as fractions were collected (30 drops in first tube; 10 drops in the remaining 25 tubes, all containing 0.5 mL 1% BSA-PBS). Ten μL of each fraction was transferred to a clean tube and counted in a gamma counter. Fractions containing the highest counts on the declining edge of the first peak were kept as iodinated mPRL (*mPRL).

Radioimmunoassay: mPRL

RIA tubes were labeled in duplicate or triplicate in preparation for the assay. A reference preparation of mPRL (NIDDK AFP, provided as 4 μg in 1mL of 1% BSA in PBS, lyophilized) was dispensed for a standard curve as follows: 1 ng/mL (10, 20, 30, 40, 50, 70, and 100 μL); 10 ng/mL (10, 14, 20, 25, 30, 40, 50, 70, and 100 μL); 100 ng/mL (10, 14, 20, 30, 45, 70, 100 μL). Unknowns were dispensed at a volume predicted to fall along the center of the standard curve. All samples were made up to a total volume of 250 μL with assay buffer [1% BSA (Fraction V, Sigma A-8022) in 0.01M PBS (containing 0.01% Na-merthiolate, pH 7.0)].

After standards and unknowns were dispensed, 100 μL of primary antibody [NIDDK anti-mouse prolactin from rabbit; 1:90,000 working dilution in 1/400 NRS-PBS EDTA (Normal Rabbit Serum, Antibodies Inc. Cat. No. 1430; diluted with 0.01M PBS-EDTA (50mM EDTA plus 0.01% Na-merthiolate in 0.01M PBS))] was dispensed in each. The radioactive labeled mPRL (*mPRL) was dispensed in each sample at 50 μL. Alternatively, to increase sensitivity, *mPRL could be added 24 hours following primary antibody addition.

To detect total counts, a total sample with 50 μL of *mPRL plus 450 μL assay buffer was included. To detect non-specific binding, a normal rabbit serum (NRS) standard (1/400 NRS-PBS EDTA) was dispensed at 100 μL with 250 μL of assay buffer and 50 μL of *mPRL (no primary antibody included). To determine total binding, a sample with 100 μL of primary antibody, 250 μL of assay buffer, and 50 μL of *mPRL was included. Specific binding was calculated as total binding less NRS binding.

Samples were incubated with primary antibody and *mPRL for 24 to 48 hours at room temperature. Secondary antibody (1:110 ARγG with PBS-EDTA; Anti-rabbit gamma globulin from goat, Antibodies Inc. Cat. No. 1156) was added at 100 µL to each sample except the total tubes, and incubated for 24 hours at room temperature.

100 µL of PEG (polyethylene glycol solution) was added to each sample (except totals). Samples were vortexed and then spun at approximately 2500 rpm in Beckman Allegra at 4^{o}C. Supernatant was decanted into an appropriate waste container and tubes inverted on an absorbent surface for 1 minute. Excess fluid was wiped from the tubes.

First, standard samples were counted in a gamma counter, and counts per minute (cpm) recorded. These values were then plotted as a function of the log of mPRL (picograms) in each sample that had been dispensed from the reference preparation, to produce a standard curve. Six points across the curve to represent an abbreviated standard curve that could be run with the unknown samples. This abbreviated standard and the unknowns were then counted using a program designed to interpolate picogram amounts of mPRL in the unknowns from the standards. The values given for each sample were then corrected to the volume dispensed in each, and the total amount of mPRL (nanograms) in each sample was calculated.

PCA Extraction

Unstressed mice were sacrificed by rapid decapitation. The posterior pituitary was removed and discarded. Anterior pituitaries were dissected and a wet weight taken. Tissue was then gently homogenized using ground glass microhomogenizers in 100 µL of 0.2N perchloric acid (PCA). PCA extracts were centrifuged at 4^{o}C for 10 minutes at 12,000 rpm. Supernatant was transferred and aliquoted in two clean tubes and stored at -80^{o}C. The protein pellet was dissolved in 50 µL of 1N NaOH and stored at -20^{o}C.

Radioimmunoassay: Dopamine

DA RIA kit was used (Rocky Mountain Diagnostics) to measure the amount of DA in PCA extracts. Cpm for each standard and sample was obtained. A standard curve was plotted and the amount of DA, in picograms, was calculated for each sample from the standard curve and normalized to the volume measured for each sample.

Statistical Analysis

A one-way ANOVA using Bonferroni's multiple comparison test or Tukey's multiple comparison test was used to determine statistical significance between groups, with significance indicated by a P value of less than 0.05.

Chapter 3

RESULTS

Estrogen Responsiveness in vivo: Lactotroph Proliferation

An *in vivo* approach was taken to investigate E_2 responsiveness in the selective knockout mice. ERα$^{fl/fl}$-pitPRL-Cre^{+} and ERα$^{fl/fl}$-pitPRL-Cre$^{0/0}$ mice were crossed onto a mouse PRL$^{-/-}$ background to observe changes in lactotroph proliferation due to the presence or absence of ERα in these cells. AP wet weight was measured, following cardiac perfusion and dissection, and results showed that there was no change in abnormal growth due to the loss of ERα in the lactotrophs (Figure 1). AP weight increased significantly with the loss of PRL in both male and female mice, regardless of the presence or absence of ERα.

Hypothalamic Dopamine

APs from ERα$^{fl/fl}$-pitPRL-Cre^{+} and ERα$^{fl/fl}$-pitPRL-Cre$^{0/0}$ mice, either on a mouse PRL wild type (PRL$^{+/+}$) or mouse PRL$^{-/-}$ background, underwent a PCA extraction and were analyzed in a DA radioimmunoassay (RIA) to determine the amount of DA reaching the AP in these animals. The amount of DA was significantly less in PRL$^{-/-}$ mice as compared to PRL$^{+/+}$ mice (Figure 2). There was a significant decrease in DA seen in in PRL$^{+/+}$ mice upon the loss of ERα, and this response was mostly seen in males (Figure 2B and C). Overall, there was no significant difference between ERα$^{fl/fl}$-pitPRL-Cre$^{0/0}$- PRL$^{-/-}$ and ERα$^{fl/fl}$-pitPRL-Cre^{+}-PRL$^{-/-}$ in males or

females. DA was normalized to AP weight. Weights showed significant differences among groups identical to that shown in Figure 1.

Estrogen Responsiveness in vitro: mPRL Synthesis

Because no significant differences were observed *in vivo*, an *in vitro* approach was taken to determine direct E_2 responsiveness at the level of the pituitary by determining mouse PRL synthesis with various treatments. APs were dissociated and treated in culture with the natural ligand and ERα agonist 17β-estradiol (E_2), the ERα antagonist ICI 182780, and a combinatory dose of E_2 and ICI. Real time RT-PCR was used to determine changes in PRL synthesis, and results showed no difference among the treatment groups in WT mice (Figure 3).

As an alternative to real time analysis, mouse PRL protein from cell lysates was measured by RIA, following the same *in vitro* experiment (Figure 4). First, this was done on cell lysates from WT mice to see if there was a significant response to treatment. There was a significant increase in PRL levels in response to E_2 treatment. Protein levels decreased when treated with the ERα antagonist ICI, and these levels were recovered just below baseline when E_2 was added with ICI. After demonstrating this technique as efficient for evaluating direct changes in PRL at the level of the pituitary, the same experiment was performed using dispersed AP cells from $ER\alpha^{fl/fl}$-pitPRL-Cre^{+} mice. As compared with WT animals (Figure 4, left), there was no response to treatment with E_2 or ICI in the selective knockout mice (Figure 4, right), and PRL levels were below baseline of WT mice.

This *in vitro* experiment was repeated in ERα$^{fl/fl}$-pitPRL-Cre$^{0/0}$ and ERα$^{fl/fl}$-pitPRL-Cre^{+} mice in a side-by-side preparation, in 1.5 month old females and also males. In control female mice, treatment with ICI significantly decreased PRL content in lactotrophs, and treatment with E_2 significantly increased PRL content as expected (Figure 5A, left). In selective KO females, there was no significant response to ICI, however there was a significant increase in PRL content in response to E_2 treatment (Figure 5A, right). The same result was seen in male mice (Figure 5B).

PRL Promoter Sensitivity

Because there was a response to treatment in lactotrophs from ERα$^{fl/fl}$-pitPRL-Cre^{+} mice when repeating the *in vitro* experiment, an *in vivo* approach was taken to investigate these results further. The PRL promoter is E_2 sensitive, and it was possible that the 1.5 month old females had not been predisposed to enough circulating E_2 to activate the PRL promoter and drive efficient Cre recombinase activity. To test this, 1 month old female ERα$^{fl/fl}$-pitPRL-Cre^{+} mice received subcutaneous pellets of vehicle or E_2 (0.05mg), were sacrificed after 21 days of treatment, and their APs dissected and dispersed. Primary cells were treated *in vitro* as done previously, and PRL content from cell lysates was analyzed by RIA. Mice treated *in vivo* with vehicle had a significant response to E_2 *in vitro* with an increase in PRL as seen previously (Figure 6, left). Mice treated *in vivo* with E_2 did not show a significant response to E_2 *in vitro* (Figure 6, right).

Chapter 3

DISCUSSION

Two parameters of lactotroph function were examined in ERα$^{fl/fl}$-pitPRL-Cre^{+} mice in order to examine the role of direct estrogen action on the lactotroph. *In vivo* studies showed no difference in lactotroph proliferation, as assessed by AP weight, between control mice and the selective knockout mice bred onto a genetic background with spontaneous formation of prolactinomas. DA levels were substantially lower in the AP of mice on the PRL$^{-/-}$ background, regardless of the presence or absence of ERα in the lactotrophs. *In vitro*, repeated experiments revealed an unexpected response of lactotrophs from ERα$^{fl/fl}$-pitPRL-Cre^{+} mice to treatment with E_2. However, further investigation determined this may be a result of E_2 sensitivity of the PRL promoter that was used to drive Cre recombinase expression.

A study by Scully et. al. examined prolactinomas in rats, and found pituitary tumors induced by E_2 to significantly increase pituitary wet weight. They showed this increase in weight correlated with pituitary cell number and DNA content. AP weight proved to be a useful marker of lactotroph proliferation in this study as well. Other studies examining pituitary adenomas, particularly in mouse PRL$^{-/-}$, have shown pituitaries to enlarge over a period of weeks to months, with aged mice forming large pituitary tumors, and females showing enlargement as early as 6 to 8 weeks of age (77). In this study, mice were sacrificed at 6 months of age, and those crossed onto a PRL$^{-/-}$ background showed a significant increase in AP weight up to as much as 10 milligrams.

Although measuring wet weight proved to be a sufficient marker of pituitary growth and cell proliferation, and there was a significant increase in $PRL^{-/-}$ mice, one might expect pituitary growth to be less in mice lacking ERα in the lactotrophs because stimulation by E_2 at the pituitary is removed. On the other hand there is the possibility that in the absence of ERα, there is a decrease in PRL feedback on the TIDA neurons, resulting in decreased DA inhibition at the AP, and one may see an increase in pituitary weight. However, there was no difference when comparing $ERα^{fl/fl}$-pitPRL-Cre^{+} mice and $ERα^{fl/fl}$-pitPRL-$Cre^{0/0}$ mice. With no difference seen *in vivo*, this may have implications that E_2 action at the level of the pituitary has less or no effect on lactotroph proliferation. PRL itself, and its regulation of DA, may play a more significant role in the regulation of cell proliferation, however further investigation is necessary to determine this.

Studies have shown that in $PRL^{-/-}$ mice, DA input into the pituitary decreases, and loss of tonic inhibition by DA results in cell proliferation (1,11). In order to determine the amount of DA input into $ERα^{fl/fl}$-pitPRL-Cre^{+} and $ERα^{fl/fl}$-pitPRL-$Cre^{0/0}$ mouse pituitaries on a $PRL^{+/+}$ or $PRL^{-/-}$ background, a DA RIA measured protein from PCA extracts. Results showed that DA decreased substantially and equally in mice with and without ERα on a $PRL^{-/-}$ background. This was to be expected with the absence of stimulation by PRL on TIDA activity. More interestingly, there was a significant decrease in DA input with the deletion of ERα in mice on a $PRL^{+/+}$ background, although this was observed mostly in males. This may have implication that in the absence of ERα in the lactotrophs, E_2 may have more of an effect at the level of the hypothalamus or on other factors involved in the regulation of PRL. Although these results show changes in E_2

responsiveness in terms of DA content at the AP of ERα$^{fl/fl}$-pitPRL-Cre^{+} mice, decreased DA inhibition of proliferation was not reflected in the pituitary wet weights. If DA input was less in mice lacking ERα in the pituitary lactotroph, there would be an expected increase in pituitary weight and this was not observed. Again, this could indicate that PRL is the more significant regulator of DA release and proliferation. Because DA did not decrease to minimum levels as seen in the PRL$^{-/-}$ mice, this could indicate it maintained a level able to inhibit significant proliferation over time.

In addition to whole animal studies, it was important to look at E_2 action directly at the level of the pituitary. Other investigations have shown the effects of E_2 and receptor antagonists on PRL synthesis by culturing dispersed AP cells and treating with various agents *in vitro*. These studies have shown treatment of primary cultured cells increases PRL synthesis and mRNA (43, 44, 89, 92). Real-time RT-PCR is a fluorescence based technique widely used to quantify steady state mRNA levels in different samples. Although there are many advantages to using real-time analysis, the sensitivity of the assay to many of the steps involved including template preparation, primer design, reverse transcription, instrumentation, enzymes and reagents, and the analysis of the data itself leaves room for error and misinterpretation (93). Using real-time analysis to determine mouse PRL synthesis *in vitro* showed no difference among treatment groups in this case. This may have been due to a high background caused by the large storage pool of PRL mRNA, obscuring the ability to observe dynamic changes. In addition, because there were several complications with primer design and variability in data analysis, mouse PRL synthesis had to be measured using an alternate assay.

As an alternative to measuring transcript, RIA was used to determine protein synthesis following treatment of cultured primary cells. And, in fact, this is directly measuring the final product of synthesis, which is the biologically relevant substance. This method proved to be more efficient, showing E_2 responsiveness as expected in WT mice. E_2 stimulated PRL synthesis, and ICI proved to be a sufficient antagonist, decreasing PRL synthesis. It has been well established that E_2 stimulates mouse PRL synthesis and does so by binding to ERα (12, 51, 52, 53), therefore when determining E_2 responsiveness in the selective knockout mice, it would be expected that PRL synthesis would decrease. At first, results of this study indicated no response to treatment with E_2 and ICI, with low PRL below baseline and ICI treated levels seen in W.T. mice. However, when this experiment was repeated, results from $ER\alpha^{fl/fl}$-pitPRL-Cre^{+} preparations resembled that seen in WT mice.

The $ER\alpha^{fl/fl}$-pitPRL-Cre^{+} mice were generated using transgenic animals with the integrated construct of the rat pituitary PRL promoter driving the expression of Cre recombinase. The PRL promoter is regulated by and sensitive to the pituitary specific transcription factor, Pit-1. The ER has been shown to bind to Pit-1 to regulate the transcription of PRL and promote expression (1, 42, 94). The binding of Pit-1 and ligand-bound ER to the PRL promoter in turn mediates E_2 stimulation (99). Therefore, the expression of Cre recombinase may be sensitive to circulating E_2.

Because the PRL promoter is sensitive to E_2, the expression of Cre recombinase and its efficiency may be sensitive to circulating E_2 in the $ER\alpha^{fl/fl}$-pitPRL-Cre^{+} mice. This explains the response seen in the selective KO mice used in the repeated *in vitro* experiments (Figures 5),

because these mice were significantly younger than those used in the initial experiment (Figure 4) and may have not been exposed to enough circulating E_2 to induce sufficient Cre recombinase activity. To test this hypothesis, an *in vivo* approach in conjunction with the *in vitro* experiment was implemented. $ER\alpha^{fl/fl}$-pitPRL-Cre^{+} mice received subcutaneous pellets of vehicle or E_2 for 21 days before their APs were dissociated and primary cells treated with agonist or antagonist *in vitro*. When PRL content from the cells were analyzed by RIA, results showed that Cre recombinase efficiency is in fact sensitive to circulating levels of E_2 *in vivo* in the selective KO mice as evidenced by the decreased response to *in vitro* treatment. These results also explain the low level of ERα transcript observed in RT-PCR analysis of single lactotroph samples (Chapter 1, Table 1), as these were from young mice as well.

Because the E_2 pellets used provide only a mid-range physiological dose of E_2, it will be necessary to repeat this experiment using increased doses of E_2 *in vivo* and observing if a complete KO phenotype is induced. It will be important in the future to evaluate circulating E_2 levels in $ER\alpha^{fl/fl}$-pitPRL-Cre^{+} mice used for experiment, and it will be necessary to use aged mice or mice with reproductive experience in future experiments to ensure the complete deletion of ERα in the pituitary lactotrophs.

The *in vivo* and *in vitro* studies demonstrate further the stimulatory effects of E_2 on pituitary lactotrophs by acting through the classical E_2 receptor, ERα. In the $ER\alpha^{fl/fl}$-pitPRL-Cre^{+} mice , PRL synthesis decreases with lowered response to exogenous treatments in aged and E_2 dosed mice, but there is no obvious change in cell proliferation or DA content in APs of these mice.

Further investigation into the proliferative effects of E_2 at the hypothalamus versus the pituitary and the possible mechanism involved is needed.

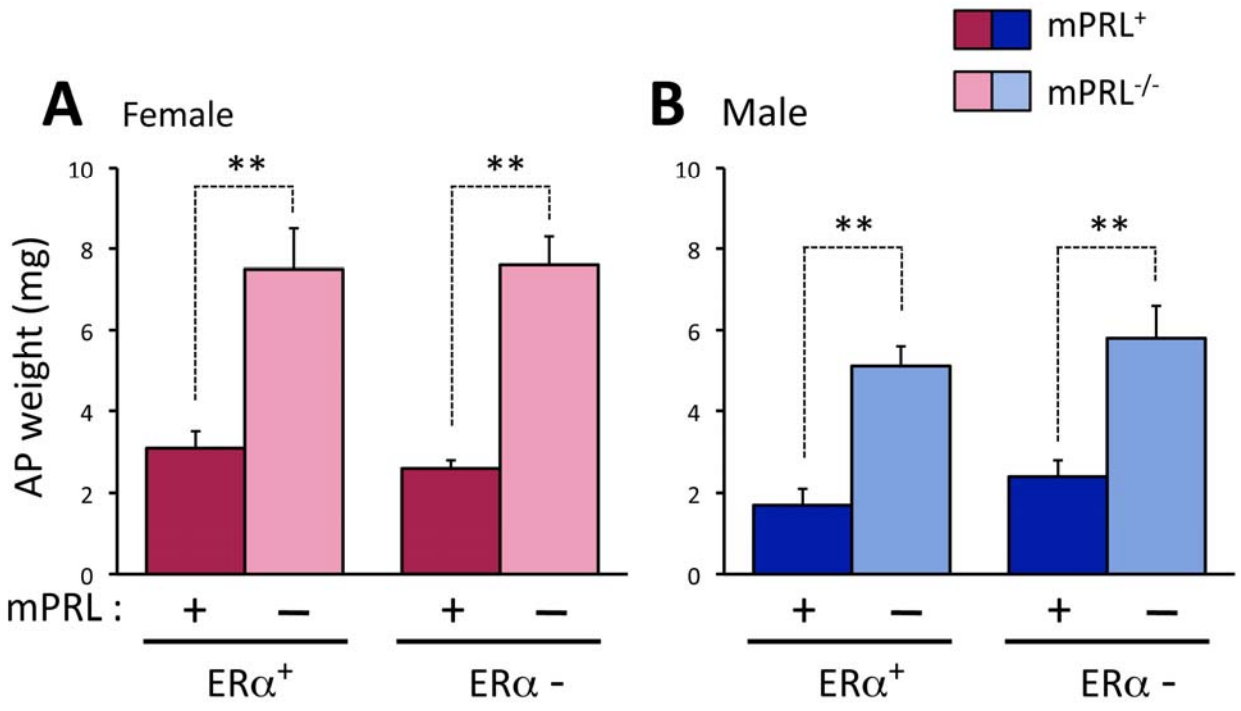

Figure 1. Knock-out of ERα in pituitary lactotrophs does not prevent abnormal growth of AP in mPRL$^{-/-}$ mice.

AP glands were rapidly dissected from 6-month old female (***A***) and male (***B***) mice and weighed. All pituitaries from mPRL$^{-/-}$ mice were significantly larger than those from mPRL^{+} mice, regardless of sex and regardless of presence of ERα in the lactotrophs. Note that overall, AP in females tend to be larger than their counterpart male. Data analyzed with one-way ANOVA followed by Tukey's multiple comparison analysis. **p<0.001.

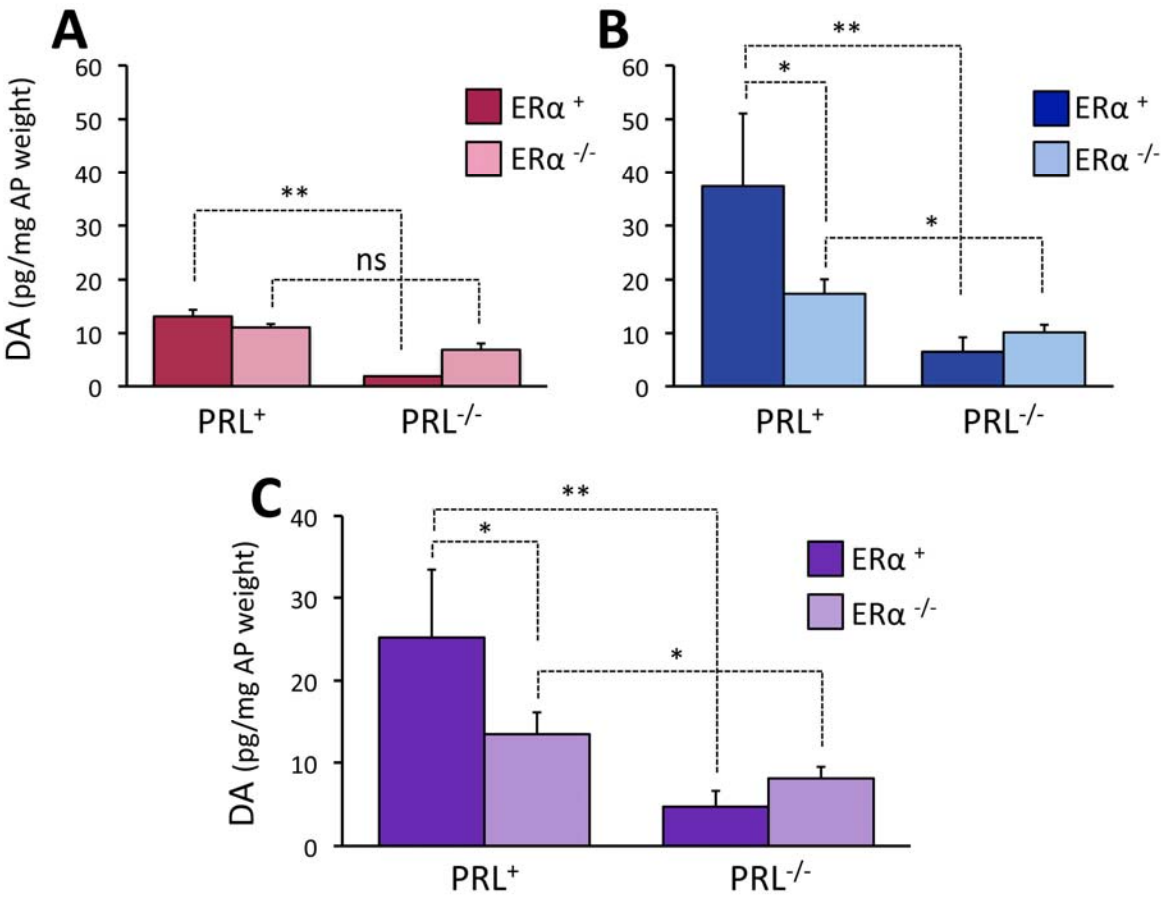

Figure 2. DA concentration is lower in APs of mPRL$^{-/-}$ mice regardless of presence or absence of ERα in lactotrophs.

AP glands were dissected from female (***A***) and male (***B***) mice that either expressed PRL (PRL^{+}) or did not (PRL$^{-/-}$), and either did (ERα^{+}) or did not (ERα$^{-/-}$) express ERα in pituitary lactotrophs. Assignment to groups was based on genotyping, with ERα$^{fl/fl}$-pitPRL-Cre^{+} mice representing the ERα$^{-/-}$ labeled group. DA was extracted from APs in 0.2% PCA and measured by RIA. ***C.*** Female and male data combined. One-way ANOVA with post analysis by Tukey's multiple comparison test. *p<0.01; **p<0.001

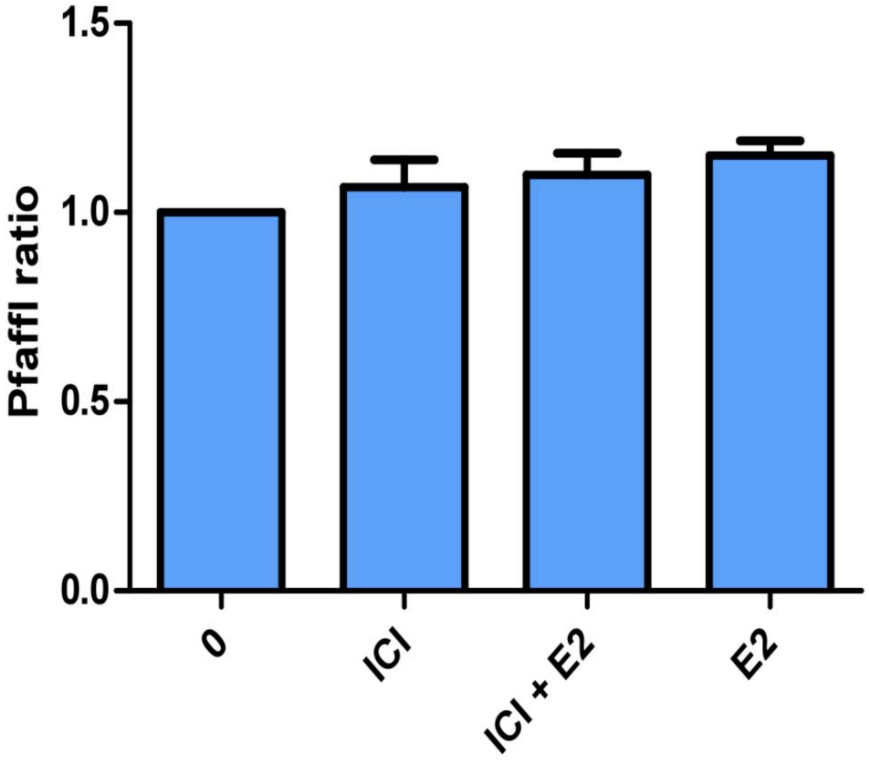

Figure 3. Real-time analysis of changes in mPRL synthesis *in vitro*.

Real-time RT-PCR analysis of changes in mPRL transcript with various treatments in a wild type mouse: 0 (baseline), 10 nM ICI, 100 pM estrogen (E2), and 10 nM ICI plus 100 pM E2. Mice were sacrificed at 3 months of age. Analyzed by pfaffl method, baseline is set to 1 with the ratio of treatments changing relative to baseline.

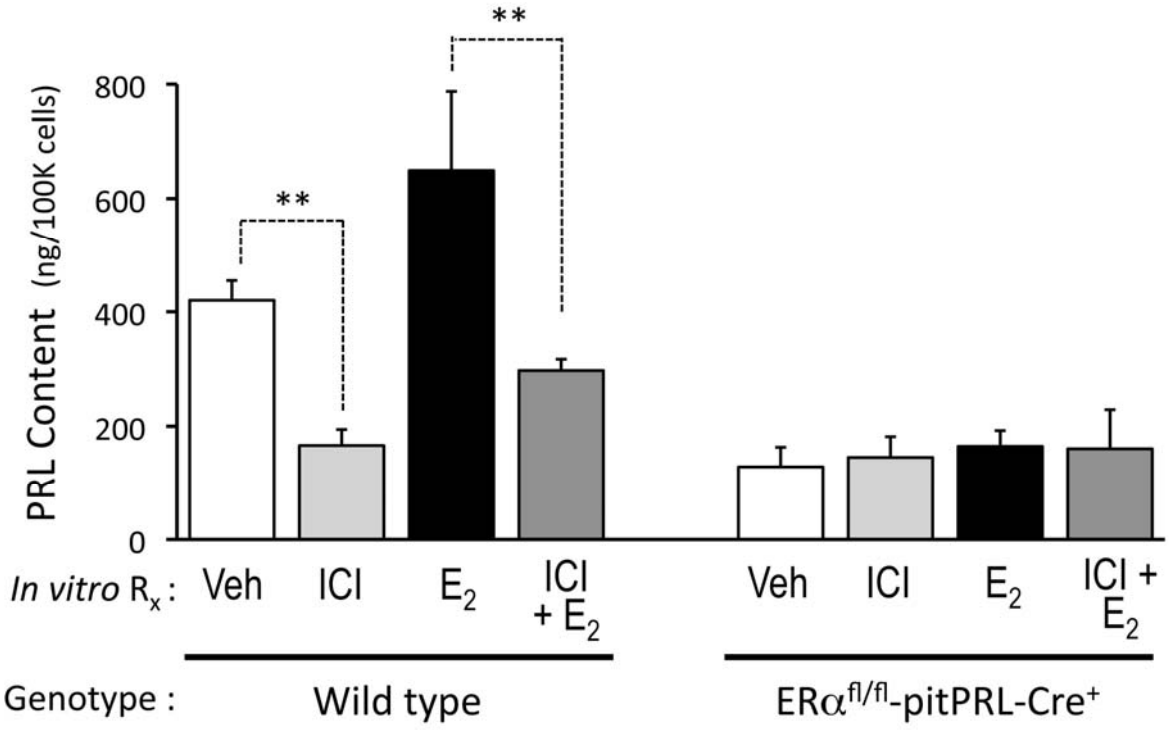

Figure 4. PRL synthesis is not affected by E_2 or ICI in cultured AP cells derived from pitPRL-Cre$^+$-ER$\alpha^{fl/fl}$ female mice.

AP glands were dissected from 3.5-4 month old female mice that were either wild type or with the pitPRL-Cre$^+$-ER$\alpha^{fl/fl}$ genotype, and, therefore, should have the ERα deleted from their lactotrophs. AP cells were dissociated and cultured for six days in the presence of vehicle (veh), E_2 (100 pM), ICI (10 nM), or both E_2 and ICI. At the end of treatment, cells were collected and PRL content determined by RIA. **p<0.01.

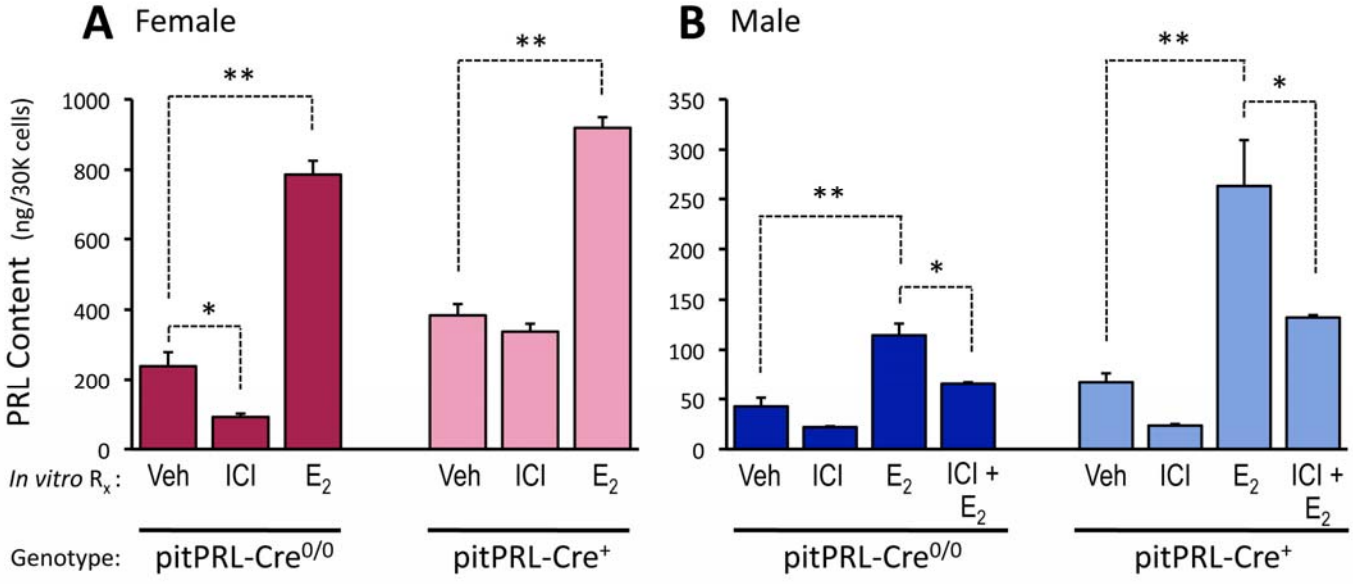

Figure 5. Estrogen stimulation of PRL content occurs in AP cells of young (1.5 month-old) mice that should have ERα deleted in the lactotrophs.

All animals used in this experiment were homozygous for floxed ERα and were either negative for Cre (pitPRL-Cre$^{0/0}$) or positive for Cre (pitPRL-Cre^{+}). The latter group should have ERα deleted in the lactotrophs. AP glands from 1.5-month old female (***A***) or male (***B***) mice were dissociated and cultured for six days in the presence of vehicle (veh), E_2 (100 pM), ICI (10 nM), or both E_2 and ICI. At the end of treatment, cells were collected and PRL content determined by RIA. One-way ANOVA with post analysis by Tukey's multiple comparison test. * $p<0.01$; ** $p<0.001$

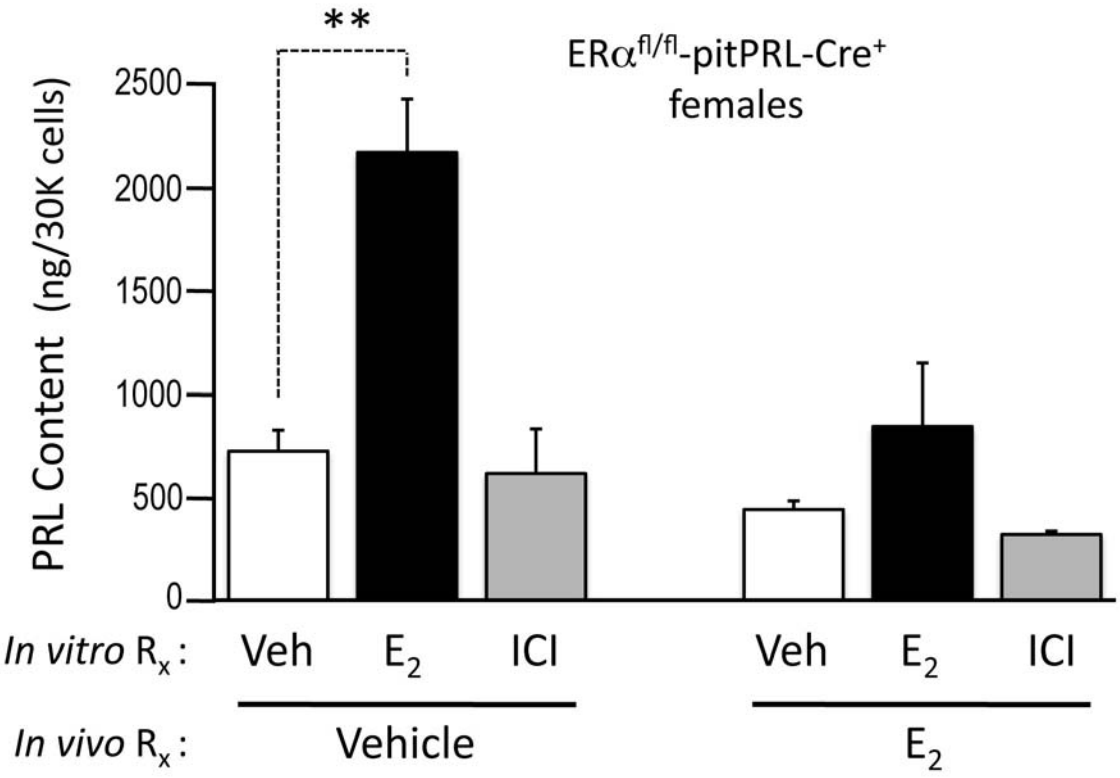

Figure 6. *In vivo* E_2 treatment of young female ERα$^{fl/fl}$-pitPRL-Cre^{+} mice reduces the effect of *in vitro* E_2 treatment on PRL content in cultured AP cells.

One month-old female mice with ERα$^{fl/fl}$-pitPRL-Cre^{+} genotype received two subcutaneous pellets of vehicle or E_2 (0.05 mg/pellet; 21-day release). Twenty-one days later, mice were sacrificed and the APs dissociated and cultured for six days in the presence of vehicle (veh), E_2 (100 pM), or ICI (10 nM). At the end of treatment, cells were collected and PRL content determined by RIA. One-way ANOVA followed by Dunnett's multiple comparison to control test. **p<0.001

Chapter 4

Future Directions

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pitPRL Promoter Sensitivity Assays

Results from the PRL promoter synthesis experiment (Chapter 2, Figure 6) demonstrate Cre recombinase expression may be dependent upon E_2 stimulation of the pitPRL promoter. In order to fully characterize the ERα$^{fl/fl}$-pitPRL-Cre^{+} mice and prove them as a sufficient model in which to study the regulation of PRL, it will be important to determine if Cre expression can be driven to provide the complete deletion of ERα in the pituitary lactotrophs. The *in vivo* treatment of ERα$^{fl/fl}$-pitPRL-Cre^{+} mice and corresponding controls will be repeated using increased doses of E_2, and E_2 responsiveness will again be determined *in vitro*. Also, it will be important to repeat several experiments, including single cell RT-PCR, using aged ERα$^{fl/fl}$-pitPRL-Cre^{+} mice that have gone through many reproductive cycles or mice that have gone through pregnancy. These mice will have experienced high circulating levels of E_2 and should have increased Cre recombinase activity.

Immunocytochemistry

An on-going process has been troubleshooting ICC on dissociated AP cells, and co-staining for mouse PRL and ERα. Using this method, it would be possible to visualize the presence or absence of ERα in the PRL-expressing lactotrophs. Dispersed AP cells from ERα$^{fl/fl}$-pitPRL-

Cre^{+} mice with a positive PRL signal should be negative for ERα if the receptor is truly deleted. Currently conditions have been optimized for PRL staining (data not shown). However, there have been complications in visualizing ERα protein, and troubleshooting is required. Future experiments would include optimizing the conditions for ERα staining, followed by co-staining for PRL and ERα.

Proliferation Studies

Additional experiments are needed to determine the extent of proliferation occurring in the selective knockout mice. Measuring AP wet weights showed no difference between $ER\alpha^{fl/fl}$-pitPRL-Cre^{+} mice and $ER\alpha^{fl/fl}$-pitPRL-$Cre^{0/0}$ mice on $PRL^{-/-}$ background. Although wet weight has been shown to be a good indicator of proliferation (41), it is important to verify proliferative activity is due to the lactotroph cells, and it is important to visualize and quantify these effects.

Ki67 is a protein that is expressed during all active phases of the cell cycle. Antibodies raised against this protein are reactive exclusively to proliferating cells, whether normal or tumorigenic, and have been used as diagnostic tools in different carcinomas (95). To determine the mitogenic properties of the APs of the selective knockout mice, APs from animals among the same groups studied in the AP *in vivo* proliferation experiment measuring AP weight, will be fixed and sectioned, followed by ICC using a Ki67 primary antibody linked to an immunofluorescent label. It would also be beneficial to co-stain with Ki67 and PRL. The fraction of PRL positive cells expressing Ki67 could then be quantified in order to determine and compare the amount of

proliferation occurring among groups. This study would more clearly illustrate the effect of the deletion of ERα on lactotroph proliferation.

Bromodeoxyuridine (BrdU) is another marker of proliferation, and is incorporated into the DNA of dividing cells. In addition to Ki67 staining of tissue sections, proliferation could be studied *in vitro*. It has been shown that direct E_2 action on the AP is required for lactotroph proliferation but not for secretory surges of PRL (45). Therefore, it would be important to determine the effect of E_2 on lactotroph proliferation at the level of the pituitary. A study by Schuff *et al*. (88) observed the effect of PRL treatment on lactotroph proliferation in primary pituitary cultures. They did so by treating with PRL *in vitro*, followed by treatment with BrdU. Cells were then fixed and co-stained for PRL and BrdU using immunofluorescence, and a proliferative index was calculated. When investigating E_2 responsiveness in the selective knockout mice, treatment with E_2 and ICI of dispersed pituitary cells was used to determine changes in PRL synthesis. This *in vitro* experiment could be repeated on primary pituitary cells from $ER\alpha^{fl/fl}$-pitPRL-Cre^{+} mice and $ER\alpha^{fl/fl}$-pitPRL-$Cre^{0/0}$ mice on a $PRL^{+/+}$ or $PRL^{-/-}$ background, and treatment with E_2 or ICI could be followed by treatment with BrdU. Cells could then be fixed and co-stained for PRL and BrdU, and a proliferation index determined. It would then be possible to make additional comparisons on proliferation of mice with and without ERα in the pituitary lactotrophs.

In vivo Studies

Following complete verification of ERα deletion in the pituitary lactotrophs, it will be important to complete an *in vivo* study in which endogenous E_2 is removed from the mouse through

ovariectomy, removing E_2 signaling at the hypothalamus in addition to the lack of E_2 stimulation at the pituitary. Following ovariectomy, E_2 can be replaced through treatment to restore E2 action at the hypothalamus, or remain absent, and the phenotype can then be observed. In this manner, E_2 responsiveness could be investigated further, determining any effects of the selective KO occurring due to changes in E_2 action at the hypothalamus. Taken with the *in vitro* analysis determining PRL regulation at the level of the pituitary, and the proliferation studies, this experiment could expand the understanding of E_2 regulation of PRL synthesis and lactotroph proliferation at the pituitary versus the hypothalamus.

The ERα$^{fl/fl}$-pitPRL-Cre^{+} mice are fertile and able to breed. They develop healthy offspring that survive to adulthood with no apparent defects. However, although these mice are able to reproduce and their offspring survive, it would be important to determine if there are any physiological effects of the selective ERα KO on lactation. PRL is essential for lactation in mammals and estrogen-induced lactotroph proliferation and PRL synthesis during pregnancy is believed to be necessary for fully developed suckling-induced PRL secretion during lactation (11). If PRL secretion during lactation is compromised in ERα$^{fl/fl}$-pitPRL-Cre^{+} mice, particularly in multiparous dams, these mice may produce less milk than their corresponding controls. This could be determined by measuring pup weights of litters from ERα$^{fl/fl}$-pitPRL-Cre^{+} mice, and comparing them to litters from W.T. and ERα$^{fl/fl}$-pitPRL-Cre$^{0/0}$ mice. The growth curves of the pups in the former group may be lower. In addition, weigh-suckle-weigh tests should be performed throughout lactation. In these studies, pups are separated from the dam for 4 hours. The litter is weighed and then returned to the dam and allowed to suckle for 30 minutes. The litter is again weighed and the increase in weight reflects the volume of milk produced.

Estrogen Receptor Beta

Although it has been shown ERβ levels are negligible in pituitaries of the mouse and this receptor does not play a significant role in PRL regulation (42, 56), it is important to verify these findings and explore the possibility that other factors, including ERβ, may be upregulated in the absence of ERα.

Nested primers have been designed against ERβ transcript and optimized for use in single cell RT-PCR. Future analyses will include collection of single lactotroph cells identified by RHPA, followed by RT-PCR on extracted RNA from these cells. In addition to screening for PRL, ERα, and mGPH, cells will be screened for the presence of ERβ. If there is an ERβ product detected, contrary to previous findings, it will be important to investigate ERβ activity in the regulation of PRL.

GPR30

The existence and function of GP30 as an ER is controversial, however, because GPR30 expression has been found in tissues and cell types that are active in the regulation of PRL, it will be important to investigate whether there is a role for this receptor in PRL regulation in the $ER\alpha^{fl/fl}$-pitPRL-Cre^{+} mice. A study by Lebesgue et al. investigated the role of GPR30 in neuroendocrine actions as a response to E_2 in the hypothalamus, and they found that the membrane receptor was important in short latency PRL secretion.

With a proposed role for GPR30 in hypothalamic regulation of PRL secretion, where ERα also plays a significant role in mediating E_2 action on hypothalamic activity and regulation of PRL, it will be important to investigate the possible activity of this receptor in the absence of direct E_2 action at the pituitary. As with ERβ, it will be essential to identify the presence of GPR30 in the lactotrophs through single cell RT-PCR analysis. It will also be important to investigate GPR30 expression in the hypothalamus, and the possibility of compensatory action in the absence of ERα.

Non-classical Signaling

Not only are E_2, DA, and PRL important regulators of PRL synthesis, secretion, and cell proliferation, but there are other factors involved. Stimulatory factors on PRL include various growth factors, TRH, oxytocin, vasoactive intestinal peptide, galanin, serotonin, opioids, Pit-1, and many more. Inhibitory factors include somatostatin, calcitonin, endothelin-1, and TGFβ (1). For example, studies have shown the growth factor, IGF-1 is a partial mediator of E_2 action in lactotrophs and influences differentiation and proliferation of cells, as well as E_2 induced PRL synthesis (96). In contrast, another study demonstrated, in primary cell culture of rat lactotroph cells, that E_2 inhibits cell proliferation in the presence of IGF-1 (97).

There is also a proposed "cross-talk" model, in which the role of classical ERα action in proliferation and release of PRL is mediated by growth factor receptor activation in the absence of circulating E_2 (57). It has been shown that EGF has the ability to stimulate PRL gene

expression and release by inducing S118 phosphorylation of ERα by ERK1/2 (98). Also, investigations have shown that E_2 induced PRL expression requires an intact MAPK/ERK pathway. This pathway is important in the induction of cell proliferation. Blocking this pathway in cultured pituitary cells inhibited the ability of E_2 to promote PRL transcription and synthesis (69).

It is clear that E_2 action on PRL regulation is complex. It will be important to investigate these additional PRL-regulating factors and alternative pathways, in addition to ERβ and GPR30, to determine if they come into play and influence E_2 responsiveness in the absence of ERα in lactotrophs.

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