ANALYSIS OF GnRH AS A CENTRAL REGULATOR OF FERTILITY: EXPLORING THE MULTIPLE ROLES OF ERK SIGNALING

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ANALYSIS OF GnRH AS A CENTRAL REGULATOR OF FERTILITY: EXPLORING THE MULTIPLE ROLES OF ERK SIGNALING

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Extracellular signal-regulated kinase (ERK) signaling is required for function of the hypothalamic-pituitary-gonadal axis. This axis is regulated by interconnected hormonal feedback loops, permitting reproduction. Gonadotropin releasing hormone (GnRH) is secreted by the hypothalamus to act on the pituitary, resulting in gonadotropin secretion. The gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH) are produced and secreted by pituitary gonadotropes, and act on the gonads, promoting steroidogenesis and gametogenesis. This dissertation focuses on two isoforms of ERK, ERK 1 and ERK 2. Although they do appear to have some redundant functions, ERK 1 is not able to compensate for loss of ERK 2. ERK1 null mice are viable and fertile, whereas loss of ERK2 is embryonic lethal. Therefore, ERK 2 has to be knocked out in a tissue or time dependent manner. For the studies included here, we utilize a mouse model of GnRHR associated ERK loss. This model allows us to investigate the role of ERK in pituitary gonadotropin production and secretion. ERK loss significantly reduced gonadotropin production, and this model allowed us to characterize the effects of hypogonadotropism as animals aged. We followed those studies with an investigation into GnRHR localization and function in the murine placenta, and the effects of ERK loss on placentation, gestation, and parturition. These experiments revealed abnormal histology and vascularization,

prolonged gestation and dystocia, and absolute fetal mortality. Finally, we utilized unbiased screening techniques (RNA sequencing) to identify novel targets of GnRH signaling downstream of the ERK cascade. This revealed a bile acid receptor, TGR5, which has a functional role in gonadotropin production in the pituitary. Female TGR5 knockout are subfertile, with a marked delay in the onset of puberty. The studies in this dissertation describe the role of ERK in multiple aspects of the HPG axis. All of the studies have clinical implications, either in the understanding and treatment of idiopathic hypogonadotropic hypogonadism (IHH) or in understanding links between puberty, nutrition, metabolism and fertility.

BIOGRAPHICAL SKETCH

Jess grew up in a small town outside of Philadelphia, PA. She had a strong interest in animals and veterinary medicine, fostered by her experiences riding horses and volunteering at University of Pennsylvania's New Bolton Center. She attended University of Maryland, College Park for her undergraduate studies, where she graduated with a B.S. in Animal Science and a B.A. in History. During her time at University of Maryland, she worked as an intern at Smithsonian's Conservation Biology Institute, the research arm of Smithsonian's National Zoo. She investigated cheetah reproductive endocrinology, focusing on estrous cyclicity in older females. After graduation from University of Maryland, she completed an internship in Zoo Nutrition at Disney's Animal Kingdom. She next joined a summer research program for veterinary students at Cornell, working with Dr. Mark Roberson, and started veterinary school at Cornell that fall. She was accepted into the Combined DVM/PhD program during her first year of veterinary school, and continued working in Dr. Roberson's lab throughout the next few years. She graduated with her veterinary degree in 2014, and began her thesis research full time at that point. She balanced clinical responsibilities in the veterinary hospital with her research in Dr. Roberson's lab, the results of which are presented in this thesis.

Thanks beyond measure to my parents, Tom and Cheri, and grandparents, Gerry and Sylvia, whose unwavering support and love made this crazy adventure possible

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LIST OF ABBREVIATIONS

GnRH : Gonadotropin-releasing hormone
GnRHR : gonadotropin-releasing hormone receptor
PLC : phospholipase C
IP_3 : inositol 1,4,5-trisphosphate
DAG : diacylglycerol
PKC : protein kinase C
MAPK: mitogen-activated protein kinase
LH : luteinizing hormone
FSH : follicle stimulating hormone
αGSU : common glycoprotein alpha subunit
IHH : idiopathic hypogonadotropic-hypogonadism
HPG : hypothalamic pituitary gonadal axis
JNK : c-Jun N-terminal kinases
ERK : extracellular signal–regulated kinases
ARP3 : Actin related protein 3
ABP : actin-binding protein
PI3K : phosphatidylinositol-3-kinase
PI4K : phosphatidylinositol 4-kinase
pERK : phosphorylated ERK
ICER : inducible cAMP early repression
CREB : cAMP response element binding protein
PKA : Protein kinase A
MEK: mitogen activated kinase kinase
ROS: reactive oxygen species
NOX: NADPH oxidase
DUOX: dual oxidase
GnIH: gonadotropin inhibitory hormone
GRIC: GnRH Receptor IRES Cre
GPCR: G-Protein coupled receptor
HPG: hypogonadal mouse
IHH: Idiopathic hypodonatropic hypogonadism
BMP: Bone morphogenic protein
FGF: Fibroblast growth factor
MAPK: Mitogen activated protein kinase
DAG: Diacylglycerol
MAPKKK: Mitogen activated protein kinase kinase kinase
MAPKK: Mitogen activated protein kinase kinase
JNK: c-Jun n-terminal kinase
BMK: Big MAP kinase
CBP: CREB bind protein
ERKdko: ERK double knockout ($ERK1^{-/-}$, $ERK2^{f/f}$)
ATF3: Activating Transcription Factor 3

EGR1: Early growth response 1
IRES: Internal ribosome entry site
BSA: Bovine serum albumin
PBS: Phosphobuffered saline
RIA: Radioimmunoassay
qRT-PCR: Quantitative real-time polymerase chain reaction
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
Stra8: Stimulated by retinoic acid 8
CL: Corpora lutea
PMSG: Pregnant mare serum gonadotropin
hCG: Human chorionic gonadotropin
GFP: Green fluorescent protein
AF: Antral follicle
TUNEL: Terminal deoxynucleotidyl transferase dUTP nick end labeling
EVT: Extravillous trophoblast
PGF2α: Prostaglandin F2 alpha
CA: Cholic acid
DCA: Deoxycholic acid
FXR: Farnesoid receptor 1
GpBAR1: G-protein bile acid receptor 1
KNDY: Kisspeptin, neurokinin, dynorphin

LIST OF SYMBOLS

α: alpha
β: beta
μ: micro

CHAPTER 1

Literature Review

Introduction

The hypothalamic-pituitary-gonadal (HPG) axis controls reproduction via a series of hormones regulating gonadal function through interconnected feedback loops. Secretion of hypothalamic-derived gonadotropin-releasing hormone (GnRH) integrates inputs from higher brain centers to coordinate the activity of the pituitary gonadotrope and the biosynthesis and secretion of the gonadotropins which ultimately regulate gonadal function.[1] Failure of GnRH to serve as the central integrator of this system has been associated with idiopathic hypogonadotropic-hypogonadism (IHH) and clinical infertility, while pharmacological application of GnRH analogs and gonadotropins has important implications of the treatment of such infertility.[2] IHH is relatively rare, with a prevalence of 1 in 10,000 men.[3] However, it has been estimated that up to 16 percent of couples suffer from infertility, causing huge economic and social implications.[4–6] Further, the GnRH-GnRH receptor (GnRHR) system has been characterized in several types of cancer and may offer therapeutic possibilities in their treatment. Given the central role of GnRH action in the control of fertility, it is of paramount importance to understand the molecular basis of control of GnRH action in the pituitary gonadotrope, including new and novel alternate ways to modulate GnRH action and gonadotropin secretion. The goal of this review is to summarize established knowledge regarding the HPG axis and to discuss several new findings in this field focusing on novel regulators of GnRH action.

(A portion of this Chapter has been accepted as an invited review for publication in *Seminars in Reproductive Medicine* in a manuscript entitled "Novel insights into gonadotropin-releasing hormone action in the pituitary gland".[7]

Development and function of the pituitary in the hypothalamic-pituitary-gonadal axis

The hypothalamic-pituitary-gonadal (HPG) endocrine axis regulates reproductive function. Hypothalamic neurons produce the decapeptide gonadotropin-releasing hormone (GnRH), which is released by nerve terminals at the median eminence and travels through the hypophyseal portal vasculature to the anterior pituitary.[8] The anterior pituitary (adenohypophysis) develops in conjunction with the intermediate and posterior pituitary (neurohypophysis), to form the functional pituitary unit. As a discrete endocrine organ, the anterior pituitary serves to control numerous physiological functions such as control of growth, stress, basal metabolism, lactation and reproduction. This is accomplished through the development of five endocrine cell lineages (gonadotropes, somatotropes, thyrotropes, lactotropes and corticotropes) that collectively function to control systemic homeostasis. Interestingly, these tissues derive from different embryonic origins; the neurohypophysis from neural ectoderm, and the intermediate and anterior pituitary from oral ectoderm.[9] Rathke's pouch, the embryonic origin of the anterior and intermediate pituitary, begins to form at embryonic day (e)7.5 in the mouse.[10] There is a rudimentary pouch by e8.5, and the pouch is fully established at e12.5.[11–13] This anatomic feature was first described in 1830, by H. Rathke, and was further investigated by E. Frazer in 1911.[14,15] Originally,

Rathke's pouch was noted to be derived from both brain and gut tissue. Several of these researchers described the neuro- and adenohypophysis developing separately and fusing as the embryo develops.[9,16] Further differentiation of specific cell lineages and expansion of the gland continues throughout gestation, regulated by factors such as bone morphogenic protein 4 (BMP4), fibroblast growth factor (FGF), sonic hedgehog, Wnt, BMP2, and others.[17,18] Other transcription factors regulate the development of specific cell lineages. For pituitary gonadotropes, these include expression of NR5A1, Lhx3 and Lhx4, Egr-1, Pitx1, and several others.[19–22] Gonadotropes, along with lactotropes, somatotropes, thyrotropes, and corticotropes make up the cell types within the anterior pituitary.[23]

About 10-15 percent of cells in the anterior pituitary are gonadotropes.[24,25] These cells can be identified by the expression of 4 genes: the two unique gonadotropin subunits, luteinizing hormone β (*LHβ*) and follicle stimulating hormone β (*FSHβ*), their common glycoprotein hormone subunit (*αGSU*), and the *GnRHR*.[26] This specific signature is utilized in subsequent chapters of this dissertation to help define effects of various genetic interventions and treatments used in these studies. GnRHR, a G-protein coupled receptor (GPCR) is expressed on the surface of the gonadotrope cell lineage in the anterior pituitary.[27] The receptor couples with GnRH released from the hypothalamus, in a highly evolutionarily conserved pairing. [28]

The GnRHR and GnRH signaling

As mentioned previously, GnRHR is a rhodopsin-like GPCR with high specificity and affinity for its ligand, GnRH.[29,30] Like most GPCRs, GnRHR has 7

transmembrane domains, with three intracellular and three extracellular loops. Broadly, there are three groups of GnRHRs, with only the first two being found in mammals.[31,32] GnRHR I binds with GnRH I, and is the main regulator of the HPG axis. GnRHR II binds to GnRH II, but binds very poorly to GnRH I. Humans functionally only express GnRHR I; GnRHR II is not functional as it contains a frameshift mutation, leading to a stop codon.[31–33] Unlike other GPCRs, GnRHR lacks an intracellular carboxyl terminal tail.[1,32,34] These tails play a role in organizing receptor localization and desensitization following ligand occupancy. In other GPCRs, phosphorylation of the carboxyl terminal tail is involved in membrane internalization and desensitization of GPCRs; however, given the consipcious lack of a c-terminal tails, the GnRHR has been described as resistant to desensitization and downregulation.[35–37] Additionally, only one percent of GnRHRs are found on the cell surface, but addition of a C-terminal tail increases this percentage between 10 and 50 fold suggesting this portion of GPCRs coordinates trafficking and subcellular localization.[38,39] The majority of GnRHRs are found in the ER. [39,40] Those receptors that are found on the cell surface are found exclusively in the membrane rafts. Membrane rafts are small domains within the plasma membrane that are enriched in cholesterol and sphingolipids.[41] In normal conditions, the presence of GnRHR in the raft compartment is exclusive and constitutive. Further disruption of raft association uncouples GnRHR to normal cell signaling suggesting this localization is critical for GnRHR function. GnRHRs can be relocated to non-raft compartments of the plasma membrane if a C-terminal tail from a non-raft associated GPCR is fused to the GnRHR.[42] GnRHR associates with many signaling complexes in the membrane raft,

such as Gαq, c-Raf kinase and the mitogen-activated protein kinase (MAPK) ERK1/2, confirming its involvement in this signaling cascade.[43] GnRH/GnRHR involvement in MAPK signaling is described below.

GnRH binds to the GnRHR, activating the heterotrimeric G protein $G\alpha_q$ which activates phospholipase Cβ (PLC).[38,44,45] This leads to accumulation of PLC-derived diffusible second messengers inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG).[46] These signaling intermediates facilitate calcium (Ca^{2+}) release from endoplasmic reticulum and activation of protein kinase C (PKC), respectively, and PKC activation and membrane depolarization leads to activation of voltage-dependent L-type Ca^{2+} channels leading to influx of extracellular calcium.[47] Release of internal calcium stores and calcium derived from extracellular sources via L-type Ca^{2+}channels along with PKC activation activate key MAPK pathways necessary for the downstream effects of GnRH. These include the biosynthesis of gonadotropin subunits and modulators of MAPK signaling such as dual specificity phosphatases thought to regulate the activation kinetics (magnitude and duration) of MAPKs.[48–50]

MAPK signaling in the HPG axis

MAPKs are a large family of serine-threonine kinases that play important roles in the regulation of cell proliferation, differentiation, migration and apoptosis depending upon developmental and physiological context. They participate in well-established phosphorylation cascades, starting with MAPKKK, which activate MAPKK, and finally phosphorylation of MAPK. The terminal kinases of the four main signaling cascades include extracellular signal-regulated kinase (ERK1/2), c-Jun N-terminal kinase (JNK), big MAPK (BMK), and the p-38 stress-activated protein

kinase.[51–54] Our lab, and research reported within this dissertation, focuses on ERK1/2 signaling. The canonical ERK signaling pathways is comprised of cRaf protein kinase, which phosphorylates MAP-ERK (MEK) kinases 1 and 2, which phosphorylates ERK1/2 on threonine and tyrosine residues to induce activation of these kinases.[55] After phosphorylation by MEKs, ERKs translocate from the cytoplasm to the nucleus, where they phosphorylate and activate transcription factors such as CREB, c-Fos, c-Jun, Elk1, and Egr1.[56] Despite a wealth of data supporting this canonical cascade, work in our lab has shown that other MAPKKKs may be involved in ERK1/2 activation, as cRaf kinase phosphorylation is not required for ERK activation by GnRH.[57] Thus, a definitive understanding of this signaling pathway is not yet complete. However, an intact MAPK cascade is necessary for proper function of the gonadotropes and gonadotropin release.[26,58]

GnRH stimulation of pituitary gonadotropes is required for the biosynthesis of LH and FSH. These two glycoprotein hormones are heterodimers composed of αGSU and their respective β-subunits; the β subunits are requisite for the specific biological actions of the two gonadotropins. LH and FSH act upon the gonads to regulate gametogenesis and steroidogenesis and ultimately ovulation.[27] Specific evidence for GnRH requirement comes from studies of the hypogonadal (HPG) mouse, a model with a naturally occurring loss-of-function mutation in the GnRH gene. The HPG mouse is infertile, with arrested germ cell development, and lacks GnRH, LH and FSH.[59] Restoration of the GnRH gene restored gonadotrope cell function and fertility to these mice.[60] This mouse has been a commonly used model system to understand the role of specific aspects of the HPG axis.[61,62]

GnRH and gonadotropin pulsatility

LH is secreted by the pituitary gonadotropes in a pulsatile fashion coincident with pulsatile secretion of GnRH.[63] A GnRH surge, and subsequent LH surge is required for ovulation.[64] Several labs have shown the importance of the signaling cascades initiated by GnRH, including ERK, on LH production and secretion. Important factors include CREB binding protein (CBP), ERK, FOXP3, Pitx1, and Egr1[65–69]

Another important signal in controlling gonadotropin secretion stems from steroid hormones and the feedback loops they participate in. Androgens inhibit LHβ transcription, while estradiol promotes LHβ transcription, the latter a likely function of estradiol's ability to upregulate the GnRHR within the gonadotrope at times important to preparation for ovulation.[70,71] GnRH stimulation has a direct link to LH release; pulsatile stimulation of GnRH from the hypothalamus in pituitary causes a LH pulse event in sheep.[72] Multiple other factors contribute to this relationship, such as post-partum interval and estradiol concentration.[73] Fasting also decreased LH pulsatility, but leptin administration restored normal LH secretion, indicating a link between metabolic status, energy balance, and gonadotropins.[74] Other factors that alter GnRH and LH secretion include endogenous opioid concentrations and photoperiod.[75]

Like LH, FSH is impacted by GnRH pulse frequency, transcription factor expression, such as SMAD, Lhx3, Pitx1 and 2, and steroid hormone concentration.[76–79] However, it is also regulated by other hormones, such as inhibin, activin, and follistatin. Inhibin and activin are secreted locally in the pituitary, and act in both paracrine and autocrine fashions. They colocalize in granules with LH and FSH, and

are secreted simultaneously.[80] Inhibin and activin are also secreted from the gonads, regulating the gonadotropes through systemic circulation as well as locally.[81] Thus, while LH and FSH share a dependence on GnRH stimulation of the gonadotrope, FSH clearly requires additional integration by secreted factors that do not control LH biosynthesis and secretion. Studies in this dissertation focus on novel factors controlling the secretion of gonadotropins.

For both gonadotropins, GnRH pulse frequency is a significant regulator of production and release. Early studies in primates by Knobil and colleagues revealed that continuous exposure to GnRH resulted in diminished gonadotropin synthesis and secretion while pulsatile administration elevated production and secretion of gonadotropins. Later studies by the Kaiser group and others more fully appreciated the impact of slow versus rapid GnRH pulses and the impact of variable interpulse interval on gonadotropin subunit gene expression; consistent with pulse frequencies observed during the menstrual and estrous cycles, slow GnRH pulse frequency favored FSHβ expression while more rapid pulses favored LHβ expression.[82–87] More recently, GnRH-induced signals derived from such intermediates as Protein kinase A (PKA), cAMP response element binding protein (CREB), inducible cAMP early repression (ICER), PI3K and PI4K were found to greatly impact how differences in GnRH pulse frequency affected gonadotropin synthesis and secretion.[88] These signaling intermediates are activated by GnRH stimulation at variable pulse frequency, and can be influenced by other hormones such as inhibin, activin, and steroid hormones which helps to produce and control gonadotropin production and secretion.[89] The signaling relationship between PKA, CREB and ICER was

particularly intriguing since PKA activates CREB and ICER is a CREB repressor. Thompson and colleagues demonstrated that within the GnRH signaling network, slow GnRH pulsatility favored FSHβ expression and specific inhibition of PKA catalytic activity decreased GnRH-induced CREB phosphorylation, and subsequent FSHβ transcription.[90] GnRH stimulation also increased ICER levels in gonadotropes; however, this increase was blocked with PKC and MEKI/II inhibitors suggesting GnRH-induced ICER levels were ERK dependent.[88] Inhibition of PKA and CamKII did not inhibit ICER induction.[88]

To determine a potential role of ICER on the impact of PKA/CREB, studies were conducted in LβT2 cells using variable pulse frequencies. Rapid pulses stimulate *Lhb* expression, and low pulse frequency known to regulate *Fshb* expression through CREB activation. ICER accumulation in gonadotropes was higher with high GnRH pulse frequency compared to low pulse frequency, and this response was blunted with MEKI/II-ERK pathway inhibition. It may be that low frequency GnRH pulses preferentially induce PKA activation and CREB phosphorylation, leading to preferential FSH biosynthesis and secretion.[90] Rapid GnRH pulse frequency preferentially increases LHβ and ICER accumulation, effectively blocking a role for CREB on FSHβ under these conditions.

Recent advances in our understanding of gonadotrope biology and GnRH action

Due to the vital and dynamic role of the anterior pituitary-derived gonadotropins in controlling the reproductive axis, understanding gonadotrope regulation and function by GnRH and other factors are areas of active research. This is

an enormous field of study that has been reviewed extensively by many including our lab.

Clinical conditions such as IHH in humans may best characterize the absolute requirement for GnRH and GnRH signaling in the support of fertility. This condition is often caused by a failure in GnRH neuron migration from the olfactory placode to the hypothalamus, and when combined with anosmia, is referred to as Kallmann's syndrome.[91] This syndrome has been associated with multiple genetic mutations including those in *FGF8, FGFR1, KAL1, SEMA-3A, GNRHR, PROK2, PROKR2, and KISSR*, among others.[2,92] Failure of the GnRH neuronal migration leads to a lack of GnRH stimuli to the gonadotrope and subsequent failure of the reproductive axis. These patients suffer from infertility and often are insensitive to GnRH stimulation.[93] However, the variation in phenotypes can even occur between siblings with the same genetic mutation.[94] IHH patients who are treated for fertility often have variable responses to exogenous GnRH and gonadotropin treatment.[95–100] Phenotypes involved include everything from infertility to weight gain, to miscarriage, deafness and anosmia.[91,101,102] With multiple known mutations in GnRH and GnRHR that result in IHH, understanding the mechanisms and pathways of GnRH related signaling in multiple tissues can help lead to understanding and treatment of this condition.[95,101,103] Appropriately, GnRH and its agonists have come to play important roles in both fertility treatments and contraceptive regimens.[104,105]

In addition to the role of GnRH/GnRHR in controlling fertility, more recent studies provide evidence that GnRH and GnRHR are important outside of the HPG axis. Tran et al recently explored local effects of FoxL2 deletion in the pituitary and

the testes, using a GnRHR associated Cre line.[76] GnRHR is alsoexpressed in the ovary, oviduct and lymphocytes.[106–108] In addition, GnRHR has recently been characterized in the placenta of multiple species, including humans, mice, and dogs.[109–111] Knowledge of these extra-pituitary sites of GnRHR expression have important implications for my dissertation research and will be examined in subsequent chapters of this thesis. GnRH and GnRHR are associated with multiple types of cancer, including prostate, ovarian cancer, benign prostate hyperplasia, adrenal adenoma, leiomyoma, nasopharyngeal carcinoma, and gastric cancer. GnRH and its receptor may have roles in treatment, metastasis, prognosis and other aspects of cancer biology, both within and outside of the HPG axis.[112–118] With these novel and emerging functions of GnRH, understanding its signaling and function is of paramount importance.

GnRHR and spatially restricted Ca^{2+} signaling

As described above, GnRH-induced calcium signaling in gonadotropes has long been established as a potent modulator of gonadotrope function and gonadotropin secretion.[119–121] Calcium flux induced by GnRH signaling induces secretion of gonadotropins and activation of MAPK cascades, which lead to LH and FSHβ subunit gene and GnRHR transcription via extracellular signal–regulated kinases (ERK) and c-Jun N-terminal kinases (JNK), as well as changes in the actin cytoskeleton. These Ca^{2+} responses are particularly integral to ERK activation and subsequent gonadotropin release.[120,122] GnRH-induced ERK activity is required for fertility in female mice, and genetic loss of both ERK isoforms conditionally in the gonadotrope

leads to an anovulatory infertility in female mice (a focus on my dissertation research).[65] Two separate pools of calcium appear to govern the ability of GnRH to discriminate activity of MAPK signaling pathways; GnRH-induced ERK activation is regulated by calcium influx through L-type Ca^{2+} channels, while activation of JNK isoforms appears to require a much larger Ca^{2+} flux from intracellular stores.[120] Interestingly, these calcium spikes are not all equivalent but appear to correlate with the magnitude and duration of GnRH input to the gonadotrope. Low levels of GnRH cause small, irregular Ca^{2+} transients, but larger amplitude GnRH pulses cause larger changes in Ca^{2+} signaling.[121] Variation in frequency and amplitude of these transients help differentiate signals for FSH and LH production. For example, LH biosynthesis is tightly regulated by these Ca^{2+} signals, with an intense peak of production at the GnRH/LH surge, inciting ovulation. In contrast, FSH is produced at a more constant, basal level, without the supraphysiologic drive of a large secretory event.

The hypothesis that local membrane-associated Ca^{2+} transients were necessary for GnRH-induced ERK activity was originally substantiated by pharmacological studies that disrupted the L-type channel; however, these studies did not provide important evidence that new elegant imaging strategies can provide.[48] Understanding how calcium transients are controlled at local, spatially restricted levels within the cell would potentially inform pharmacological interventions that would be useful in the control of fertility. Using novel total internal reflection fluorescence (TIRF) microscopy, Dang and colleagues were able to visualize Ca^{2+} influx via L-type Ca^{2+} channels following activation of GnRHR signal transduction. Recall that TIRF microscopy takes advantage of strategies of imaging of fluorescent activities directly

at the cell surface offering the opportunity to examine spatially restricted fluorescent signals rather than Ca^{2+} transients that occur more deeply within the cytosol. The spatially restricted Ca^{2+} flux was appropriately termed calcium "sparklets" that occurred at the surface of the gonadotrope when stimulated with GnRH.[120] These sparklets were recapitulated with an L-type Ca^{2+} channel agonist FPL 64176, which also resulted in ERK activation reminiscent of GnRH stimulation. If these L-type Ca^{2+} channels were blocked using nicardipine, or the cytoskeleton was stabilized pharmacologically, the sparklets and subsequent ERK activation was prevented, indicating spatially restricted calcium influx through L-type channels is a key calcium signal induced by GnRHR activation leading to ERK activation.[120] ERK activation is known to occur through direct PKC activation to mimic GnRH action, and in accordance with that, a PKC agonist activated calcium sparklets in a manner consistent with GnRH or FPL 64176. Interestingly, GnRH-induced cytoskeletal actin reorganization also appears to be required for the induction of spatially restricted calcium sparklets and ERK activation.[120] Pretreating cells with an actin stabilizer (jasplakinolide) effectively preventing actin reorganization followed by treatment with GnRH inhibited calcium sparklets and ERK pathway activation. Both intra-and extracellular calcium stores play key roles in gonadotrope function.

GnRH action and the actin cytoskeleton

An important role for GnRH-induced actin reorganization and LH secretion was recently described by our group.[123] With GnRH stimulation, the actin-associated protein cortactin migrates to the leading edge of gonadotropes, localizing with actin

and actin-related protein 3 (Arp3), an effect mediated by phosphorylation of tyrosine kinases.[123] Blocking either cortactin or ARP resulted in the loss of actin-dependent lamellipodia and membrane ruffles, as well as gonadotropin secretion. The actin remodeling necessary for response to GnRH is dependent on cortactin re-localization within the cytoskeleton, as is gonadotropin secretion.[123,124]

Recent studies by Edwards and colleagues extended our understanding of the mechanisms of the role of actin cytoskeleton in GnRH signaling via L-type Ca2+ channels and ERK activation.[125] Dynamin is a membrane-associated GTPase that is involved in membrane constriction, vesiculation and cell wall formation.[126] Disrupting dynamin signaling in gonadotropes has long been known to disrupt GnRH signaling to the ERK pathway[127–129], however, the mechanistic basis for this has not been fully appreciated. By creating a dynamin-GFP fusion protein and expressing it in clonal gonadotrope cells (αT3-1 cells), Edwards and colleagues were able to visualize the distribution and changes in localization of dynamin with or without GnRH stimulation. Dynamin localizes in the lamellipodia and membrane protrusions with actin and actin binding protein (ABP) after GnRH stimulation. Inhibiting the GTPase activity of dynamin via dynasore decreased ERK activation, but did not alter JNK phosphorylation. Dynasore treatment also led to inhibition of actin reorganization after treatment with GnRH, and subsequent decreases in L-type Ca^{2+} channel activity, as measured by TIRF microscopy described above. Either inhibiting dynamin or the L-type Ca^{2+} channels decrease calcium influx at the membrane. Actin cytoskeletal remodeling, necessary for gonadotropin secretion, is dependent on cortactin

localization and these studies implicate dynamin signaling and calcium influx through L-type Ca^{2+} channels in this process.[125]

A role for PI3K/PI4K signaling within the GnRH signaling network

The role of phosphatidylinositol-3-, and phosphatidylinositol 4-kinases (PI3K and PI4K, respectively) in ERK activation was recently investigated by several groups.[130,131] Clonal αT3-1 and LβT2 cells treated with phorbol ester, a PKC agonist, showed persistent ERK1/2 phosphorylation (pERK) consistent with a known role for PKC signaling in ERK activation in gonadotropes. Interestingly, pharmacological inhibition of PI3K or PI4K resulted in reduced ERK phosphorylation following GnRH and EGF treatments.[130] The gonadotropin subunits were variably affected by inhibition of PI3K/PI4K. Common glycoprotein αGSU production was inhibited by PI3k/PI4K inhibition, LHβ production was unaffected, and FSHβ production was increased, underscoring the gonadotrope's ability to differentially regulate FSH and LH production, likely via separate pathways.[130] While complex, these studies supported the conclusion that PI3K/PI4K is involved in the regulation of ERK activity and gonadotropin production via the GnRHR in important gonadotrope cell lines and it will be critical to examine the role and requirement of these pathways in vivo using genetically modified mice to fully realize the impact of these pathways on fertility.

A role for DICER in the gonadotrope

Another important factor in gonadotrope cell function is the intracellular enzyme DICER. DICER is a member of the RNAse III family and functions to cleave

double stranded RNA molecules involved in the production of small interfering- and micro-RNAs in cells. Wang and colleagues created a novel gonadotrope-specific CRE mouse, using a *Fshb* promoter-Cre recombinase fusion to specifically target gonadotropes. Mating this CRE line with a conditional $DICER^{f/f}$ allele resulted in the specific deletion of *DICER* in murine gonadotropes.[78] Loss of DICER in gonadotropes reduced gonadotropin production, testes size, and resulted in complete infertility. In female mice, this deletion resulted in hypoplastic uteri and smaller ovaries; the ovaries lacked corpora lutea suggestive of ovulation failure; and these mice exhibited abnormal estrous cycles and low circulating levels of progesterone. The FSHβ-cre mouse line provides an important opportunity for specific Cre expression and conditional gene deletion in the pituitary gonadotropes since other gonadotrope-specific Cre lines have the potential for mis-expression in tissues within the reproductive axis other than gonadotropes. Further, this new mouse model implicates an important role for DICER and RNA metabolism within the gonadotrope where loss of DICER results in a marked infertility.[78] The implications for how DICER and RNA metabolism affect reproductive function is likely to be far-reaching due to the complexity of how individual microRNAs impact differentiated gonadotrope cell function.

Relative effects of GnRH and a common agonist, Buserelin

Both human and veterinary medicine utilize buserelin, a GnRH agonist, for treatment of infertility.[132–135] However, there are known off-target effects, such as neuropathic enteritis associated with the use of this agonist in vivo.[135,136] Nederpelt

and colleagues used whole cell impedence, as well as inositol phosphate accumulation assays to understand the differences between the effects of the native GnRH and the pharmacologically super active agonist buserelin. As anticipated, these studies revealed buserelin had a higher efficacy and potency, when compared to GnRH.[137] Along with higher efficacy, buserelin also had a longer binding duration than GnRH. When cells were treated with either ligand and washed, approximately 80% of buserelin treated cells remained active after washing, while only 30% of GnRH treated cells remained active.[137] Understanding the differences between the pharmacokinetics of the endogenous ligand and the agonist can aid in creating treatment protocols and informing clinical decisions. Recent work has shown buserelin has a role in treatments besides fertility, and understanding its mechanism of action can help improve treatments for conditions such as cancer, embryonic mortality and cryptorchidism.[138–145]

Novel modulators of GnRH action

Phoenixin

GnRH is the primary regulator of LH biosynthesis and secretion while GnRH and activin are important regulators of FSH.[81,146] More recently other modulators of GnRH action have come to light. A potential role for Phoenixin was recently described by Yosten and colleagues.[147] Using a genome-wide screen, this group identified novel protein sequences that were conserved across species. Phoenixin was identified in this screen, found in highest abundance within the magnocellular and parvocellular paraventricular nucleus, as well as the supraoptic nucleus in the

hypothalamus. When combined with GnRH administration, Phoenixin increased LH production and release, ostensibly through increasing expression of the GnRHR.[147] *In vivo*, rats treated with siRNA to knock down Phoenixin expression displayed disrupted estrous cycle behavior and reduced GnRHR within the pituitary gland suggesting this peptide may play an important role within the neuroendocrine axis.[147]

Treating hypothalamic derived GT1-7 cells with Phoenixin increased both the GnRH and GnRHR expression, indicating its potential importance within the HPG axis. Although speculative, Phoenixin appears to act on GPR173 in the hypothalamus, which also couples with a metabolite of GnRH (GnRH-(1-5)) to increase GnRHR expression.[148,149] GPR173 is a G-protein coupled receptor linked to Gαs and cAMP signaling. Consistent with this observation, the Belsham group linked Phoenixin/GPR173 signaling to PKA/CREB and C/EBPβ activation and up-regulation of a number of gene targets including GnRH, GnRHR, Kisspeptin and Oct-1 expression in hypothalamic neuronal cell lines.[150] Coupled with evidence from knockdown studies described above, Phoenixin is emerging as an important regulator of reproductive function and a potential modulator of GnRH action.

Intracellular reactive oxygen species (ROS)

Kim and Lawson demonstrated in both the gonadotrope –derived LβT2 cell line and in primary pituitary cell cultures that GnRH stimulation increased intracellular reactive oxygen species (ROS) such as superoxide radicals, hydroxyl radicals and hydrogen peroxide.[151] These free radicals are produced from NADPH oxidase (NOX) and dual oxidase (DUOX) enzyme family, which use NADPH as an

electron donor, and create ROS as a by-product.[152] ROS signaling and NOX family members have been linked to control of the reproductive axis; however, their specific role(s) in the pituitary gonadotrope had not yet been elucidated.[152–155] NOX/DUOX subunit mRNAs and protein levels were increased by GnRH stimulation in mouse pituitary cells in primary culture and gonadotrope cell lines.[151] Pharmacological inhibition of NADPH oxidase decreased GnRH-induced ROS production indicating that the NOX/DUOX pathway is activated through GnRH signaling. Interestingly, NOX/DUOX enzymes appear to be necessary for JNK and ERK activation and subsequent regulation of the gonadotropin subunit genes; inhibition of NOX/DUOX or the use of a ROS scavenger decreased GnRH-induced JNK and ERK activation. DUOX1 and DUOX2 are both regulated by intracellular Ca^{2+} stores and inhibition of DUOX enzyme activity reduced calcium-induced ERK activation; interestingly, inhibition of NOX activity did not affect MAPK activation state induced by GnRH suggesting that DUOX activity may hold primary importance. DUOX inhibition also decreased GnRH-induced immediate early gene responses, which led to decreased gonadotropin subunit production and secretion.[151] *FSHβ* appears to be preferentially mediated by Duox2. This important link between cellular metabolism and gonadotrope cell function may have important implications on how the reproductive system perceives and manages adverse metabolic conditions.[151]

The GnRHR and extracellular ATP production

The GnRHR is present within discrete plasma membrane domains termed membrane rafts within gonadotropes. Productive signaling between the GnRHR and

the ERK pathway requires localization of the GnRHR in rafts.[41,43,156] Recently, our own lab carried out proteomic studies examining the co-localization of the GnRHR with other potential signaling proteins within the raft domain using immunoprecipitation of the GnRHR and flotillin 1 (a raft marker) followed by mass spectrometry.[157] These studies revealed that the GnRHR and flotillin 1 colocalized with the F_0F_1 ATP synthase complex in the membrane rafts. The ATP synthase complex is normally found in the inner mitochondrial leaflet and is central to ATP production with a cell; the presence of the ATP synthase complex and subunits of the electron transport chain at the cell surface were unexpected. Using αT3-1 cells, subunits of the ATP synthase complex and electron transport chain were confirmed by immunoprecipitation, flow cytometry and cell surface biotinylation studies leading to the determination that the catalytic surface of the complex pointed outward into the extracellular space.[157] This spatial configuration was confirmed by analyzing the production of extracellular ATP in the media when αT3-1 cells and whole mouse pituitary explants were treated with ADP as a substrate for synthase activity. Extracellular ATP synthesis could be inhibited by a number of inhibitors to the synthase, perhaps the most revealing was removal of inorganic phosphate from the cell culture media or with prolonged GnRH exposure which appeared to downregulated the ATP synthase from the cell surface of the gonadotrope. Using a sheep model, substrate for the ATP synthase (ATP/ADP) was detected in plasma from the hypothalamic-pituitary portal vasculature indicating that hypothalamic input into the system was possible. Extracellular ATP synthesis appears to plays a modulatory role in gonadotropin secretion. GnRH-induced LH secretion was increased in the presence

of extracellular ADP/ATP suggesting that extracellular ATP "tone" could modulate the LH secretion.[157] While the *in vivo* implications of this finding are not entirely clear, extracellular ATP levels may be an important modulator of GnRH-induced LH secretion.

Gonadotropin inhibiting hormone

Gonadotropin inhibitory hormone (GnIH) is a hypothalamic decapeptide that inhibits the synthesis and release of gonadotropins, acting on both the hypothalamic GnRH neurons as well as pituitary gonadotropes.[158,159] Originally, this peptide was found to be regulated by melatonin, and implicated in seasonal reproductive differences, especially in avian species.[158,160] As research progressed, a link between environmental stimuli such as weather, stress, and GnIH was uncovered. This link was expanded to include poor reproductive performance during times of stress, where GnIH levels is an important modulator of the reproductive axis.[161] This correlation was confirmed in an ovine model examining secretion of GnIH into the hypothalamic portal system. Release of GnIH from the median eminence (ME) occurs at higher levels during the nonbreeding season, ostensibly inhibiting reproduction.[162] Testing the direct relationship between environmental stress and GnIH secretion, Clarke and colleagues subjected ewes to both long and short term stressors. Long term administration of adrenocorticotropin decreased LH secretion, increased the number of GnIH-containing neurons and GnIH mRNA levels in individual GnIH-neurons but did not affect the levels of GnIH detected in plasma at the ME. Moreover, these stressors appeared to increase the number of GnIH nerve fibers that occurred in proximity to

GnRH neurons. Interestingly, short-term stressors such negative auditory stimuli (dog barking) followed by induction of hypoglycemia with insulin or a separate stressor induced by LPS administration did not change GnIH levels at the ME, but increased neuronal activation of GnIH neurons and the contact fibers between GnIH and GnRH cells.[163] These new studies provide compelling evidence that GnIH is a potent negative regulator of reproduction during times of chronic and acute stressors. Manipulation of the GnIH systems has clear and important implications as a pharmacological target for GnRH inhibition.

CONCLUSIONS

Understanding and controlling reproduction is critical in the face of a growing global population and sustainability of food supplies to support such a population. Conversely, issues related to infertility also strain health care, particularly in developed countries. These issues span human medicine and species barriers; for example, controlling pet populations and those of invasive species, while improving reproduction of critically endangered species has important relevance. Moreover, insuring adequate production of food and fiber often relies upon efficient reproductive strategies. GnRH, a potent regulator of reproduction as an integral and required part of the HPG axis; GnRH and the GnRHR are critical targets for manipulating reproduction. Thus, understanding the potential modulators and inhibitors of GnRH action in the pituitary and hypothalamus, as well as the function and intricacies of the intracellular signaling pathways increases the ability to modulate gonadotrope cell behavior. Similarly, understanding non-canonical signaling pathways from outside of

the HPG axis has helped to identify and characterize novel targets for pharmacological/medical intervention in the control of fertility.

This introduction has worked to highlight recent advances in research involving GnRH and GnRHR action. This work has uncovered novel binding partners, activation mechanisms, functional behavior and signaling mechanisms regarding GnRH. However, more areas of research exist, especially in understanding the clinical implications and treatment of IHH. Specifically, understanding the progression of IHH and the result of long term loss of gonadotropin stimulation has been under investigated to this point. Additionally, the role of GnRH and GnRHR in the placenta has not been thoroughly explored, especially in the context of ERK signaling. IHH patients have difficulty with conception and throughout pregnancy, for reasons that have not been thoroughly elucidated (Chapter 3). In addition to understanding different facets and clinical implications of the loss of GnRH signaling, gonadotropins, and ERKs in various physiologic time points and tissues, we hoped to elucidate additional targets and modulators of GnRH signaling in the context of the ERK1/2 deficient mouse model system (Chapter 2). My dissertation research seeks to define physiological mechanisms both in the context of the HPG axis and other aspects of reproduction, as well as modulators of GnRH action related to other areas such as metabolism and metabolic regulation of reproductive potential (Chapter 4). We hypothesize ERK1/2 signaling in GnRHR expressing cells is a central regulator of reproduction in multiple areas, including aging, pregnancy and metabolism.

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CHAPTER 2

Sex- and age-specific impacts of ERK loss within the pituitary gonadotrope

ABSTRACT

ERK (extracellular signal-regulated kinase) signaling regulates the reproductive axis, but specific mechanisms have yet to be completely elucidated. Conditional gene targeting allows isolation and analysis of ERK-dependent mechanisms controlling gonadotrope cell function. In the present study, an ERK1 null and ERK2 floxed mouse was combined with gonadotrope-specific Cre (GRIC) driver. The mice were characterized for reproductive abnormalities to understand the role of ERK signaling in the gonadotrope during aging. As observed previously using a different pituitary-specific Cre driver, female ERK double knockout (ERKdko) animals were hypogonadotropic, leading to infertility through altered estrous cyclicity and anovulation. Transcript levels of four gonadotrope specific genes (*GnRHR*, and the three gonadotropin subunits) were reduced in pituitaries at estrus. Further, post-castration response to endogenous GnRH stimulation was blunted. As females aged, they exhibited abnormal ovarian histology, as well as increased bodyweight. ERKdko males were initially less affected, showing moderate subfertility up to 6 months of age. Male ERKdkos also displayed a blunted response to endogenous GnRH following castration. By 12 months of age, ERKdko males had reduced testicular weight and sperm production. By 18 months of age, the ERKdko males retained only 23% of sperm production occurring coincident with reduced testis and seminal vesicle weight, and marked seminiferous tubule degeneration. These studies support speculation that hypogonadotropism secondary to loss of ERK signaling within the reproductive axis has important impacts on reproductive competence in both sexes and shed new light

on the pathophysiology of aging in idiopathic hypogonadotropic hypogonadism (IHH).

INTRODUCTION

The hypothalamic-pituitary-gonadal (HPG) axis regulates reproduction through multiple interconnected endocrine feedback loops.[1] The hypothalamus secretes gonadotropin releasing hormone (GnRH) from the median eminence, which travels through the hypophyseal portal system to act upon gonadotrope cells in the anterior pituitary.[2,3] These gonadotrope cells are characterized by expression of GnRH receptor (GnRHR), and comprise approximately 10- 15% of the pituitary cells.[4] In response to GnRH stimulation, the gonadotrope produces two peptide hormones: luteinizing hormone (LH) and follicle stimulating hormone (FSH).[5] They are made from two gonadotropin specific subunits, LHβ and FSHβ, which combine with a common glycoprotein subunit (αGSU), to create bioactive gonadotropins.[6] In turn, these hormones act upon the gonads to control steroidogenesis and gametogenesis.[7,8]

Isolating the role of specific signaling pathways and genes in discrete tissues or cell types in the HPG axis aids in understanding both the function of the axis and can lead to increased ability to manipulate, augment or restrict reproductive endocrine function. To this end, we focused on the role of extracellular signal-regulated kinase 1 and 2 (ERK1/2) in the pituitary gonadotropes. Whole body knockout of ERK2 results in embryonic lethality, but ERK1 null animals are viable and fertile.[9–11] Therefore, understanding the role of ERK2 in reproduction requires a tissue specific knockout. In the pituitary, ERK is necessary for immediate early gene activation. Activation of

factors like early growth response protein 1 (Egr1), c-Fos, activating transcription factor 3 (ATF3), and Nur77, along with the gonadotropin hormone subunits, is crucial for appropriate response to GnRH stimulation and are all ERK signaling-dependent.[12,13] Thus, ERK1/2 signaling is an integral regulator of gonadotrope cell function, as well as the function of multiple parts of the HPG axis.[14–18]

Multiple labs, including our own, have used gonadotrope specific models to understand the role of genes or signaling intermediates in the neuroendocrine axis.[19–21] Previously, Bliss et al used the *αGSU* promoter regulating Cre recombinase expression to understand the role of ERK1/2 in pituitary gonadotropes.[20] While very useful, expression of *αGSU* is not specific to pituitary gonadotropes, as the Cre was also expressed in thyrotropes. Alteration of the thyroid hormone axis has clear effects on reproductive potential leaving open questions of how exactly to interpret the role of ERK signaling within the reproductive axis. A novel gonadotrope specific Cre, the *GnRHR* IRES Cre (GRIC) mouse, has more recently been used to study ablation of specific genes in the pituitary gonadotropes.[4,22] This Cre driver was knocked in downstream of the GnRHR coding region, along with an IRES sequence. This results in a bicistronic mRNA, and independent translation of the *GnRHR* and the Cre recombinase.[4] *GnRHR* is activated at e13.5, markedly later than e9.5, when the *αGSU* Cre was activated, potentially accounting for some of the differences between the two models. [20,22]

Utilization of the GRIC model, in combination with an $ERK1^{-/-}$, $ERK2^{fl/fl}$ background, provides specific ablation of *ERK2* localized to the gonadotrope within the pituitary. However, this Cre has been shown to be expressed in the testes and

placenta as well[19] (Unpublished data, Roberson lab). The current studies more fully characterize the reproductive phenotype of the ERK deficient males regarding the impact of ERK signaling loss on gonadotrope cell function and fertility. ERK signaling is altered in cellular aging, senescence, and oxidative damage, and alteration in ERK expression can change cellular response to these insults.[23–29] Based upon these observations we investigated sex and age-related impacts of ERK1/2 loss within the reproductive system.

Both males and females show changes in the HPG axis as they age. Changes in gonadotropins, steroid hormones and gonadal function have been documented in aging animals and humans. In rodent models, serum gonadotropins have been shown to have altered secretion and cyclicity in aging female animals, with decreased LH and increased FSH, [30,31] as well as altered steroidogenesis and decreased serum progesterone.[25] Interestingly, these effects could be mitigated by repeated pregnancy or progesterone supplementation.[33] Males also display the same alterations in gonadotropin levels, decreased quality and quantity of LH and increased FSH, corresponding inversely to sperm count.[33–35] Although ERK's role in reproduction has been well characterized, understanding the effects of ERK loss in the pituitary, and subsequent hypogonadism, as animals age could help to understand and mitigate the effects of IHH.

We investigated the effects of ERK1/2 loss in aged animals, as well as those of reproductive age.[23–27] The present studies show that loss of ERK signaling clearly impacts the timing of reproductive senescence in males and female that is likely attributable to loss of gonadotropic stimulation and or local effects of GRIC-medicated

ERK deletion in the testis. Collectively our studies provide valuable insights into the effect(s) of hypogonadatrophic hypogonadism on gonadal competence as mice and potentially human patients age.

MATERIALS/METHODS

ANIMALS

ERK1 null (*ERK1*$^{-/-}$), ERK2 floxed (*ERK2*$^{fl/fl}$) and *GnRH* receptor IRES Cre (GRIC) mice have been described previously.[4,19,20] ERK1/2 knockout animals were designated ERKdko (*ERK1*$^{-/-}$, *ERK2*$^{fl/fl}$, *Cre*$^{+/-}$) and compared with Control animals (*ERK1*$^{-/-}$, *ERK2*$^{fl/fl}$, *Cre*$^{-/-}$). Animals were handled in compliance with the Cornell University Institutional Animal Care and Use Committee. For breeding challenge, males of both genotypes were paired with one control and one ERKdko female. Females were checked daily for copulatory plugs, and monitored for changes in body weight and signs of pregnancy and parturition.

GENOTYPING

Genomic DNA was isolated from tail snips (3 mm), or an equivalent quantity of other tissues as indicated, using a E-Z Tissue DNA Kit (Omega Biotek, Norcross, GA) per manufacturer's instructions. Routine PCR genotyping was performed on animals as previously described.[36] PCR confirmation of ERK1 knockout, ERK2 flox, Rosa26 reporter, Stra8 cre, and GnRHR Cre alleles were performed with primers listed below.

Primer		Sequence (5’ to 3’)
Rosa26 Reporter	Rosa26 Forward	TAA GCC TGC CCA GAA GAC TC
	Rosa26 Reverse	AAA GTC GCT CTG AGT TGT TAT
	Rosa26 Common	TCC AGT TCA ACA TCA GCC GCT ACA
ERK1	ERK1 Forward	AAG GTT AAC ATC CGG TCC AGC A
	ERK1 Reverse	AAG CAA GGC TAA GCC GTA CC
ERK2	ERK2 Forward	AGC CAA CAA TCC CAA CCC TG
	ERK2 Reverse	GGC TGC AAC CAT CTC ACA AT
GnRHR	GnRHR Forward	GAA CTA CAG CTG AAT CAG TC
	GnRHR Reverse	CTC TAA CAA ACT CTG TAC A
	GnRHR Homozygous	CGG AAT TCA TCG ATC ATA TCA GAT CC
Stra8	Stra8 Forward	GTG CAA GCT GAA CAA CAG GA
	Stra8 Reverse	AGG GAC ACA GCA TTG GAG TC

HISTOLOGY

Tissues were fixed in 10% formalin, paraffin embedded, serially sectioned at 4μm, and stained with hematoxylin and eosin using standard histological techniques. Sections were scanned and digitized using an Aperio Scanscope (Vista CA), and analyzed using ImageScope (Leica Biosystems, Buffalo Grove, IL). For characterization of the ovarian follicular population, every third section was examined microscopically for identification of luteal tissue in both ERKdko and control females. For characterization of the testicular tissues, every third section was examined microscopically for gross evaluation. The largest sections were chosen, choosing 4 slides total, and 20 seminiferous tubules were chosen for analysis from each section on all slides in both ERKdko and control males. Tubule diameter was averaged between all slides and animals from each group.

VAGINAL CYTOLOGY

The vaginal vault was swabbed to make a cytological smear. The smear was stained with Wright's Giemsa stain, and examined with light microscopy. 100 cells were counted, and epithelial cells and leukocytes were differentiated on the basis of morphology. An animal was deemed to be in estrus with >85% superficial epithelial cells.

EPIDIDYMAL SPERM COUNT

After euthanasia, testes and epididymis were dissected free. The epididymis was placed in 1ml 4 percent BSA in PBS, and tubules extracted. The preparation was incubated (32^{o}F) for 20 minutes. 480ul of 10% formalin was mixed with 20ul of the preparation, and placed on a hemocytometer, and sperm were counted to determine sperm numbers and assessed for morphology.

GONADECTOMY

Ovariectomy and castrations were performed under Avertin (Tribromoethanol, Sigma-Aldrich, St. Louis, MO) general anesthesia, with standard aseptic techniques. The castrations were performed with ventral midline incisions and the ovariectomies were performed with flank incisions. They were given ketoprofen postoperatively for pain control. The animals were euthanized 7 days post-operatively, and blood and pituitaries were collected.

SERUM PEPTIDE HORMONE ANALYSIS

The blood was allowed to clot for 15 minutes, then centrifuged for 10 minutes at 2500 rpms. The serum was collected and frozen at -80 F. Pituitaries were snap frozen. Serum was analyzed at University of Virginia Ligand Core through RIA multiplex, in duplicate or using in-house FSH and LH assays as previously described.[37] Testosterone was assayed using a commercially available kit (IBL, Minneapolis, MN) per the manufacturer's instructions.

RNA ISOLATION AND QUANTITATIVE PCR

Tissues were collected and Trizol (ThermoFischer, Waltham, MA) extraction was performed per manufacturer's instructions to isolate total RNA. Reverse transcription in 1000ug reactions was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer directions. qRTPCR was performed using SYBRGREEN (ThermoFisher, Waltham, MA) and primers listed below. Amplifications were carried out using a BioRad CFX96 Touch Real-Time OCR Detection System (BioRad, Berkeley, CA). RNA levels were standardized using the internal control Gapdh and assessed using ddCT methodology.[20]

Primer		Sequence (5' to 3')
Glyceraldehydes-3-phosphate dehydrogenase	*Gapdh* forward	ATGTTTGTGATGGGTGTGAA
	Gapdh reverse	ATGCCAAAGTTGTCATGGAT
Gonadotropin Releasing Hormone Receptor	*GnRHR* forward	TGCTCGGCCATCAACAACA
	GnRHR reverse	GGCAGTAGAGAGTAGGAAAAGGA

Luteinizing Hormone β-subunit	*LHβ* forward	CTGAGCCCAAGTGTGGTGTG
	LHβ reverse	GACCATGCTAGGACAGTAGCC
Follicle Stimulating Hormone β-subunit	*FSHβ* forward	GCCATAGCTGTGAATTGACCA
	FSHβ reverse	AGATCCCTAGTGTAGCAGTAGC
α-Glycoprotein Subunit	*α-GSU* forward	TCCAGGGCATATCCCACTCC
	α-GSU reverse	CATTTCCCATTACTGTGGCCTTA

B-Galactosidase *in vitro* assay

For β-galactosidase *in vitro* assays, tissues were fixed in 4% paraformaldehyde/PBS for 1 hour at 4°C, then rinsed 3 times for 30 minutes each in a rinse buffer (100 mM sodium phosphate (pH 7.3), 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP-40 (by volume)). Sections were stained overnight in staining buffer (rinse buffer with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/ml X-gal). Sections were fixed overnight in 10% formalin, then washed with distilled water twice for 30 minutes. They were dehydrated by sequential ethanol washes (70%, 95%, twice in 100%, then washed in methyl salicylate until the tissue cleared.

STATISTICS

Pairwise comparisons were made by Student's *t*-test for most data. When appropriate, a one-way analysis of variance was used, with Tukey's post-hoc test. All data are expressed as means ± standard error of the mean. A p value of <0.05 was considered statistically significant.

RESULTS:

ERKdko females are infertile, hypogonadotropic, and anovulatory

Female control and ERKdko mice were paired with control males and monitored daily for copulatory plugs. While 100 percent of control females exhibited copulatory plugs within the first 3 days of pairing, only 33 percent of ERKdko females exhibited copulatory plugs when paired with males for 30 days. All control females had litters at approximately 20 days post plug, while no ERKdko animals were observed to have litters (Table 1).

Due to the complete infertility of ERKdko animals, vaginal cytology was used to assess dynamics of the estrous cycle. Control animals had a cycle length of 5.3 ± 0.26 days. ERKdko animals had significantly longer interestrus interval, with 9.2 ±1.01 days between estrus (Table 1). The ERKdko animals showed normal diestrus and estrus cytology (Figure 1A and Table 1); however, both diestrus and estrus intervals were significantly prolonged, with a clear lack of normal periodicity seen in control females[20]. To better understand the relationship between estrous cycle behavior and ovarian activity, ovaries were collected at estrus and examined by histology. While both control and ERKdko animals showed ovarian follicles in various stages of maturation, control ovaries displayed an average of 6.3 corpora lutea per ovary compared to a conspicuous absence of CLs in the ERKdko ovaries, indicating an anovulatory phenotype (Figure 1B and Table 2). Body weight, uterine wet weight and ovarian weights at estrus were not significantly different between control and ERKdko animals at 6 months of age (Table 2).

To assess additional impacts of ERK deletion on gonadotrope cell function in female mice, we utilized qPCR to determine the abundance of the four genes known to define the gonadotrope cell lineage (*LHβ*, *FSHβ*, *αGSU*, and *GnRHR*). These studies demonstrate decreased mRNA expression of all four gonadotrope genes at estrus in ERKdko females, compared to control females (Figure 1C). This was consistent with basal levels of FSH and LH in circulation in ERKdko and control females (Figure 2A & B; sham).

To determine the response of ERKdko and control females to endogenous hyper-stimulation by GnRH, animals were sham operated or ovariectomized and then euthanized after 5 days to analyze changes in serum gonadotropins. There was a significant increase in serum FSH and LH following ovariectomy in control animals which did not occur to the same extent in ovariectomized ERKdko females (Figure 2A, B). These findings were generally consistent with previously published data from our group using the *αGSU* Cre driver with the following exception.[20] Current use of the GRIC Cre driver resulted in a more robust inhibition of response of FSH following castration which may reflect greater penetrance of the GRIC Cre in this model.

TABLE 1: ERKdko animals have significantly longer estrous cycles, spend more time in both diestrus and estrus, have smaller litters and are anovulatory compared to control animals

	Control	**GRIC ERKdko**
Estrous cycle length (d)	**5.3 ± 0.3 [a] (n=5)**	**9.2 ± 1.0 [b] (n=5)**
Mean Litter size	**7.0 ± 0.6 [a] (n=3)**	**0 [b] (n=6)**
Time in estrus (d)	**1.4 ± 0.1 [a] (n=5)**	**3.2 ± 0.5 [b] (n=5)**
Time in diestrus (d)	**1.9 ± 0.2 [a] (n=5)**	**4.3 ± 0.5 [b] (n=5)**
CL/ovary	**6.3 ± 1.3 [a] (n=6)**	**0 [b] (n=6)**

Figure 1: ERKdko are infertile due to hypogonadotropism and anovulation A) ERKdko animals exhibit irregular and prolonged estrous cycles compared to control animals. **B)** Ovaries from ERKdko animals showed no CLs, compared to approximately 6 per ovary for control animals. **C)** ERKdko animals had significant reduction in all four signature gonadotrope gene transcript levels (αGSU, LHβ, FSHβ, GnRHR) compared to control animals. ($p<0.05$) **D)** These transcript levels remained significantly lower in aged ERKdko animals (12 months), when compared to aged control littermates ($p<0.05$).

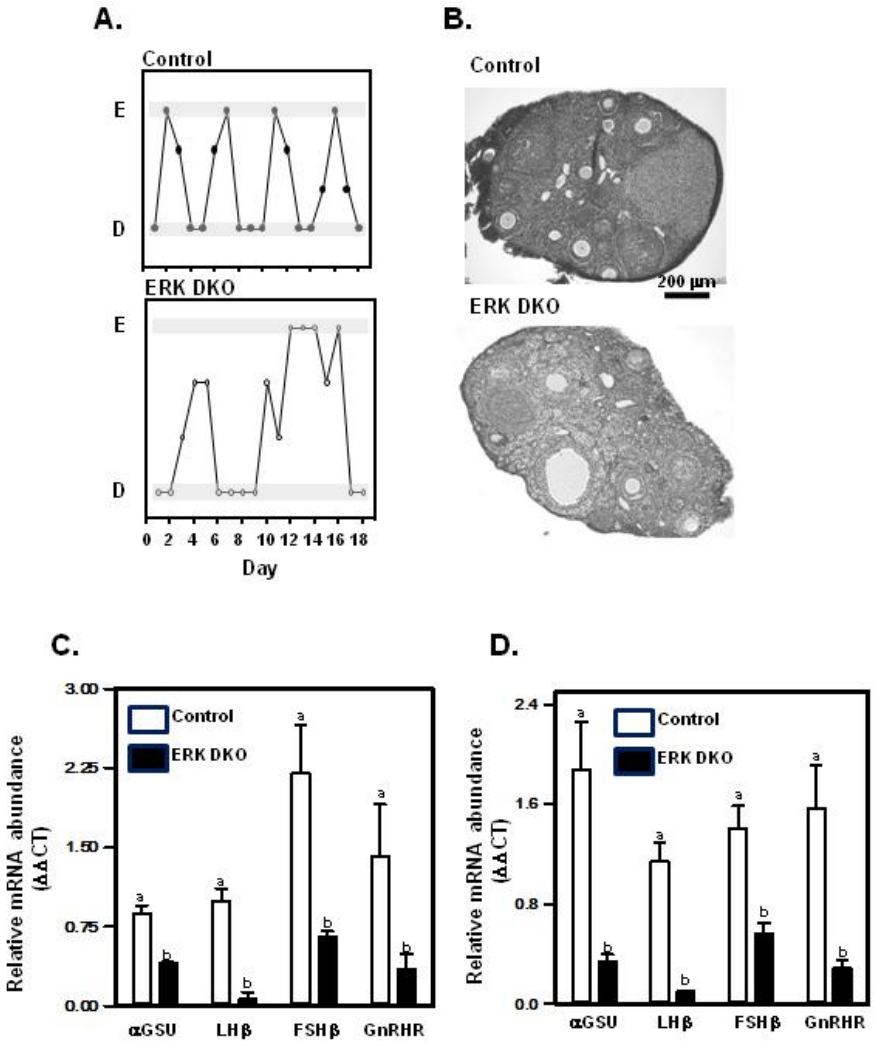
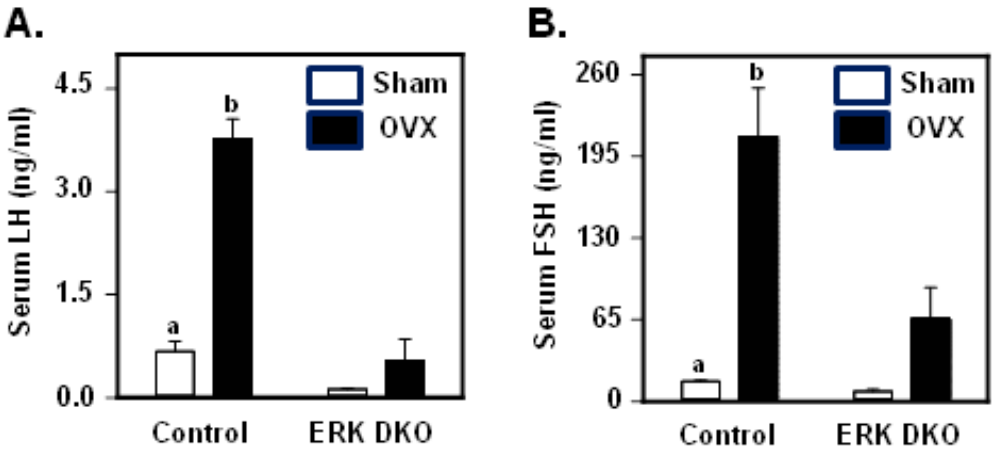

A.
Control
E
D
ERK DKO
E
D
0 2 4 6 8 10 12 14 16 18
Day
B.
Control
200 μm
ERK DKO
C.
Relative mRNA abundance (ΔΔCT)
3.00
2.25
1.50
0.75
0.00
Control
ERK DKO
a
b
αGSU
LHβ
FSHβ
GnRHR
D.
Relative mRNA abundance (ΔΔCT)
2.4
1.6
0.8
0.0
Control
ERK DKO
αGSU
LHβ
FSHβ
GnRHR

Table 2: Aged (12 month) ERKdko animals showed increased body weight, but decreased uterine and ovarian wet weight as a percentage of body weight

	Control	**GRIC ERKdko**
Body weight (gm; 6 mos)	24.3 ± 1.4^{a} (n=14)	27.4 ± 1.3^{a} (n=21)
Body weight (gm; 12 mos)	28.9 ± 0.6^{a} (n=23)	36.3 ± 1.2^{b} (n=12)
Ovarian WT/BW (mg; 6 mos)	$3.0 \times 10^{-3} \pm 0.1 \times 10^{-3a}$ (n=4)	$2.2 \times 10^{-3} \pm 0.1 \times 10^{-3a}$ (n=4)
Ovarian WT/BW (mg; 12 mos)	$1.2 \times 10^{-3} \pm 0.09 \times 10^{-3a}$ (n=13)	$0.8 \times 10^{-3} \pm 0.1 \times 10^{-3b}$ (n=7)
Uterine WT/BW (gm; 6 mos)	0.05 ± 0.1^{a} (n=4)	0.03 ± 0.003^{a} (n=4)
Uterine WT/BW (gm; 12 mos)	0.04 ± 0.004^{a} (n=13)	0.02 ± 0.001^{b} (n=7)

Figure 2: ERKdko animals have a blunted gonadotropin response to castration. A) Control animals showed significant increases in LH following castration and maximized endogenous GnRH. This effect was blunted in ERKdko animals, who did not show a significant difference in LH levels between castrated and sham op females. B) Similarly, ERKdko animals showed a blunted FSH response to endogenous GnRH following castration. ($p<0.05$).


A.
Serum LH (ng/ml)
4.5
3.0
1.5
0.0
a
b
Sham
OVX
Control
ERK DKO
B.
Serum FSH (ng/ml)
260
195
130
65
0
a
b
Sham
OVX
Control
ERK DKO

Female ERKdko animals show altered age-related changes in body weight and ovarian histology

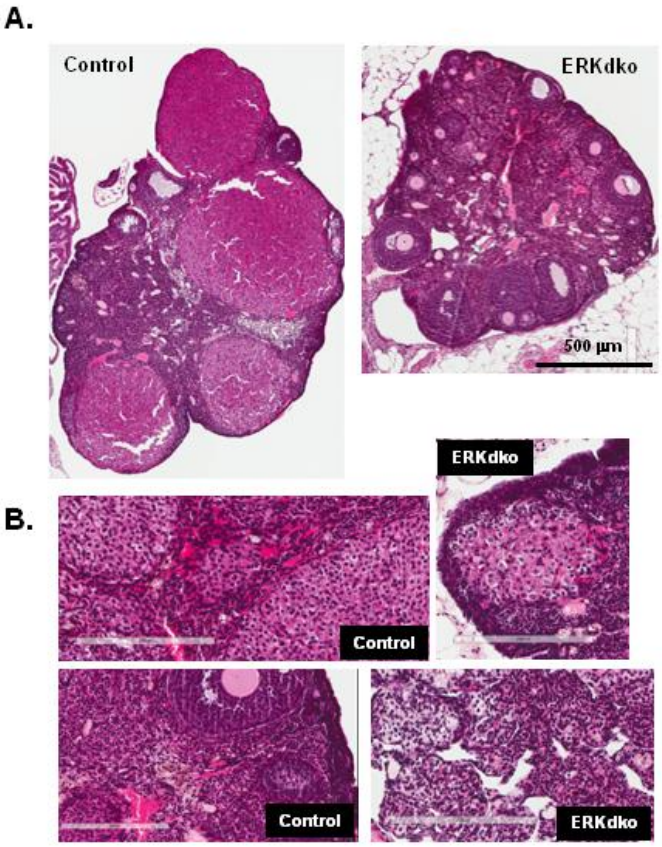
Female control and ERKdko animals were maintained until approximately 12 months of age. These animals received identical access to food and water and were not given the opportunity to reproduce. At the time of weaning through 6 months of age, there were no differences in body weight between genotypes. At 12 months of age, body weight was ~25% higher in ERKdko females compared to control females (Table 2). Absolute ovarian and uterine weights were reduced in ERKdko older females and this was amplified when presented as ovarian and uterine weight/unit body weight compared to the control genotype (Table 2).

Aged ERKdko females (12 months) also showed signs of abnormal ovarian histology presumable reflecting premature reproductive aging and senescence. Consistent with the younger ERKdko females, aged ERKdko animals showed an absence of CLs. Further, aged females showed loss of normal ovarian architecture with abnormal accumulations of extra-cellular matrix and regions of marked acellularity. Ovarian histology in control animals appeared unremarkable with multiple CLs present and normal architecture (Figure 3A & B). Comparison of uterine histology between genotypes in the aged females was unremarkable (data not shown) suggesting that changes in ovarian architecture were specific within the reproductive axis.

To understand the changes in pituitary gonadotrope function, we performed qRT-PCR on pituitaries from 12 months old females. Transcript levels of the four

gonadotrope genes were not significantly different between 6 month old and 12 month old control animals. However, all of these transcript levels were significantly reduced in aged ERKdko animals. LHβ showed the most significant reduction, with an 11-fold decrease compared to control animals (Figure 1D).

Figure 3: Aged ERKdko animals show alterations in ovarian histology. A) ERKdko animals show abnormal accumulations of extra-cellular matrix and regions of marked acellularity. B) Higher magnification of areas of acellularity and ECM accumulation


A.
Control
ERKdko
500 µm
B.
ERKdko
Control
Control
ERKdko

Male ERKdko animals show moderate subfertility

Males of both genotypes were capable of producing copulatory plugs, and there was no difference between control and ERKdko males in days to first plug or number of copulatory plugs needed to produce a pregnancy (data not shown). However, ERKdko males sired smaller litters compared to control males (Figure 4A). There was no significant difference in testis weight between control and ERKdko males at 6 months of age, even when normalized to body weight (Table 3). However, ERKdko males displayed a mild, but statistically significant reduction in sperm count at 6 months of age (Table 3). This corresponded with a modest reduction in seminiferous tubule area (Figure 4B). Sperm morphology was grossly normal for both genotypes (data not shown).

To assess the effect(s) of ERK deletion on gonadotropin subunit and GnRHR mRNA levels, qPCR was performed on pituitaries from control and ERKdko males at 6 month of age (Figure 4C). Consistent with responses in ERKdko females at estrus (Figure 1C), ERKdko males displayed reduced expression of LH and FSH β mRNAs compared with control males. Common glycoprotein hormone α subunit and GnRHR mRNAs were not statistically different between genotypes.

Figure 4: ERKdko males show reduced fertility. **A)** ERKdko animals had a significantly reduced litter size compared to control animals (4.8 versus 7.1 pups per litter) **B)** ERKdko animals have significantly reduced seminiferous tubule area compared to control animals. **C)** 4 month old ERKdko animals have significantly reduced transcript levels of LHβ and FSHβ, compared to control animals. Reductions in αGSU and GnRHR were not significant. **D)** 18 month old ERKdko animals had significant reduction in αGSU, LHβ, and FSHβ transcript levels compared to controls. ($p<0.05$ for all significant differences)

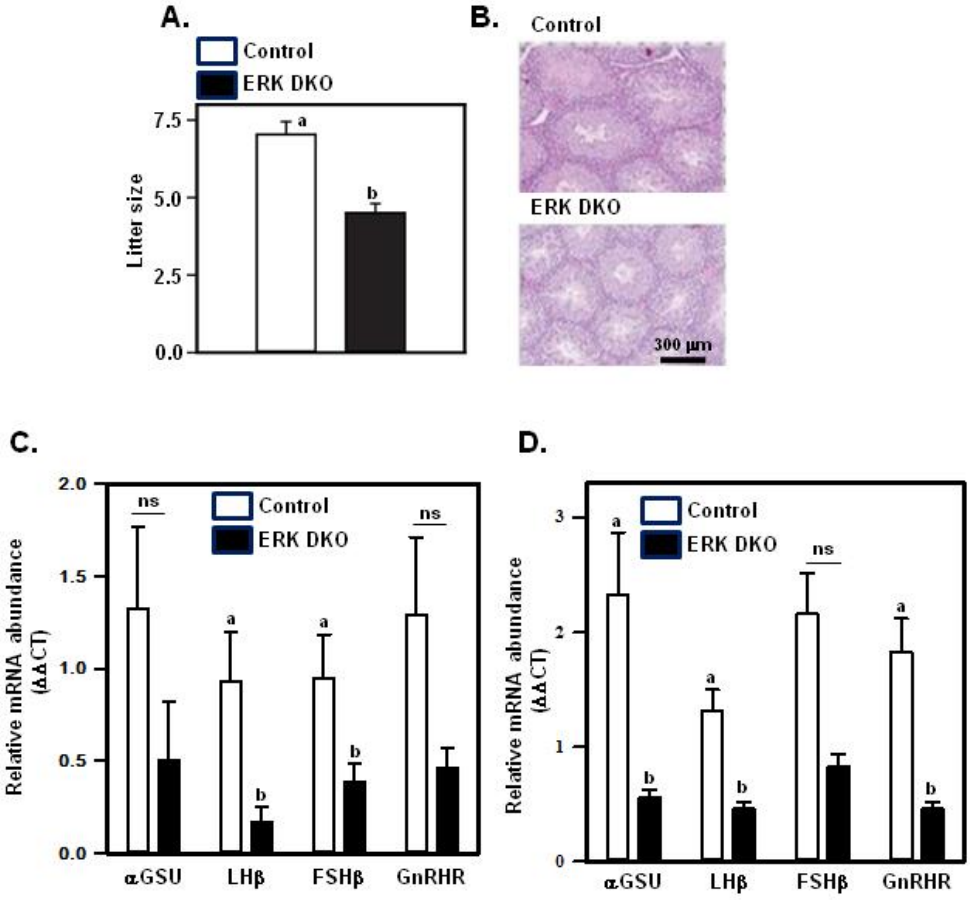
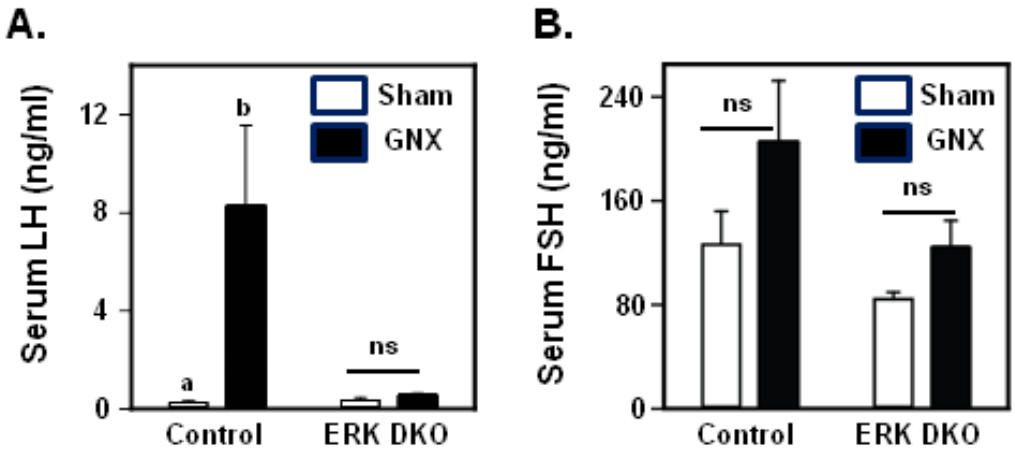

A.
Control
ERK DKO
Litter size
7.5
5.0
2.5
0.0
a
b
B.
Control
ERK DKO
300 µm
C.
Relative mRNA abundance (ΔΔCT)
2.0
1.5
1.0
0.5
0.0
ns
Control
ERK DKO
a
b
αGSU
LHβ
FSHβ
GnRHR
D.
Relative mRNA abundance (ΔΔCT)
3
2
1
0
Control
ERK DKO
ns
αGSU
LHβ
FSHβ
GnRHR

Table 3: Male ERKdko animals show reduced testis to body weight ratio, seminal vesicle weight and sperm count as they aged, when compared to age matched controls

	Control	GRIC ERKdko	STRA8 ERKdko
Testis (mg)/BW (g) 6 mos	$3.6x10^{-3} \pm 0.1x10^{-3a}$ (n=7)	$3.4x10^{-3} \pm 0.4x10^{-3a}$ (n=6)	
Testis (mg)/BW (gm) 12 mos	$3.8x10^{-3} \pm 0.1x10^{-3a}$ (n=14)	$2.5x10^{-3} \pm 0.1x10^{-3b}$ (n=12)	
Testis (mg)/BW (gm) 18 mos	$4.1x10^{-3} \pm 0.1x10^{-3a}$ (n=14)	$2.8x10^{-3} \pm 0.2x10^{-3b}$ (n=12)	$3.7x10^{-3} \pm 0.1x10^{-3c}$ (n= 6)
Seminal Vesicle (mg) 6 mos	288.2 ± 22.3^{a} (n=7)	250.2 ± 16.6^{a} (n=6)	
Seminal Vesicle (mg) 12 mos	395.3 ± 54.3^{a} (n=14)	258.4 ± 29.6^{a} (n=12)	
Seminal Vesicle (mg) 18 mos	362.7 ± 24.8^{a} (n=14)	257.8 ± 8.2^{b} (n=12)	429.3 ± 60.5^{c} (n= 6)
Total epididymal sperm 6 mos	$20.6x10^{6} \pm 0.8x10^{6a}$ (n=7)	$17.0x10^{6} \pm 1.1x10^{6b}$ (n=6)	
Total epididymal sperm 12 mos	$14.7x10^{6} \pm 0.4x10^{6a}$ (n=14)	$7.8x10^{6} \pm 0.2x10^{6b}$ (n=12)	
Total epididymal sperm 18 mos	$13.4x10^{6} \pm 0.6x10^{6a}$ (n=14)	$3.8x10^{6} \pm 0.4x10^{6b}$ (n=12)	$5.3x10^{6} \pm 0.3x10^{6c}$ (n= 6)

To assess the impact of hyper-stimulation with GnRH, male control and ERKdko animals were castrated or underwent a sham surgery and then euthanized after 7 days and serum concentrations of LH and FSH were measured. Again, consistent with control females, castration resulted in a marked increase in LH secretion compared to sham operated controls (Figure 5A). While castration resulted in a numerical increase in FSH in circulation, this change was not statistically significant (Figure 5B). Similar responses to castration on LH secretion were not evident in the ERKdko males (Figure 5A).

Figure 5: ERKdko males show blunted LH response to maximized endogenous GnRH stimulation post castration A) Control animals show a significant increase in serum LH concentration following castration. However, this response was blunted in ERKdko animals. **B)** Neither control nor ERKdko animals showed a statistically significant FSH increase in response to castration.

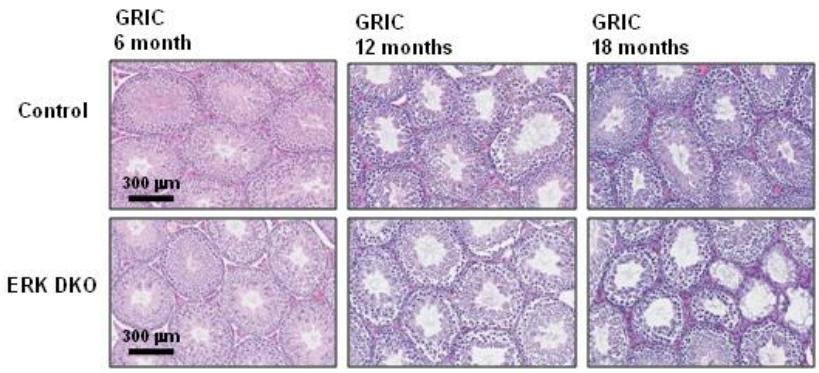

A.
Serum LH (ng/ml)
12
8
4
0
a
b
ns
Sham
GNX
Control
ERK DKO
B.
Serum FSH (ng/ml)
240
160
80
0
ns
ns
Sham
GNX
Control
ERK DKO

Males show premature reproductive aging, characterized by testicular dysplasia

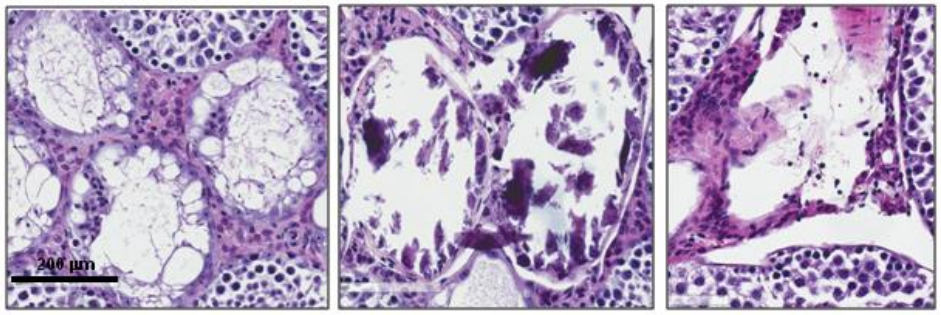
Control and ERKdko males were assessed at 6, 12, and 18 months of age for body weight, testis size, sperm count, and seminal vesicle weight (Table 3). By 12 and 18 months of age, ERKdko animals had significantly lower sperm count and testis weight. Seminal vesicle weights in ERKdko animals were reduced in the 18 month group compared to controls. Aging related abnormalities were also evident in testicular histopathology (Figure 6). Testicular histology revealed evidence of testicular dysplasia in ERKdko animals at 18 months, but not in age matched control testes. The testes showed areas of marked testicular degeneration, calcification, aspermatic tubules and giant spermatid cells (Figure 6).

Figure 6: Aged ERKdko animals show loss of normal testicular histology. A) Testes of ERKdko animals showed grossly normal morphology until 18 months of age, where they display signs of testicular degeneration and dysplasia, such as calcification, aspermatic tubules, and giant spermatid cells. **B)** High magnification showing degeneration, calcification and loss of tubules.

A.

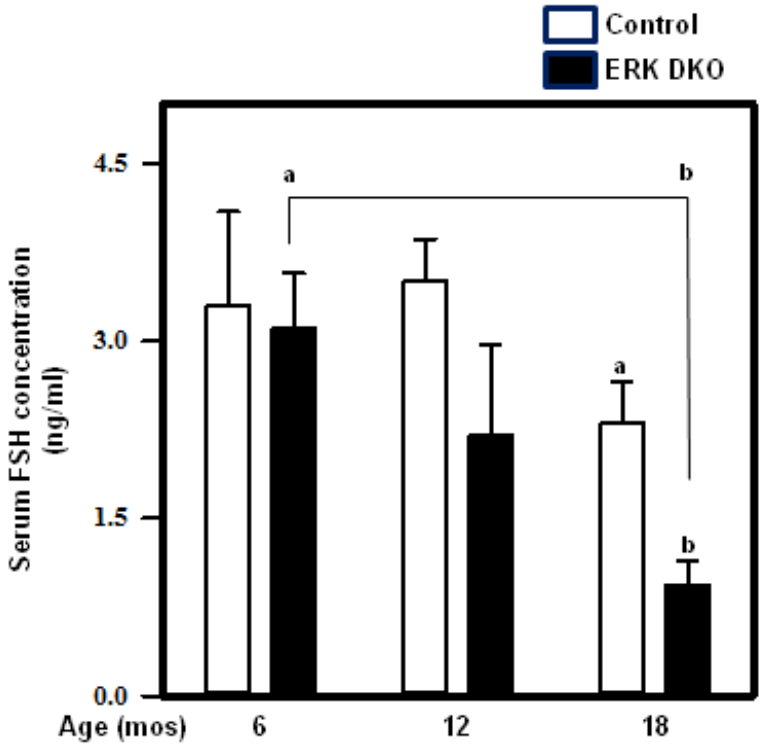

GRIC
6 month
GRIC
12 months
GRIC
18 months
Control
ERK DKO
300 µm
300 µm

B.


200 µm

The four signature gonadotrope gene transcript levels were assessed in pituitaries of males at 18 months of age. Neither control nor ERKdko animals showed significant changes in transcript levels within genotypes between 6 and 18 months. However, there was a significant decrease between Control and ERKdko animals at 18 months in *LHβ*, *αGSU* and *GnRHR*.(Figure 4D) Interestingly, we found no significant differences between genotypes or ages in circulating testosterone levels indicating that despite low levels of LH, these were sufficient to maintain testosterone levels in ERKdko males (data not shown). Serum LH and FSH assays were also performed. FSH levels were not significantly different between genotypes or ages at 6, 9, or 12 months. However, aged ERKdko animals (18months) had a significant reduction in serum FSH, approximately a 3 fold decrease compared with 6 month animals and control animals at 18 months of age (Figure 7).

Figure 7: ERKdko animals showed a significant decrease in serum FSH at 18 months of age ($p<0.05$).


Control
ERK DKO
Serum FSH concentration (ng/ml)
4.5
3.0
1.5
0.0
a
b
a
b
Age (mos)
6
12
18

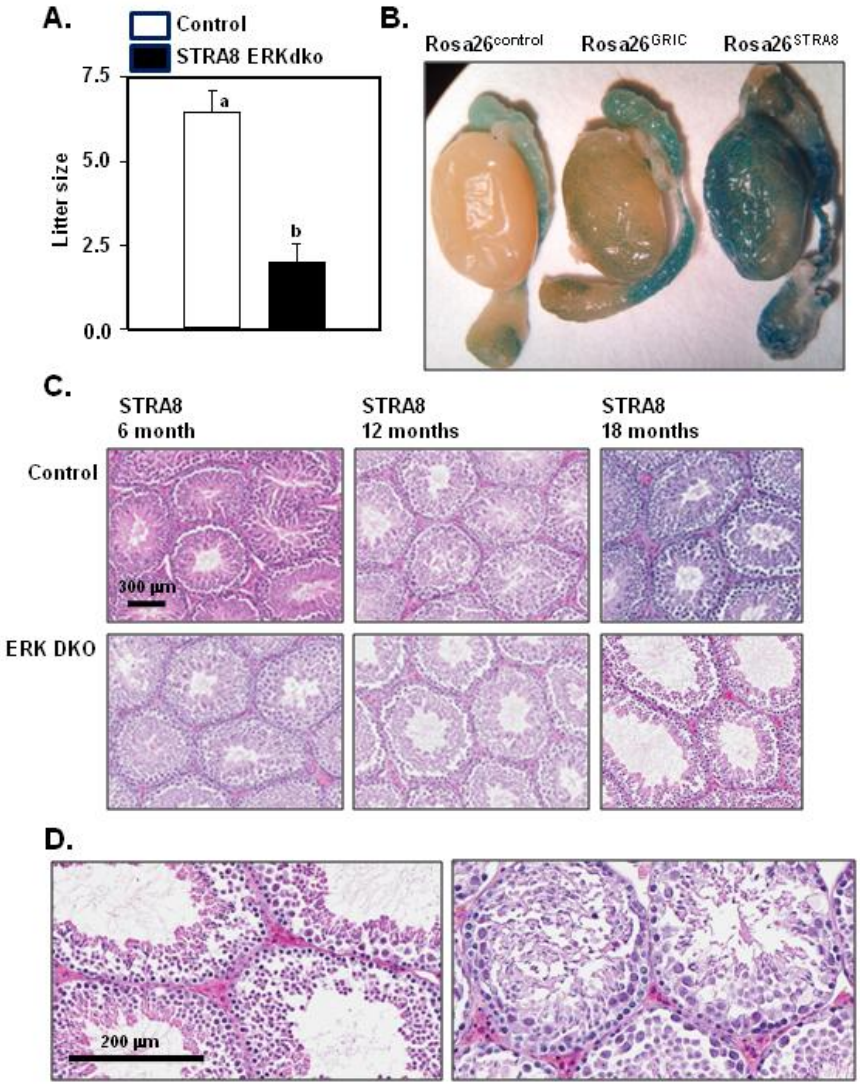
Phenotype of aging ERKdko males is likely due to hypogonadotropism, not loss of testicular ERK signaling

Other labs utilizing the GRIC Cre driver have reported Cre expression within the testes, specifically in the germ cell lineage.[19] In order to parse out potential effects of gonadotrope-specific ERKdko versus lesions that might be attributable to loss of ERK signaling directly within the testes, we examined reporter activity in Rosa 26 mice expressing a GRIC Cre allele using *in situ* β-galactosidase staining (Figure 8B). These studies supported the prediction from others that GRIC Cre activity is present within the testes but not the ovary.

To further understand the differential effects of ERK signaling in the pituitary and testes, we characterized aged males from a *Stra8* Cre line on the same ERKdko background. Consistent with the GRIC-Cre driver, the *Stra8* Cre driver is expressed in the spermatogonia, as well as in later stages of spermatogenesis.[38] Analyzing testicular histology, as well as reproductive phenotype, of the *Stra8* ERKdko males would allow us to understand whether the phenotype seen in the GRIC Cre ERKdko animals was specifically due to testicular or gonadotrope loss of ERK signaling. Histology of *Stra8* Cre ERKdko animals revealed minor degenerative changes at 18 months of age compared to control animals, consistent with the advanced age of the animals. By comparison, testicular degeneration was markedly more severe in the GRIC Cre ERKdko animals.(Figure 8C) Loss of ERK signaling in the Stra8 males showed a moderate decrease in sperm count, a severe decrease in litter size, but interestingly no significant change in testicular size, testes to body weight ratio, body weight or seminal gland weight compared to control animals. (Table3, Figure 8A) Aside from

the reduced sperm count, Stra8 ERKdko animals more strongly phenocopied the control animals than the GRIC ERKdko males, indicating the likelihood that the testicular degenerative phenotype seen in the GRIC ERKdko males was due to prolonged hypogonadotropism, not a direct effect of ERK signaling loss within the germ cell lineage of the testis.

Figure 8: Testicular degeneration in ERKdko animals is due to hypogonadotropism, not loss of testicular ERK A) Stra8 animals show significantly smaller litter sizes than control animals (7.1 versus 2 pups/litter). **B)** Both GRIC and STRA8 Rosa26 animals showed β- galactosidase expression *in situ* in the tubules of the testes. **C)** 18 month old Stra8 animals did not show the testicular degeneration seen in 18 month old ERKdko animals


A.
Control
STRA8 ERKdko
7.5
5.0
2.5
0.0
Litter size
a
b
B.
Rosa26control
Rosa26GRIC
Rosa26STRA8
C.
STRA8
6 month
STRA8
12 months
STRA8
18 months
Control
ERK DKO
300 µm
D.
200 µm

DISCUSSION:

Previous work in our lab has shown the role of ERK signaling within the pituitary is less essential to reproduction in males than in females; however, given the potential contributions of the thyrotrope lineage in these studies, the absolute role of ERK loss in the gonadotrope was unclear.[20] We and others have attempted to understand the mechanisms and pathways involved in gonadotropin production using more specific gonadotrope Cre-mediated gene excision[19–21]. In the current studies, GRIC-mediated ERKdko animals of both sexes were hypogonadotropic, with a reduced response to endogenous GnRH stimulation. Both also showed reduced fertility at 6 months of age, with males being subfertile and females being infertile and anovulatory. We hypothesize this difference is due to the variation in LH and FSH requirements between males and females. In males, LH and FSH levels in ERKdko animals appear to be sufficient to maintain fertility, however, this is suboptimal. Females, on the other hand, have a notably more complex requirement for gonadotropins in reproduction, particularly in LH biosynthesis leading to the preovulatory surge and ovulation.[20] Loss of ERK signaling in females precludes appropriate LH accumulation necessary for the LH surge. Although ERK signaling has been shown to be the primary pathway for gonadotropin biosynthesis, other signaling cascades have been proven to be able to create small amounts, which is likely how the ERKdko animals retain gonadotropin function. [39–41]As the animals aged in this hypogonadotropic model, both sexes showed loss of normal gonadal histologic architecture, which was not seen in control aged animals or *Stra8* Cre ERKdko aged males.

The requirement for pituitary ERK signaling in female mice in the present studies was generally similar to previous work done before in our lab by Bliss et al.[20] However, several points of comparison are noteworthy. The GRIC Cre excision appears to be exclusive to the pituitary gonadotrope, without expression in other pituitary cell lineages such as the thyrotrope[4]. Much like the *αGSU* Cre ERKdko animals, the loss of the ERK2 function in gonadotropes ablates the ability of female mice to produce a functional LH surge, capable of inducing ovulation. This was confirmed by the blunted LH serum levels in ERKdko females following castration. Interestingly, although FSH production was also blunted, females appeared to have similar numbers of follicles, including large antral follicles compared with control females suggesting that FSH levels were sufficient for normal ovarian folliculogenesis. As mentioned before, though the males also show blunted gonadotropin production in response to endogenous GnRH production, they only demonstrate moderate subfertility as young animals. There were some important differences between the models. The GRIC ERKdko females proceed through all phases of the estrous cycle, and are acyclic, instead of displaying anestrous similar to the *αGSU* females. One of the primary differences between the αGSU and GRIC models of ERK deletion was in FSH production. Both males and female ERK deficient models, reduction in FSH transcript levels and secretion was more robust in the GRIC model. While the reasons behind this difference are not completely clear, this may be due to the timing of the onset of Cre recombinase activity and/or Cre penetrance. The *αGSU* was activated at e9.5, while the GRIC Cre is not active until e13.5.[42,43] Multiple markers of pituitary and gonadotrope differentiation and function,

such as *FGF*, *BMP4*, *BMP2*, and the *LIM* homeodomain transcription factors, *Isl1*, *Lhx3*, and *Lhx4*, are expressed during that window, so loss of ERK in those cells could have broad impacts on pituitary lineage specification.[44]

Loss of ERK signaling, and subsequent hypogonadotropism caused age-related changes in gonadal histology, suggesting long term hypogonadotropism is detrimental to gonadal health and function. Females began showing abnormal ovarian histology at 12 months of age, but the changes were not completely consistent with hypogonadotropism. Though they lacked CLs, one would expect a loss of mature follicles as the females aged, which was not seen.[30] This was unsurprising, since the *FSHβ* levels were grossly unchanged from 6 months of age. Histology from young animals showed antral follicles as well, indicating sufficient FSH was present for follicle recruitment and maturation. By contrast, aged (18 month old) males displayed a severe reproductive phenotype including marked loss of sperm production, reduced testicular and seminal vesicle weight, as well as areas of moderate to severe testicular degeneration. This degeneration was localized to focal areas within the testis, not generalized throughout the testis. Interestingly, the testis weight and sperm count decreased between 6 and 12 months in males, but the decrease in seminal vesicle weight and the histological changes did not occur until 18 months of age. Although seminal vesicle weight is usually correlated with testosterone, it has been shown to have LH receptors, and the weight loss is likely associated with the precipitous drop in LH in 18 month old ERKdko males.[45] At 18 months, males had only 22% of the sperm production seen at 6 months. While these males showed dramatic effects of testicular degeneration, it did not affect almost 80 percent of the tubules. This loss of sperm is

more than is expected with aging males in humans.[34] Unlike human males, these mice did not show decreased testosterone with age. This is surprising, especially given the decrease in LH seen in the aged GRIC ERKdko males. In humans, blocking GnRH increases regularity of LH and testosterone secretion, as well as the feedback between them. However, age decreases these interactions, causing higher frequency of LH pulses, though lower pulse amplitude, and reduces the organization of the secretion of both hormones.[46,47] Treating with GnRH seemed to correct the effects of aging, indicating the importance of an intact hypothalamic-pituitary relationship.[48] Both male and female ERKdko animals showed increased body weight as they aged, likely due to decreased metabolic rate concomitant with loss of reproduction function. This is a well characterized phenomenon in human females during menopause, in castrated domestic animals, as well as in IHH patients[57–62]

We speculate that the phenotype seen in aged GRIC ERKdko animals is primarily due to hypogonadotropism, not hypogonadism or gonadal Cre excision. In female mice, there is no evidence the GRIC-Cre driver is expressed outside of the gonadotrope (except during pregnancy) suggesting that the anovulatory phenotype observed was due to hypogonadotropism. (Chapter III) Loss of gonadotropin production presumptively caused the absence of CLs, and studies in other chapters of this dissertation show that gonadotropin replacement is sufficient to induce ovulation and CL formation in ERKdko females. The histological degeneration within the ovary with increased age also appears to be due to the trophic effects of the gonadotropins and potentially secondary changes in ovarian steroids. Male ERKdko mice showed evidence of premature reproductive aging including a reduction in seminal vesicle

weight, and reduced testicular size. While we initially presumed this was associated with hypogonadism, testosterone levels were not significantly different between control and ERKdko animals suggesting either that the effects of ERK deletion might be local within the testes or that the reduced gonadotropins observed with the ERKdko males was sufficient to maintain testosterone production by Leydig cells.[49,50]

ERKdko males also showed evidence of premature testicular degeneration, such as tubule vacuolation and calcification with increased age.[51,52] These pathophysiological changes were not seen in littermate control males, and were apparently not associated with decreased testosterone levels.[53,54] Since the GRIC Cre driver is expressed in the male germ cell lineage, we wanted to understand whether the histological effects we were observing were secondary to testicular ERK deletion or loss of gonadotrope cell trophic input to the testes. To assess this genetically, we developed a conditional ERKdko using the STRA8 Cre driver with specific ERK excision unique to the germ cell lineage, similar to the GRIC Cre driver. ERK deficiency induced by the STRA8 Cre driver did not phenocopy the GRIC ERKdko males suggesting that testicular degeneration was due to hypogonadtropism rather than a testicular loss of ERK signaling. This makes the GRIC ERKdko mouse an interesting model to study the specific effects of chronic hypogondotropism on the gonads with increasing age. This is not an area that has been well characterized in the literature in humans or model systems. IHH patients have been characterized with genetic deficiencies in GnRH production and/or secretion and treatment with GnRH or gonadotropins is a successful method to induce fertility and treat IHH in humans.[55,56]

Our mouse model responds appropriately to gonadotropins (Chapter XX), making them a potential model for treatment as well as the disease.

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DISCLOSURES

The authors have nothing to disclose

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CHAPTER 3

Conditional loss of ERK1 and ERK2 results in abnormal placentation and delayed parturition in the mouse

ABSTRACT

Extracellular-signal-regulated kinases (ERK) 1 and 2 regulate many aspects of the hypothalamic-pituitary-gonadal axis. We sought to understand the role of ERK1/2 signaling in cells expressing the gonadotropin-releasing hormone receptor (GnRHR) using a conditional Cre allele regulated by the endogenous GnRHR gene promoter (ERK double knockout, or ERKdko). Previous studies demonstrate that female ERKdko mice were anovulatory. The present study examined if exogenous gonadotropins could rescue this phenotype, which lead to pregnancy in approximately one-third of the ERKdko females. Litters from ERKdko females were significantly smaller and pup weights at e18.5 were reduced, occurring coincident with prolonged gestation/parturition and 100% neonatal mortality. Interestingly, placental size was similar in control and ERKdko females indicating reduced placental efficiency. Based on this, we examined placental *GnRHR* expression during gestation in mouse implantation sites. *GnRHR* mRNA levels at e10.5 and e12.5 were comparable to pituitary *GnRHR* mRNA levels from adult female mice. Careful analyses of maternal decidua ate12.5 showed enrichment of *GnRHR* mRNA compared to the whole implantation site. Studies in Rosa26 reporter mice confirmed GRIC Cre activity, and ERKdko placentas showed reduced ERK2 protein levels. Histopathology revealed abnormalities in maternal vascular remodeling and architecture of the placental decidua (e18.5). Evidence of prominent regions of apoptosis at the decidual/uterine interface around the time of parturition (e18.5) was observed in control animals and reduced in ERKdko animals. These studies support a model of ERK-dependent signaling within the maternal decidua leading to loss of placental architecture, reduced

physiologic placental apoptosis necessary for placental detachment at parturition, and ultimately prolonged gestation and fetal mortality.

INTRODUCTION:

Mitogen-activated protein kinases (MAPKs) are signal transducing kinases that in general contribute to cell differentiation, survival and proliferation.[1,2] MAPKs are a part of ubiquitous signaling cascades involving multiple intermediates associated with phospho-transfer including Raf-1 kinase, MEKK1, MEKs 1 and 2, and ERKs 1 and 2 (MAPK3 and MAPK1, respectively).[3] ERK1 and ERK2 have been long known to function integrally during embryonic development and in adult animals.[4,5] ERK1 and ERK2 are believed to have divergent functions during development since ERK1 null mice are grossly viable and fertile while ERK 2 null mice are embryonic lethal.[6–8] Embryonic lethality of the ERK2 null mice appears to be strain-specific; some have reported evidence of failed early mesodermal differentiation while others report mis-regulation of placental development.[9] Both instances lead to early embryo mortality where ERK 1 does not appear to be compensatory. In adult animals, ERKs 1 and 2 appear largely redundant helping to coordinate cellular responses to growth factors, peptide hormones and other ligands via a wide array of receptor subclasses controlling cell proliferation, differentiation and survival depending on the physiological context.

Since ERK 2 null animals are embryonically lethal, a conditional approach to examining the specific role of ERK signaling in discrete cell types is necessary. In our original studies, we developed a pituitary specific conditional ERK double knockout using the *ERK1* null and *ERK2* floxed alleles combined with a Cre recombinase drive

regulated by the gene promoter for the α subunit to the glycoprotein hormones (*αGSU*).[3,10] These studies were instrumental in understanding the role and requirement for GnRH-induced ERK signaling to a cohort of immediate early response genes regulating gonadotropins in the pituitary. The resultant phenotype was female-specific anovulatory infertility due to a loss of *LHβ* subunit expression resulting in an inability to mount a preovulatory surge of LH. Further, we examined the specific role of Raf-1 kinase in the regulation of GnRH-induced ERK activation finding that Raf-1 was dispensable for activation of this pathway. Similar studies of ERK double knockout conditionally in GnRH neurons did not impact fertility while loss of ERKs in the granulosa cell compartment revealed a requirement for ERK signaling in the ovarian follicle.[11] Loss of LH-induced ERK activation in granulosa cells also resulted in infertility.[12] Our original use of the *αGSU* Cre driver was not without caveats, with *αGSU* being expressed in thyrotropes and gonadotropes. The development of the *GnRHR*-IRES-Cre (GRIC) driver helped to resolve this caveat since Cre recombinase is knocked in to the endogenous *GnRHR* loci downstream of the *GnRHR* coding sequence.[13] We have recently studied a similar ERK1/2 double knockout using the GRIC line examining the impact of this signaling loss in gonadotrope on male fertility and how these animals manage reproductive aging in a hypogonadal state (Chapter 2).

In the present studies, we make use of conditional deletion of ERK 1 and 2 using the GRIC driver to examine the hypothesis that these hypogonadotropic-hypogonadal animals would respond to exogenous gonadotropins, ovulate and establish pregnancy. This question was of particular important since the *GnRHR* mRNA has been detected in tissues and cell types beyond the gonadotrope and GnRH

neurons including human trophoblast lineages and maternal decidua.[14,15] However, it is presently unclear if Cre is expressed in these tissues in the GRIC model and if so, how specific loss of ERK signaling might impact pregnancy and parturition. GRIC-ERKdko females were responsive to a standard superovulation paradigm with a subset of treated animals establishing and maintaining pregnancy without further gonadotropin therapy; however, gestation and parturition in these animals were markedly prolonged with all of the neonates dying before postnatal day 3. GRIC-mediated gene excision was detected primarily in the maternal decidua and to a lesser extent in trophoblast cell populations later in gestation in the mouse. Loss of ERK signaling in this system resulted in dramatic histological changes in placental architecture, maternal artery remodeling and focal points of apoptosis normally associated with the initiation of parturition.

MATERIALS and METHODS:

ANIMALS

Animals were handled in compliance with the Cornell University Institutional Animal Care and Use Committee. ERK1 null (*ERK1*$^{-/-}$), ERK2 floxed (*ERK2*$^{fl/fl}$) and *GnRH* receptor IRES Cre (GRIC) mice have been described previously.[13,16,3] To create gonadotrope specific ERK1/2 knockout animals, they were crossed and designated ERKdko (*ERK1*$^{-/-}$, *ERK2*$^{fl/fl}$, *Cre*$^{+/-}$), or Control (*ERK1*$^{-/-}$, *ERK2*$^{fl/fl}$, *Cre*$^{-/-}$). For experiments involving Cre visualization, ROSA26-GNZ KI mice were purchased from Jackson Laboratory These were crossed with GRIC animals, and designated ROSA26 (*GRIC*$^{+/-}$, *ROSA*$^{+/-}$ or *GRIC*$^{+/+}$, *ROSA*$^{+/+}$). For timed matings, mice were paired and

checked for copulatory plugs daily. Pairs were separated after a copulatory plug was observed (embryonic day (e)0.5). On e10.5, e12.5 and e18.5, the dams were euthanized, and placentas were carefully dissected. For superovulation studies, females were injected with 100ug of pregnant mare serum gonadotropin (PMSG) intraperitoneally. 46-48 hours later, they were injected with 100ug human chorionic gonadotropin (hCG) intraperitoneally. For assessing CL and follicle formation, superovulated animals were euthanized 72 hours later, and ovaries were collected for histological examination. For induction of pregnancy, and assessment of parturition, superovulated control and ERKdko females were placed with a male and checked for copulatory plugs the following morning day. Following evidence of copulation, females were minimally handled until e18.5, at which point they were checked every 12 hours. They were monitored for signs of initiation of parturition, contractions, distress, hiding, blood on the shavings, and pups.

GENOTYPING

Genomic DNA was isolated from tail snips (3 mm), or an equivalent quantity of other tissues as indicated, using a E-Z Tissue DNA Kit (Omega Biotek, Norcross, GA) per manufacturer's instructions. Routine PCR genotyping was performed on animals as previously described.[17] PCR confirmation of *ERK1* knockout, *ERK2* flox, *GnRHR* Cre and *Rosa* alleles were performed with primers as listed below.

Primer		Sequence (5’ to 3’)
Rosa26 Reporter	Rosa26 Forward	TAA GCC TGC CCA GAA GAC TC
	Rosa26 Reverse	AAA GTC GCT CTG AGT TGT TAT
	Rosa26 Common	TCC AGT TCA ACA TCA GCC GCT ACA
ERK1	ERK1 Forward	AAG GTT AAC ATC CGG TCC AGC A
	ERK1 Reverse	AAG CAA GGC TAA GCC GTA CC
ERK2	ERK2 Forward	AGC CAA CAA TCC CAA CCC TG
	ERK2 Reverse	GGC TGC AAC CAT CTC ACA AT
GnRHR	GnRHR Forward	GAA CTA CAG CTG AAT CAG TC
	GnRHR Reverse	CTC TAA CAA ACT CTG TAC A
	GnRHR Homozygous	CGG AAT TCA TCG ATC ATA TCA GAT CC

IMMUNOBLOTTING

Placentas were halved, with one half placed in formalin for histological studies or snap frozen for RNA analysis, and the other half homogenized in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 130 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.5% deoxycholate, 2mM EDTA, 5mM sodium vanadate, 0.2 mM phenylmethysulfonylfluoride, and 5 mM benzamidine. Protein concentrations of lysates were determined by Bradford assay. Samples were boiled for 5 minutes in sodium dodecyl sulfate loading buffer, resolved by SDS-PAGE, and transferred to polyvinylidine difluoride membranes by electroblotting. Membranes were blocked with 5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.5; 150mM NaCl; 0.05% Tween 20) and then incubated with specified antisera (anti-ERK2, anti-βactin and horseradish peroxidase-conjugated secondary antibodies from Santa Cruz, Dallas, TX).[3] Protein bands were visualized using enhanced chemiluminescence according to manufacturer’s instructions (BioRad, Berkeley, CA) and imaged on

ChemiDoc XRS (BioRad, Berkeley, CA) They were analyzed using Image Lab software (BioRad, Berkeley, CA).

RNA ISOLATION AND QUANTITATIVE PCR

Placentas were halved, and Trizol (ThermoFischer, Waltham, MA) extraction was performed per manufacturer's instructions. Reverse transcription in 1000ng reactions was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer directions. Amplifications were carried out using a BioRad CFX96 Touch Real-Time OCR Detection System (BioRad, Berkeley, CA). RNA levels were standardized using the internal control Gapdh and calibrated to corresponding transcript levels of a control group.[3]

Primer		Sequence (5' to 3')
Glyceraldehydes-3-phosphate dehydrogenase	*Gapdh* forward	ATGTTTGTGATGGGTGTGAA
	Gapdh reverse	ATGCCAAAGTTGTCATGGAT
GnRHR	*GnRHR* forward	TGCTCGGCCATCAACAACA
	GnRHR reverse	GGCAGTAGAGAGTAGGAAAAGGA

HISTOLOGY, IMMUNOFLUORESCENCE, and β-GALACTOSIDASE ASSAYS

Mice were paired and checked for copulatory plugs daily. Pairs were separated after a copulatory plug was observed (embryonic day (e)0.5). On e12.5 and e18.5, the dams were euthanized, and placentas were carefully dissected. For histological examination, tissues were fixed in 10% formalin, paraffin embedded, serially sectioned at 4μm, and stained with hematoxylin and eosin, terminal deoxynucleotidyl

transferase dUTP nick end labeling (TUNEL), isolectin or smooth muscle actin using standard histological techniques.[18] Sections were examined with light microscopy. Sections were scanned and digitized using an Aperio Scanscope (Vista CA). Placenta area, area of decidua, junctional zone and labyrinth layers, along with luminal area of decidual vessels were quantitated using Aperio software. TUNEL was quantified using the Positive Pixel Algorithm on ImageScope (Leica Biosystems, Buffalo Grove, IL).

For immunofluorescence labeling of GFP, placentas were embedded in Tissue-Tek OCT media (Sakura Finetek, Torrence, CA) and maintained at -80°C in 2-methylbutane for 24 hours. Sections (10μM) were cut using a cryotome and stored at -80°C. Frozen sections were fixed with 4% paraformaldehyde, blocked for one hour, and stained with anti-GFP antibody (Abcam, Cambridge, United Kingdom) at 1:100 overnight followed with a FITC goat-anti-rabbit secondary antibody (Vector Laboratories, Burlingame, CA). Sections were cover slipped with SlowFade Gold with DAPI (Life Technologies, Carlsbad, CA) and imaged on an AxioVision fluorescent microscope with Zen software (Zeiss, Oberkochen, Germany). Sections were also scanned and digitized using an Aperio Scanscope (Vista CA).

For β-galactosidase *in vitro* assays, tissues were fixed in 4% paraformaldehyde/PBS for 1 hour at 4°C, then rinsed 3 times for 30 minutes each in a rinse buffer (100 mM sodium phosphate (pH 7.3), 2 mM MgCl2, 0.01% sodium deoxycholate, 0.02% NP-40 (by volume)). Sections were then stained overnight in staining buffer (rinse buffer with 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 1 mg/ml X-gal). Sections were cleared using methyl salicylate, fixed overnight in 10% formalin, then washed with distilled water twice for 30 minutes.

They were dehydrated by sequential ethanol washes (70%, 95%, twice in 100%, then washed in methyl salicylate until the tissue cleared.

For liquid β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer (PROMEGA, Madison, WI) tissues were collected, and the assay was performed according to manufacturer's instructions.

STATISTICS

Pairwise comparisons were made by Student's *t*-test. All data are expressed as means ± standard error of the mean. A p value of <0.05 was considered statistically significant.

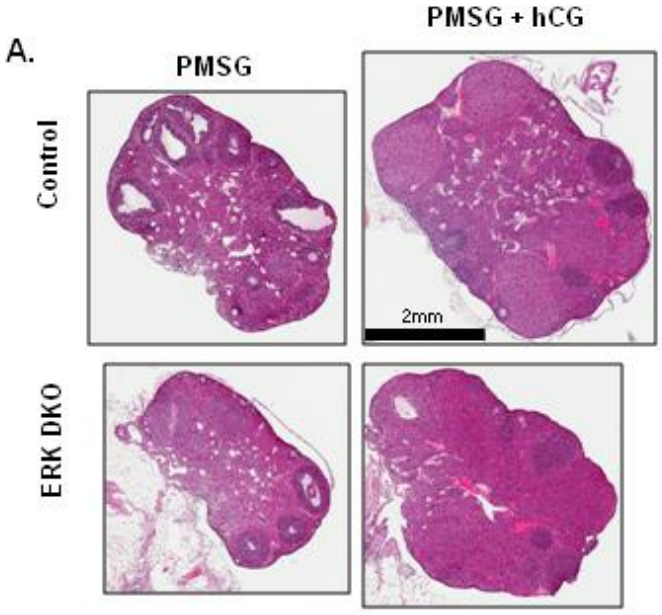
RESULTS:

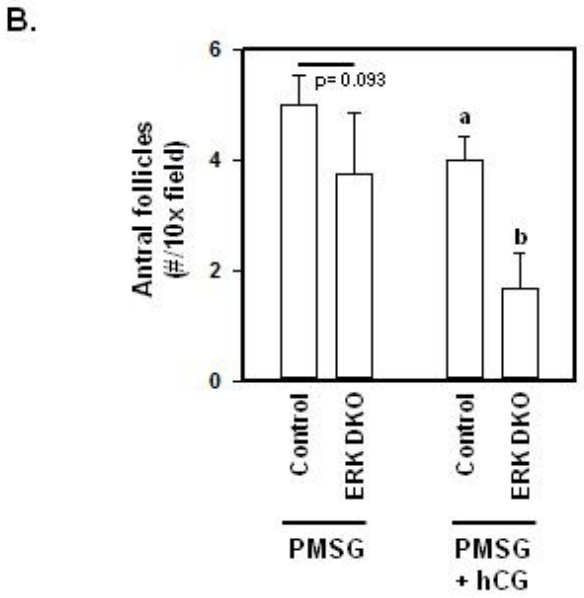
ERKdko animals can ovulate in response to exogenous gonadotropin stimulation.

We previously reported a pituitary-specific conditional knockout of ERK1 and 2 using the αGSU Cre driver resulted in an anovulatory phenotype due to loss of LH biosynthesis.[3] Those studies also characterized the ability of ERK deficient female mice to respond to exogenous gonadotropin stimulation and ovulate; however, we did not examine the possibility that gonadotropin stimulation and mating would lead to a viable pregnancy. We mated the *ERK2*$^{fl/fl}$, *ERK1*$^{-/-}$ animal with GRIC Cre animals to improve specificity of ERK ablation to the pituitary gonadotropes.[3,13] In the present studies, these animals were designated ERKdko animals while control animals were designated ERK2$^{fl/fl}$, ERK1$^{-/-}$, but Cre negative. Consistent with the αGSU model system[3], female ERKdko GRIC mice are infertile and anovulatory, with blunted

gonadotropin secretion in response to GnRH (Chapter 2). To better understand the gonadal response to exogenous gonadotropin stimulation in this model, female control and ERKdko animals were treated with either pregnant mare serum gonadotropin (PMSG) or a standard superovulation protocol of PMSG followed by administration of human chorionic gonadotropin (hCG) to induce ovulation.[19] Three days later, control and ERKdko were euthanized and ovaries were collected and the number of antral follicles (AF) and corpora lutea (CL) were counted. As expected, addition of hCG in the superovulation paradigm resulted in the presence of CLs in both control and ERKdko females compared with PMSG alone (Figure 1A). No differences were detected in the number of antral follicles comparing control and ERKdko females receiving PMSG alone (Figure 1B). However, when treated with PMSG and followed by hCG, control animals had significantly more antral follicles compared to ERKdko animals (Figure 1 A, B). These data support the conclusion that the ERKdko females are fully capable of ovulation; however, due to the hypogonadotropic hypogonadism in this model, fewer ovarian follicles were stimulated by the superovulation paradigm.

Figure 1: ERKdko females respond appropriately to exogenous gonadotropin stimulation. **A)** Photomicrographs of ovaries from Control and ERKdko female mice. Both genotypes responded to gonadotropin administration. **B)** Fewer antral follicles were seen in ERKdko animals compared to Control with PMSG administration (p=.093). Following PMSG and hCG administration, ERKdko animals had significantly fewer antral follicles (1.6) when compared to control (4). There were no changes in the number of CL's present between genotypes, following either treatment. The treatments were successful, as ERKdko animals are incapable of making CL's without hCG stimulation.


A.
PMSG
PMSG + hCG
Control
ERK DKO
2mm


B.
Antral follicles (#/10x field)
6
4
2
0
p= 0.093
a
b
Control
ERK DKO
Control
ERK DKO
PMSG
PMSG + hCG

ERKdko animals can maintain pregnancy, but show prolonged gestation and parturition

We next asked whether superovulated ERKdko females could establish and maintain a pregnancy to term with viable pups in the absence of ERK signaling within the gonadotrope. Control and ERKdko females were induced to ovulate, mated to control males and copulatory plugs were observed. Control and ERKdko females with a copulatory plug were allowed to proceed through gestation. Beginning at 19 days following detection of a copulatory plug, females were observed visually every 12 hours and assessed for signs of parturition (hiding, contractions, and visible distress) or the presence of pups. All control and approximately 70 percent of ERKdko animals exhibited a copulatory plug after superovulation and were paired with fertile experienced males. Control animals gave birth normally around gestational day 20; however, ERKdko females did not give birth until nearly gestational day 24 ($p<0.05$; Table 1). In control females, parturition was complete within a 12 hour period; while ERKdko females showed continuous signs of labor for 2.3 days ($p<0.05$; Table 1) suggesting the possibility of abnormal parturition or dystocia. Finally, control animals had an average of 9 pups/litter; while litter size in the ERKdko females was 2.4 pups/litter ($p<0.05$; Table 1) consistent with reduced antral follicle populations in the ERKdko females. Pups from ERKdko females were stillborn or died soon after birth; the percentage of live pups from the ERKdko females was zero compared with 85.7% in the control females ($p<0.05$; Table 1).

Table 1: Female ERKdko mice showed alterations in gestation and parturition, resulting in fetal mortality

	Litter size (# of pups)	% live pups on PN3	Gestation length (d)	Parturition length (d)
Control	9.0 ± 1^{a} (n=6)	85.7 ± 11.7^{a} (n=3)	20.3 ± 0.25^{a} (n=5)	1.0 ± 0.0^{a} (n=4)
ERKdko	2.4 ± 1.3^{b} (n=7)	0^{b} (n=7)	23.9 ± 0.55^{b} (n=4)	2.3 ± 0.42^{b} (n=4)

GnRHR is preferentially expressed in the placental decidua

Perusal of the literature revealed reports of normal pups routinely born to wildtype females after superovulation protocols. This suggests administration of gonadotropins to induce ovulation was not likely a cause of prolonged gestation and fetal demise in our model system.[19–22] Moreover, while ERKdko animals have been shown to display defects in gonadotropin production, no evidence in the literature directly links hypogonadotropism to delayed parturition and periparturient fetal mortality.[23–26] We considered that the abnormal pregnancies may be a consequence of expression of Cre activity and subsequent loss of ERK signaling potential in the placenta, as GnRH and GnRHR have been detected in the placenta and decidua of several species.[14,27–30]

We used qRT-PCR to detect *GnRHR* mRNA in placentas of embryonic (e)10.5 and e12.5 mice and compared this directly with female pituitaries at estrus. Levels of *GnRHR* in the whole placental disk were similar to levels of *GnRHR* found in adult female pituitaries at estrus (Figure 2). To further validate these observations, we used the *Rosa26* reporter mouse line mated to $GRIC^{+}$ males. In this model, Cre-mediated gene excision results in expression of a β-galactosidase (gal)-green fluorescent protein (GFP) fusion protein. *In-situ* β-gal staining of whole mouse pituitaries and placental disks confirmed the presence of β-gal activity within both tissues (Figure3A-C). The highest density of β-gal activity was found at the periphery of the placental disk, indicating enrichment within the maternal decidua (Figure 3B). Using placental tissues from *ROSA26* control- and GRIC-mated animals obtained at e12.5, we then carried out an *in vitro* β-galactosidase assay to quantify relative β-gal expression in a cross

section of tissues. We compared GRIC$^-$ and GRIC$^+$ placental tissues along with GRIC$^+$ pituitary, hypothalamus, ovary, pancreas, liver and muscle from the same animals. Placenta lysates from ROSA26/GRIC$^+$ animals revealed fourfold increase ($P<0.05$) of β-galactosidase activity compared with the Rosa26 animals without Cre expression. Interestingly, the placentas from ROSA26/GRIC$^+$ animals also had higher ($p<0.05$) β-gal activity compared with pituitaries from the same animals (midgestation female mice; Figure 3D).

After establishing Cre expression in placentas using the Rosa26 reporter mice, we examined localization of expression within the placental disks to better understand the results of ERK2 loss. E12.5 placentas were dissected to carefully separate the decidua from the remainder of the placental disk including the chorionic plate and labyrinth. RNA was extracted and qRT-PCR was performed, allowing us to quantify enrichment of the GnRHR mRNA in the decidua versus remainder of the disk. GnRHR mRNA was more abundantly expressed in the maternal decidua compared to the remainder of the placenta disk (Figure 2E).

Figure 2: qRT-PCR was performed on placental disks from embryonic days 10.5 and 12.5. Levels of GnRHR were comparable with those seen in adult female pituitaries collected at estrus

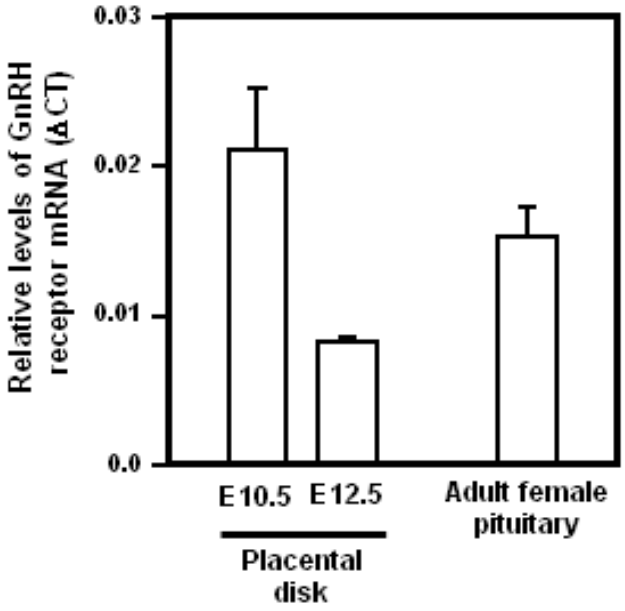
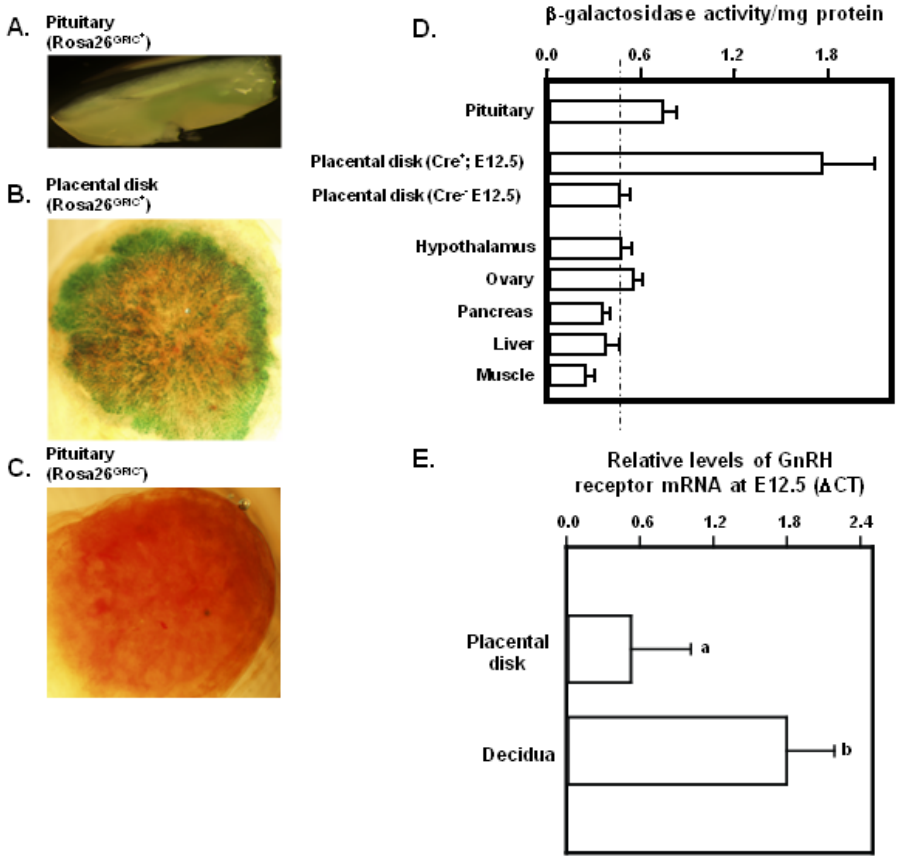

Relative levels of GnRH receptor mRNA (ΔCT)
0.03
0.02
0.01
0.0
E10.5
E12.5
Placental disk
Adult female pituitary

Figure 3: A) Adult Rosa26^{+}Cre^{+} female pituitary of stained for β-galactosidase *in situ*. β-gal staining indicates the presence of Cre activity. B) Placenta from e18.5 Rosa26^{+}Cre^{+} embryo, showing β-gal staining (indicative of Cre activity) *in situ*. C) Placenta from e18.5 Rosa26^{-}Cre^{-} embryo, showing no autologous *in situ* β-gal staining . D) Liquid β-galactosidase assay confirmed Cre activity in Rosa26^{+}Cre^{+} pituitaries and placentas, but baseline activity in Cre^{-} tissues (muscle, liver, pancreas) ($p<0.05$). E) qRT-PCR showed significant GnRHR mRNA expression in placental decidual lysates when compared to whole disk lysates ($p<0.05$). This indicates preferential expression of GnRHR and the associated Cre in the decidual compartments, instead of the entire placenta.

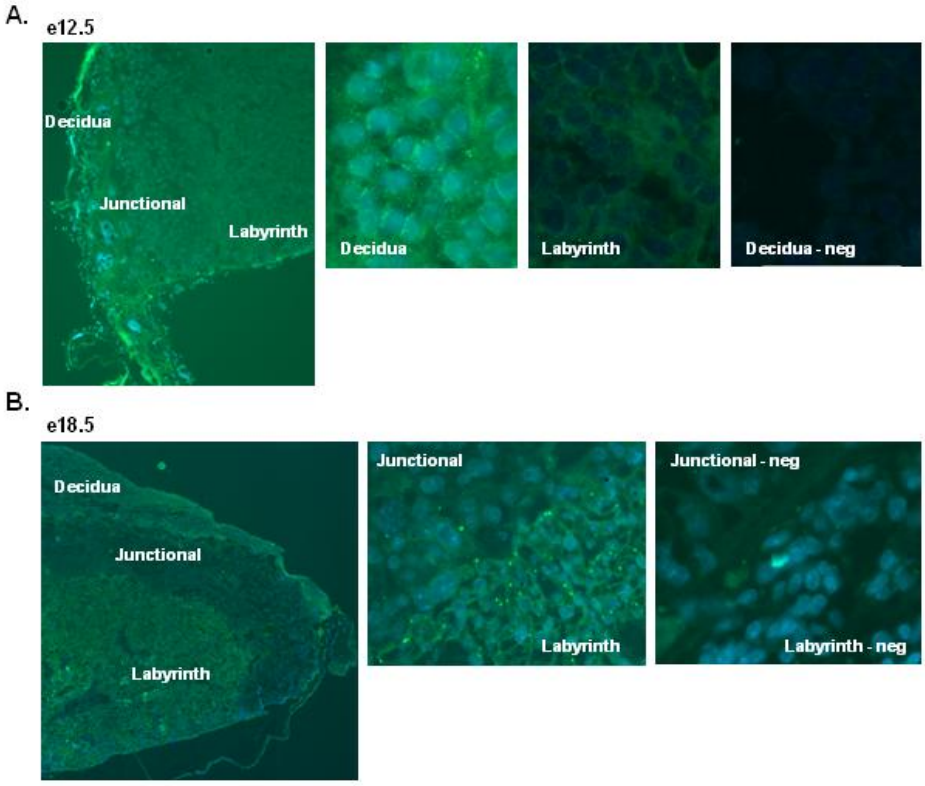

A.
Pituitary
(Rosa26GRIC+)
B.
Placental disk
(Rosa26GRIC+)
C.
Pituitary
(Rosa26GRIC-)
D.
β-galactosidase activity/mg protein
0.0
0.6
1.2
1.8
Pituitary
Placental disk (Cre+; E12.5)
Placental disk (Cre- E12.5)
Hypothalamus
Ovary
Pancreas
Liver
Muscle
E.
Relative levels of GnRH
receptor mRNA at E12.5 (ΔCT)
0.0
0.6
1.2
1.8
2.4
Placental
disk
a
Decidua
b

GnRHR is expressed throughout the placenta at e12.5 and preferentially in the labyrinth at e18.5

Rosa26 animals were time mated, and euthanized at e12.5 and e18.5. Placentas were collected, snap frozen, serial sectioned and stained for GFP expression. At e 12.5, GnRHR-GFP appears to be expressed throughout the placenta, but most strongly in the maternal decidua (Figure 4A). Punctate GnRHR expression surrounds nuclei in clusters of cell within the decidua. There is mild expression of GnRHR throughout cell cytoplasm of the labyrinth, but no areas of punctate expression were seen (Figure 4B).

At e18.5, the punctate expression seen in the decidua is no longer observed; however, the labyrinth showed marked up regulation of GnRHR expression when compared to e12.5 (Figure 4A,B). Larger foci of GFP expression are observed around the cell surface, and it additionally appears to be diffusely expressed throughout the cytoplasm (Figure 4B). Negative control showed decreased expression of both cytoplasmic and cell surface GnRHR. (Figure 4B)

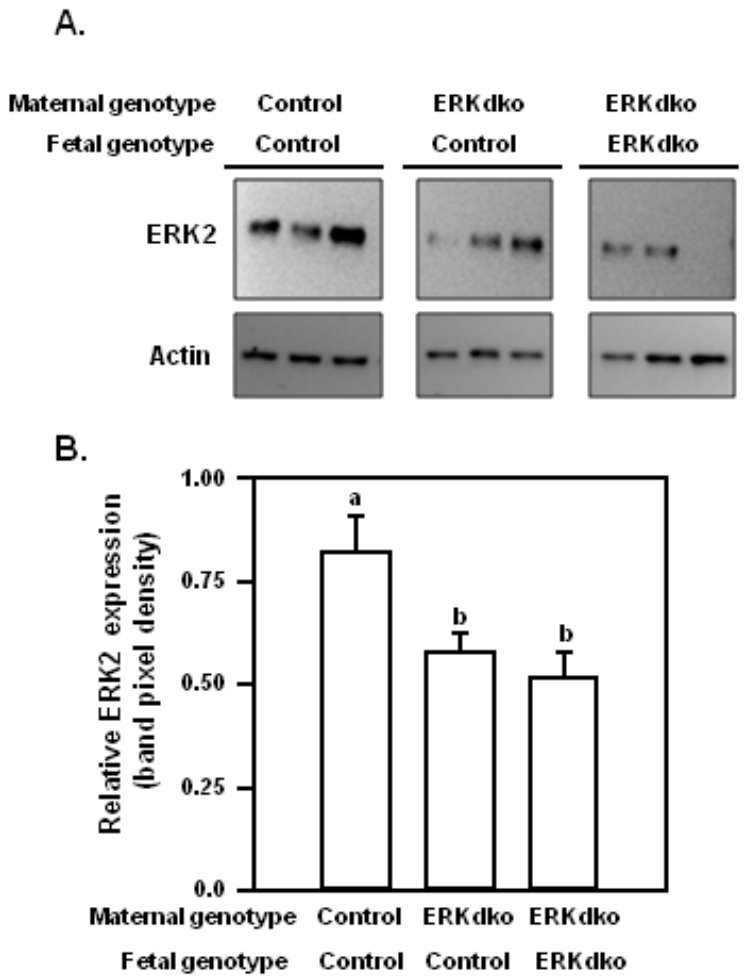
Figure 4:Localization of GnRHR-GFP in the placenta at e12.5, e18.5 A) GRIC-Rosa placentas at e12.5, stained for GFP. There is staining through out the placenta, but strongest around the periphery of the decidua and in clusters of punctate staining throughout the decidua and into the junctional zone. The labyrinth had lower basal staining, but lacked any punctate perinuclear expression. **B)** Rosa placentas at e18.5, stained for GFP. There is expression in the decidua and labyrinth, with focal areas of strong expression throughout the labyrinth. All sections are stained for DAPI.


A.
e12.5
Decidua
Junctional
Labyrinth
Decidua
Labyrinth
Decidua - neg
B.
e18.5
Decidua
Junctional
Labyrinth
Junctional
Labyrinth
Junctional - neg
Labyrinth - neg

GRIC Cre expression results in loss of placental ERK2

Western blot analyses added additional confirmation of Cre-mediated ERK gene deletion (Figure 5). We specifically assayed for the abundance of ERK2 since our genetic model is ERK1$^{-/-}$. For the remainder of the characterization, we separated the animals into three categories: control dam, control pup (control/control), ERKdko dam, control pup (ERKdko/control), and ERKdko dam, ERKdko pup (ERKdko/ERKdko). We observed ~30% reduction of ERK 2 protein levels in placentas from both ERKdko groups when compared to placentas from control dams (Figure 5). This suggests the genotype of the dam has a more significant impact on placental ERK expression than the genotype of the pup, again consistent with GnRHR localization and subsequent loss of ERK2 in maternal decidua.

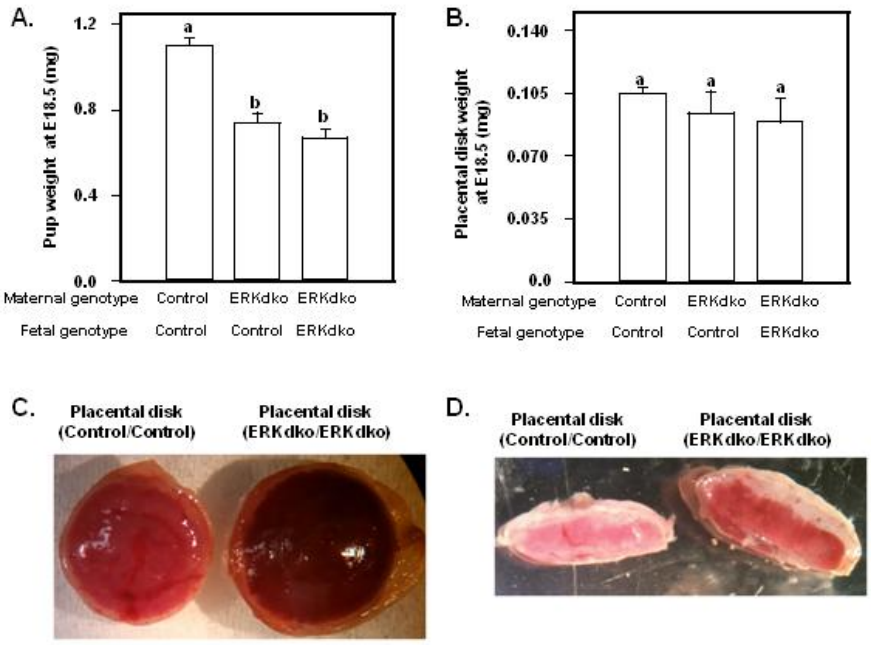
Figure 5: A) Western blotting revealed loss of ERK2 expression in placental lysates in both control and ERKdko pups from ERKdko dams. This indicates functional Cre activity, excising the floxed ERK2 locus. **B)** Quantitation of ERK 2 protein levels show significant ($p < 0.05$) decrease of ERK2 protein expression in placentas from ERKdko dams, regardless of fetal genotype. This again indicates preferential expression of the Cre in the maternal derived placental compartment (decidua) when compared to the fetal derived placental compartment (labyrinth).


A.
Maternal genotype
Control
ERKdko
ERKdko
Fetal genotype
Control
Control
ERKdko
ERK2
Actin
B.
Relative ERK2 expression (band pixel density)
1.00
0.75
0.50
0.25
0.0
a
b
b
Maternal genotype
Control
ERKdko
ERKdko
Fetal genotype
Control
Control
ERKdko

Loss of ERK2 in decidua results in gross and histological abnormalities in the placental disk

ERKdko and control animals were superovulated, mated, and euthanized at e18.5. Anecdotally, all pups from the ERKdko (and control) dams showed evidence of fetal viablity at this gestational age. ERKdko/Control and ERKdko/ERKdko pups displayed reduced fetal weight at e18.5 compared to control pups ($p<0.05$; Figure 6A). Consistent with earlier studies on ERK2 expression levels, reduced pup weight was accounted for by loss of ERKs within the decidua (maternal ERKdko). Intrauterine growth restriction seen in ERKdko/ERKdko pups could not be accounted for by changes in placental weight (Figure 6B) indicating that the fetal growth restriction observed was due to loss of relative placental efficiency, not placental size. Gross morphology of the placental disks revealed that ERKdko/ERKdko placentas appeared hemorrhagic with congested decidua and grossly exaggerated labyrinth compared to control/control placentas (Figure 6C, D).

Figure 6: A, B) e18.5 Control (left) and ERKdko/ERKdko placenta. The ERKdko placenta appears grossly congested and discolored. **C)** Pups from ERKdko dams, regardless of fetal genotype, are significantly smaller ($p < 0.05$) than those from Control dams at e18.5. This indicates a potential IUGR, and again confirms the importance of the Cre activity in the maternal compartment of the placenta, regardless of fetal Cre status. **D)** Despite changes in pup weight, placental disk weight was unaffected by maternal or fetal genotype, indicating loss of placental efficiency, instead of loss of placental mass or cellularity.


A.
Pup weight at E18.5 (mg)
1.2
0.8
0.4
0.0
a
b
b
Maternal genotype Control ERKdko ERKdko
Fetal genotype Control Control ERKdko
B.
Placental disk weight at E18.5 (mg)
0.140
0.105
0.070
0.035
0.0
a
a
a
Maternal genotype Control ERKdko ERKdko
Fetal genotype Control Control ERKdko
C.
Placental disk (Control/Control)
Placental disk (ERKdko/ERKdko)
D.
Placental disk (Control/Control)
Placental disk (ERKdko/ERKdko)

Gross placental findings described above were consistent with histological assessment of these placentas at e18.5 (Figure 7). For these studies, placentas were dissected free from the uterine wall, fixed in formalin, then sectioned and stained with hematoxylin and eosin. Pups from ERKdko dams had abnormal placental architecture regardless of fetal genotype (Figure 7). Pathological findings indicated that placentas from ERKdko dams and control fetuses appeared histologically disorganized compared to control placentas. These placentas displayed variability in decidual thickness within a placenta, with increased mitotic index in trophoblast cells suggesting increased proliferation rate. These findings were not consistent with neoplastic changes, but more aligned with exuberant tissue growth. The trophoblast cytoplasm showed grossly increased vacuolation and a mild unusual cystic development. There were also fingerlike projections of the junctional zone into the labyrinth. Loss of ERK signaling resulted in the appearance of large acellular spaces within the junctional zone and increased numbers of large vacuolated cells. These findings were further exacerbated in the ERKdko/ERKdko placentas, with more severe cystic changes, especially in the decidua and junctional zone, consistent with a more robust disorganization of the normal placental architecture seen in the control/control placentas.

Figure 7: Photomicrographs of representative placentas from control/control, ERKdko/control and ERKdko/ERKdko dam/pup pairs, stained for isolectin. Loss of ERK2 results in vacuolation of the junctional zone, with loss of normal placental architecture and invaginations of the junctional zone into the labyrinth compartment. The ERKdko placentas showed cystic development and higher rates of mitotic figures, consistent with increased proliferation. To the right are insets showing increased magnification of areas of cystic development, vacuolation and projection into the labyrinth area.

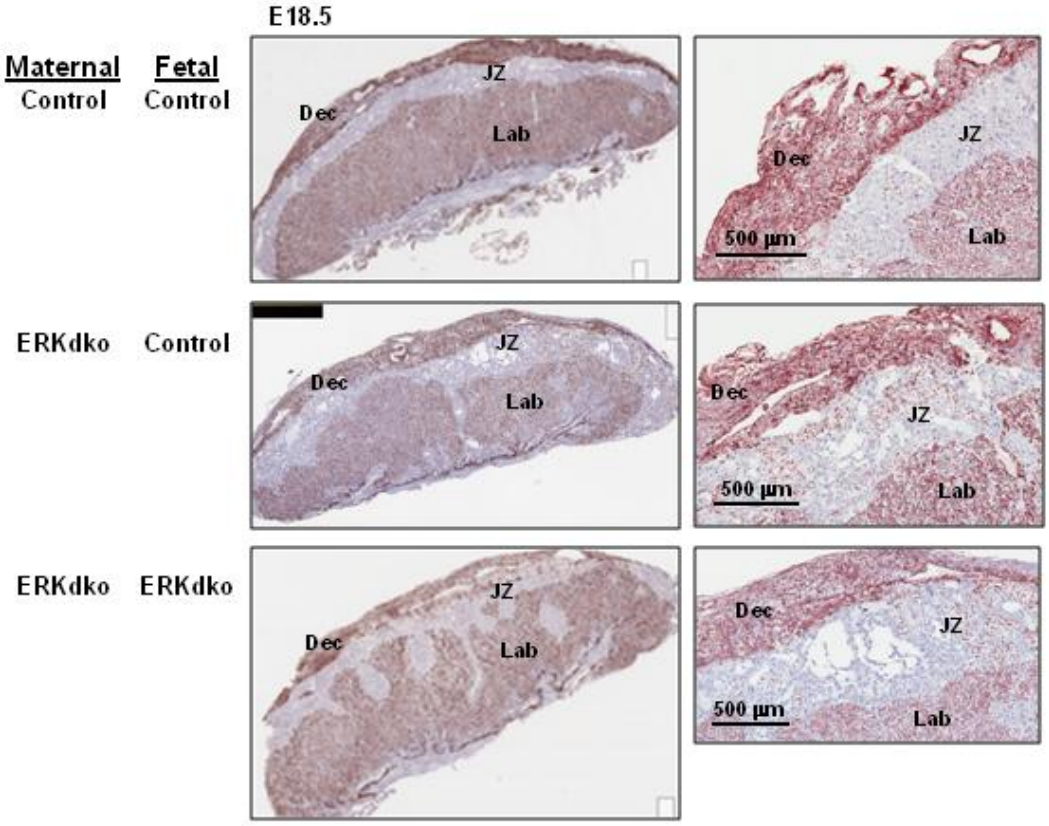

E18.5
Maternal
Control
Fetal
Control
ERKdko
Control
ERKdko
ERKdko
JZ
Dec
Lab
500 µm

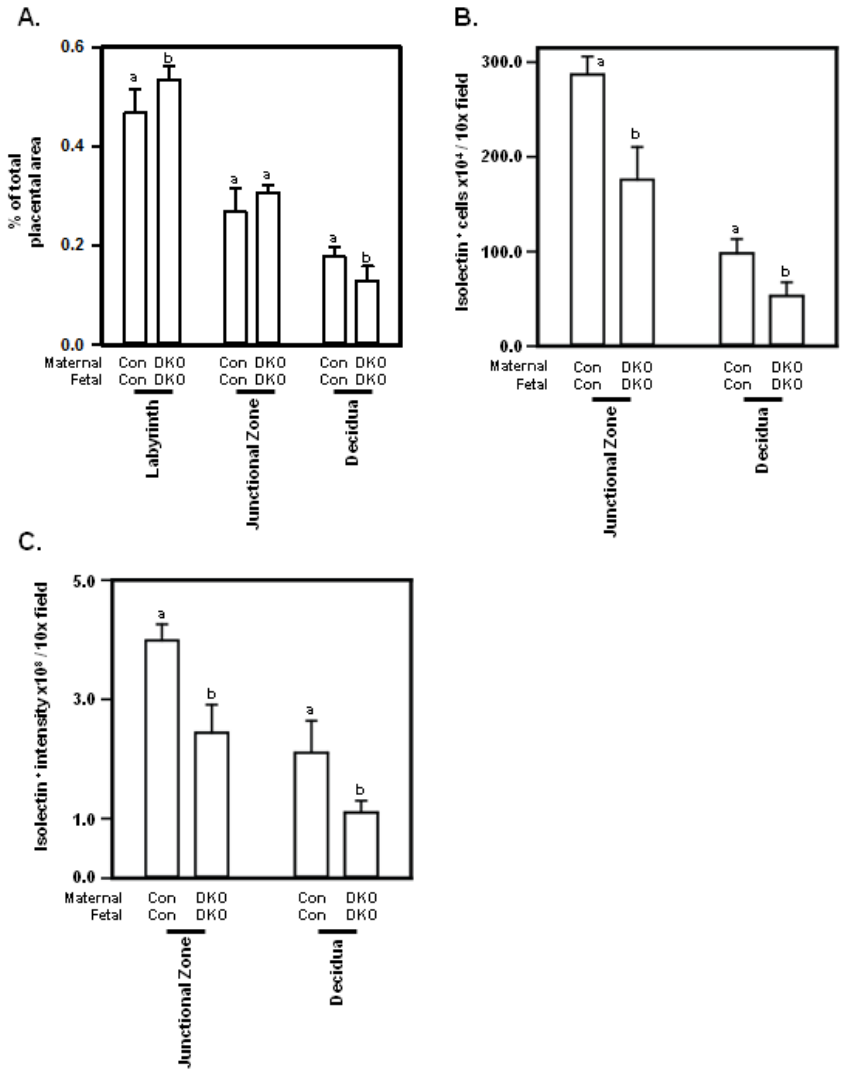
Figure 8A depicts the distribution of area of the labyrinth, junctional zone and decidua which again varied with genotype. Using Aperio image analysis software, placentas from control dams and control pups were characterized by approximately 49 percent labyrinth, 26 percent junctional zone, and 17 percent decidua (with the remainder of the area attributed to chorionic plate). Placentas of ERkdko dams and ERKdko pups showed a significantly enlarged labyrinth area and a smaller decidual area (Figure 8A), compared to the control animals. Placentas from ERKdko dams and control pups showed an intermediate phenotype that was not significantly different from either of the other two genotypes (data not shown). Junctional zone areas did not significantly vary between any of the genotypes. As these zones all have variations in functions and vasculature, we next assessed changes in placental vascularization between genotypes.

Histological changes in the placenta occur coincident with placental vasculature abnormalities

Changes in placental vasculature have long been associated with placental pathology and disease states.[31–34] We approached our assessment of the vasculature in two ways; isolectin staining to examine endothelial cell density and specific analyses of maternal arteries using smooth muscle actin staining in decidua. Staining tissues sections with isolectin (conjugated to Nova red) highlights the extracellular matrix of endothelial cells, allowing visualization of highly vascularized regions such as the labyrinth, junctional zone and the decidua.[35] Both the number of isolectin positive

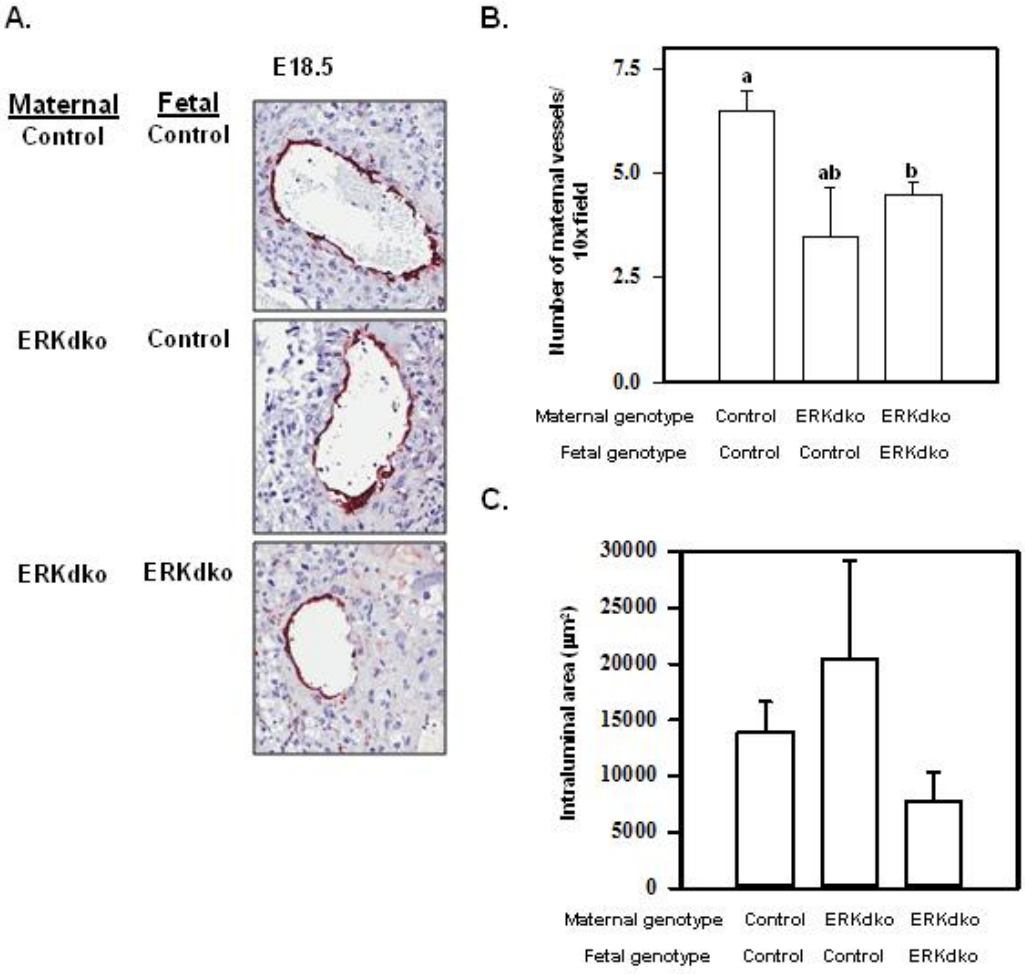
cells and the intensity of isolectin staining was blunted ($p<0.05$) in ERKdko/ERKdko placentas in both the decidua and junctional zone compared to control (Figure 8B,C).

Figure 8: A) ERKdko/ERKdko placentas show significantly smaller decidual zones and increased relative labyrinth area when compared to Control/Control placentas ($p < 0.05$). **B)** Isolectin stained cells, highlighting endothelial cells, were significantly decreased in the maternal decidual and junctional zone compartments in ERKdko/ERKdko placentas, when compared to Control/Control placentas. There was no increase in isolectin staining in the fetal labyrinth compartment. **C)** Isolectin stain intensity was decreased in both the decidual and junctional zone compartments in ERKdko/ERKdko animals when compared to Control/Control animals. ($p <0.05$). This indicates loss of endothelial cell in these compartments, likely secondary to loss of ERK2 signaling.


A.
% of total placental area
0.6
0.4
0.2
0.0
a
b
a
a
a
b
Maternal Con DKO Con DKO Con DKO
Fetal Con DKO Con DKO Con DKO
Labyrinth
Junctional Zone
Decidua
B.
Isolectin + cells x10² / 10x field
300.0
200.0
100.0
0.0
a
b
a
b
Maternal Con DKO Con DKO
Fetal Con DKO Con DKO
Junctional Zone
Decidua
C.
Isolectin + intensity x10⁶ / 10x field
5.0
3.0
1.0
0.0
a
b
a
b
Maternal Con DKO Con DKO
Fetal Con DKO Con DKO
Junctional Zone
Decidua

To assess specific changes in maternal vasculature remodeling within the decidua, E18.5 placental sections were stained for smooth muscle actin to reveal the size and location of maternal arteries in the placenta. Each artery identified was confirmed with the presence of non-nucleated erythrocytes in the lumen of the vessels, as previously described.[18] ERKdko/ERKdko and ERKdko/control placentas had approximately 50% fewer maternal arteries in the decidua than the placentas of control/control pups ($p<0.05$; Figure 9A, B). Of the arteries present, the intraluminal area of maternal vessels in the ERKdko/ERKdko placentas were approximately 60 percent smaller, than those in control/control placentas. There was no significant difference between intraluminal area of ERKdko/control placentas and either of the two other groups due to high variation in artery size (data not shown).

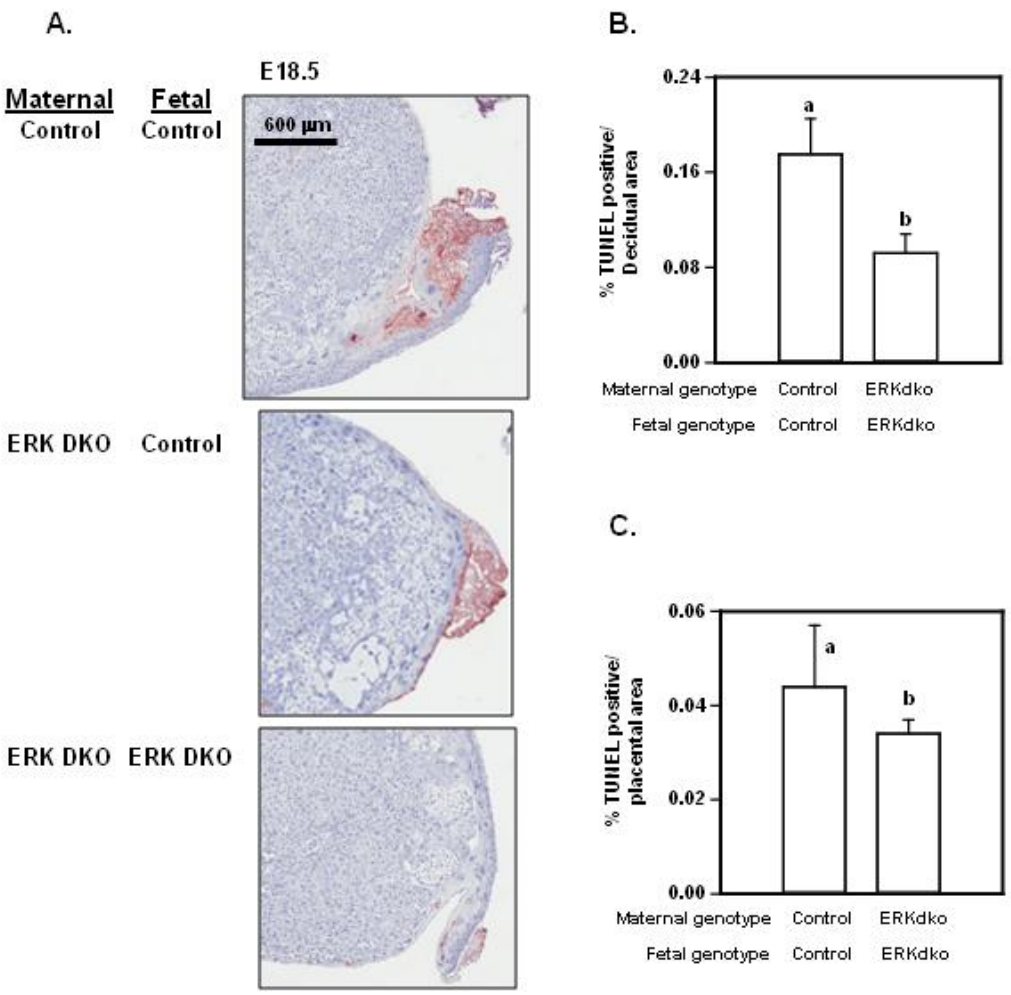
Figure 9: A) Photomicrograph showing loss of ERK2 in maternal decidua compartment results in smaller decidual spiral arteries in ERKdko/ERKdko placentas when compared to Control animals. **B)** ERKdko ERKdko placentas also show fewer maternal decidual arteries when compared to placentas from Control animals ($p<0.05$) **C)** ERKdko ERKdko placentas have significantly smaller artery lumens when compared to Control placentas ($p <0.05$, $n = 23$ arteries for Control, 26 Control/ERKdko, 30 ERKdko/ERKdko).


A.
E18.5
Maternal
Control
Fetal
Control
ERKdko
Control
ERKdko
ERKdko
B.
Number of maternal vessels/ 10x field
7.5
5.0
2.5
0.0
a
ab
b
Maternal genotype
Control
ERKdko
ERKdko
Fetal genotype
Control
Control
ERKdko
C.
Intraluminal area (μm²)
30000
25000
20000
15000
10000
5000
0
Maternal genotype
Control
ERKdko
ERKdko
Fetal genotype
Control
Control
ERKdko

Loss of ERK2 signaling in the placenta causes decreased parturition-associated apoptosis

E18.5 placentas were stained with TUNEL to examine apoptosis. As expected, control/control placentas showed focal areas of apoptosis at the interface of the decidua and uterus, allowing for separation of the maternal/placental unit during parturition.[36–39] ERKdko/control placentas had a smaller area of TUNEL positive cells around the edges of the decidua. The ERKdko/ERKdko placentas had reduced TUNEL positive staining around the edges of the decidua, indicating a loss of physiologic apoptosis associated with parturition (Figure 10A). Total placental TUNEL staining was reduced in ERKdko/ERKdko animals by nearly 25% ($p<0.05$, Figure 9B). Interestingly, most of this appeared to be localized to the periphery of the decidua. Control/control placentas showed positive TUNEL staining in approximately 17 percent of the decidua, while ERKdko/ERKdko animals showed only 9 percent of the decidua to be positive for TUNEL staining at e18.5 ($p<0.05$, Figure 10C). TUNEL staining in placentas from ERKdko/control pups were not significantly different than either of the other two groups (data not shown). The loss of pro-apoptotic tone in ERKdko/ERKdko placentas correlates with prolonged gestation and delayed parturition, indicating lack of readiness to begin parturition at e18.5 in these animals.

Figure 10: A) Photomicrographs showing TUNEL positive staining, indicating apoptosis of placental decidua. **B)** Control animals showed approximately 17 percent of decidua beginning apoptosis, indicating the initiation of the events preceding parturition. ERKdko ERKdko animals showed significantly less TUNEL staining, with only approximately 9 percent of the decidua affected ($p < 0.05$, n=5 placentas/genotype). **C)** Quantifying the entire placenta for TUNEL staining showed a modest reduction in overall apoptotic tone in Control/Control placentas, when compared to ERKdko/ERKdko placentas ($p<0.05$).


A.
E18.5
Maternal
Control
Fetal
Control
600 µm
ERK DKO
Control
ERK DKO
ERK DKO
B.
% TUNEL positive/
Decidual area
0.24
0.16
0.08
0.00
a
b
Maternal genotype
Control
ERKdko
Fetal genotype
Control
ERKdko
C.
% TUNEL positive/
placental area
0.06
0.04
0.02
0.00
a
b
Maternal genotype
Control
ERKdko
Fetal genotype
Control
ERKdko

DISCUSSION:

While ERK loss has long been shown to be detrimental to the function of the hypothalamic-pituitary-gonadal axis as well as the placenta, this is the first report of a novel model of placental ERK deletion associated with expression of the GnRHR in a mouse.[3,13] In the present studies, loss of pituitary gonadotropin production in the GRIC-ERKdko model was overcome through exogenous gonadotropin administration consistent with a standard superovulation protocol, allowing ERKdko animals to ovulate and become pregnant. The resulting pregnancy in ERKdko/ERKdko females was abnormal, characterized by prolonged gestation and parturition, and 100% fetal mortality. GnRHR-mediated Cre expression, and subsequent ERK excision was documented within the placenta. The loss of ERK signaling within the placenta also revealed abnormal developmental morphology of the decidua and junctional zones, characterized by an expanded labyrinth, reduced decidua, and reduced endothelial isolectin expression in the decidua and junctional zones. ERKdko placentas also showed reduced maternal decidual artery size and number. These placentas showed a significant reduction in physiologic apoptosis necessary for placental separation prior to parturition, consistent with the prolonged gestation phenotype observed. Collectively, these studies shed new light on the role of ERK signaling within the placenta in the mouse.

GnRHR has been localized in the placenta of multiple species, including humans and canines. In humans, GnRHR, GnRH I and GnRH II are expressed in the first trimester of human pregnancy, and regulate both trophoblast invasion and hCG secretion. GnRHR localizes to the cytotrophoblasts and extravillous trophoblasts

(EVT) of the placenta, but less in the syncytiotrophoblasts.[40] GnRH I and II localize to mononucleate villous, and EVTs, but GnRH I is also found in the multinucleated syncytial trophoblast layer in the chorionic villi during the first trimester of pregnancy. Interestingly, only GnRH I was found in the placenta at term.[41] Recently, GnRHR has been described in the canine placenta as well. Similar to the human, GnRHR was expressed at higher levels in the uteroplacental tissues than in the areas of the uterus less associated with the placenta (i.e., between implantation sites in this litter bearing species). In the canine placenta, GnRHR was found in fetal trophoblasts and maternal decidual cells, and at the surface and glandular epithelial cells in the uterus.[28] Again, this indicates the strong association with GnRHR and the maternal fetal interface in the placenta. Our studies provide a novel view of these expression profiles and the utility of the GRIC model to direct gene excision to these compartments.

GnRH and GnRHR regulate gene expression and gonadotropin secretion in the placenta.[42] This occurs through protein kinase A and C pathways, and can be blocked using inhibition of GnRH.[43,44] Treatment with GnRH or an analog has been shown to increase placental GnRHR mRNA and causes phosphorylation of PKC, ERK1/2, and JNK.[27,43,44] Activation of transcription factors increases in GnRHR transcription and hCG secretion indicates that GnRH is an important regulator of placental function, potentially acting in both paracrine and autocrine fashions.[45] Although human and rat pregnancy is affected by local placental and systemic hCG or LH secretion, respectively, no evidence exists for gonadotropin regulation of murine pregnancy.[14,23,26,46–49] This indicates the phenotype described above is likely due to loss of ERK2 signaling in the placenta, potentially affect secreted factors that regulate

placental morphogenesis and or vascularization. Other studies have shown that loss of ERK2 in the null animals causes catastrophic defects in the mouse placenta, so understanding the function of ERK signaling in a specific subset of placental cells is very informative.[50]

Loss of placental ERK2 resulted in reduced vascularization, expression of isolectins within the endothelial compartment, and placental abnormalities that may have contributed to reduced fetal weight in the current studies. We speculate that expression of GnRHR (and Cre) and subsequent loss of ERK signaling during the formation of the decidua prior to e12.5 is a likely cause of the changes in placental histology seen at e18.5.[51] Either the change in architecture or loss of ERK2 signaling could impact EVT invasion and subsequent maternal spiral artery remodeling, reducing the number and intraluminal areas of arteries seen in the decidua.[44] Additionally, matrix metalloproteases, which are also regulated by GnRH/GnRHR in the placenta, are involved in maternal spiral artery remodeling, leading to another hypothesis for reduced decidual vascularization; however, the specific role of ERK signaling in this situation is not clear.[44,52] Reduction of endothelial cell populations or function in the decidua and junctional zone likely reduces placental transfer and efficiency between mother and fetus. This results in reduction of fetal size and may account for reduction in litter size if the loss of ERK signaling at the maternal/fetal interface caused placental insufficiency. Loss of normal placental architecture, large cystic areas, general disorganization of the decidua and junctional zone, along with reduction in vascular tone could result in loss of efficient nutrient exchange between the fetus and dam, resulting in a fetal IUGR and possibly fetal distress/death.

Prolonged gestation and parturition stem from loss of placental ERK 2 and causes periparturient mortality in pups. Multiple factors initiate parturition, including fetal cortisol, maternal drop in progesterone, oxytocin, prostaglandin F2α and other endocrine facotrs.[53–56] Loss of either maternal or fetal ERK-dependent inputs could cause failure to initiate or sustain parturition, as seen in this model. This loss may stem from reduction in signaling between dam and fetus due to loss of vascular reduction or placental abnormalities. Hypogonadism in the ERKdko mice may also cause a reduction in baseline progesterone due to losses of LH support to the ovary. (Chapter 2) Progesterone withdrawal is a contributing factor to initiation of parturition and the possibility exists that the baseline reduction in progesterone in our model system does not occur at a level to initiate parturition.[57] Loss of ERK signaling in GnRHR expressing cells may ostensibly blunt or abrogate the release of PGF2α, thus contributing the delayed onset of parturition.[58] Alterations in oxytocin release is likely not related to the delayed parturition since this peptide is not implicated as an important factor in the onset of parturition in mice.[59] The loss of TUNEL staining at e18.5 indicates that ERKdko animals are not undergoing normal changes within the placenta in preparation for parturition. This indicates loss of some sort of signal to initiate focal apoptosis at the uterine/decidual interface. Future studies will need to focus on specific contribution of ERK signaling within the maternal fetal interface to determine the precise mechanisms involved.

The GRIC model holds interesting potential as a model of human disease. GnRHR has been shown to be important in placental development and function; it is even expressed at the implantation sites of ectopic pregnancy.[40] Although multiple

mouse models have been used to recapitulate idiopathic hypogonadal hypogonadism (IHH), none have focused on loss of function in cells expressing GnRHR and how ERK signaling may be functioning in these tissue compartments.[3,16,60] Up to 40 percent of IHH patients suffer from a GnRHR mutation, which could alter placental function as well.[61] Work by Janet Hall's lab has indicated that although some women with IHH respond appropriately to gonadotropin supplementation and are able to carry a normal pregnancy, others are not.[62,63] Some of these women suffer from multiple miscarriages, most often early in gestation. Not all patients with these mutations showed early pregnancy losses, indicating a non-genetic component, such as alterations in placental function as a contributing factor.[62,64,65] Further investigation of this model system may elucidate missing links and help improve assisted reproduction in women with IHH.

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CHAPTER 4

GnRH induces ERK-dependent expression of the bile acid receptor TGR5 in the pituitary gonadotrope

ABSTRACT

ERK1/2 regulates reproduction in multiple parts of the hypothalamic-pituitary-gonadal (HPG) axis. Previously, our lab has shown ERK1/2 signaling to be integral to pituitary gonadotrope function. To identify novel downstream targets of GnRH and ERK1/2, we performed RNA sequencing on pituitaries with and without GnRH stimulation. These unbiased screens revealed a novel relationship between a G-protein-coupled receptor (GPCR), *TGR5*, ERK1/2 and GnRH signaling. TGR5 is a bile acid receptor that regulates metabolic signaling including glucose homeostasis. Treatment with GnRH increased *TGR5* mRNA at both one and four hours post stimulation, which was lost with conditional loss of gonadotrope ERK1/2 signaling. This finding was validated in *in vivo* mouse models, as well as *in vitro* cell culture systems. Cholic acid (a hydrophilic bile acid) stimulation of αT3-1 cells induced CREB phosphorylation. Primary pituitary cultures treated with GnRH, cholic acid (CA) or a TGR5 agonist (INT777) all induced secretion of LH. TGR5ko mice are mildly subfertile compared to control animals. Loss of TGR5 resulted in delayed puberty, prolonged estrus cycles, smaller litters at longer inter-litter intervals, lower uterine and ovarian weights, and had lower levels of gonadotropin subunit mRNAs. In control animals, pituitary *TGR5* mRNA varied based on estrus cycle stage, and superovulation with INT777 caused ovarian luteal formation, indicating TGR5 agonists can drive an ovulatory LH surge. These studies indicate TGR5 is a modulator of the HPG axis. A known regulator of metabolism, TGR5 shows promise as a link between obesity, metabolic disorders, and reproduction, including issues of alterations in onset of puberty.

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INTRODUCTION

Work from our lab, and many others, has established the role of ERK1/2 signaling in the HPG axis, and specifically the pituitary.[1–4] Both Bliss et al, and research reported earlier in this dissertation, highlight the specific role of ERK in the pituitary gonadotrope (Chapter 2).[1] Briefly, loss of ERK signaling reduces or eliminates gonadotropin production in females, leading to infertility due to anovulation (Chapter 2). The mitogen activated protein kinase (MAPK) cascade is well established upstream of ERK1/2. [5–8] This kinase cascade activates immediate early genes, such as c-Fos, c-Jun and Egr1 and is an integral regulator of many biological processes, such as LH secretion, meiosis and placental formation.[9–13] Due to ERKs ubiquitous expression, ERK signaling is likely involved in regulating reproduction through unknown mechanisms as well as those already discovered.[13–16] To help elucidate some of these mechanisms in the pituitary, we utilized the GRIC mouse. The GRIC mouse (*GnRH* Receptor IRES Cre) allows gonadotrope specific excision of a target gene. We combined this mouse with an $ERK1^{-/-}$, $ERK^{f/f}$ mouse, to excise ERK2 specifically in the gonadotrope. We then conducted an unbiased screen to identify novel targets of GnRH stimulation that are ERK dependent.[17,18]

While a plethora of targets were identified through this screen, a few stood out as exceptionally intriguing. One of these targets, which was GnRH responsive and ERK dependent, was a bile acid receptor, TGR5, also known as GP-BAR1. This G-Protein coupled receptor (GPCR) was first identified in 2002, and has since been characterized as a regulator of metabolism.[19,20] TGR5 shows over 80 percent homology between multiple species, including humans and rodents.[21] This receptor is

preferentially activated by hydrophobic bile acids, and shows greatest response to taurine-conjugated lithocholic acid (TLCA), followed by lithocholic acid (LCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA) and cholic acid (CA).[20] Interestingly, several steroid hormones have shown activation of TGR5, including pregnandione.[20] It has been localized to multiple hepatic cell types, specifically sinusoidal endothelial cells and Kupffer cells, as well as the pancreas, macrophages, the gallbladder, the gastrointestinal tract, enteric nervous system, brown adipose tissue, muscles and others.[21–24] As expected for a widely expressed receptor, TGR5 has multiple functions. Primarily, it was recognized for its role in the gallbladder, promoting bile production, gallbladder filling, and smooth muscle relaxation.[25] It serves protective functions against hepatocellular carcinoma and cholestatic injury in the liver.[21,23] This protection is likely secondary to one of its other known effects; TGR5 reduces inflammatory reactions. Activation of TGR5 suppresses NF-κB phosphorylation and binding activity, inhibits macrophage reactivity and cytokine production, including tumor-necrosis factor α, IL-1α, IL-1β, and IL-6.[21,26–29] TGR5 activation increases metabolism and energy expenditure, without increasing activity or decreasing food intake.[21] Activation of TGR5 also increases glucose tolerance and intracellular ATP/ADP ratio. These effects are suspected to be secondary to an increase in glucagon-like peptide (GLP)-1 stimulation, which up regulates liver and pancreatic function.[24,30] Bile acid signaling through TGR5 also activates 2-iodothyronine deiodinase, which converts inactive thyroxine (T4) to tri-iodothyronine (T3), which is biologically active.[20,31–33] The improvement in metabolism following

bariatric surgery is hypothesized to be a function of increased bile acids and TGR5 stimulation.[34]

TGR5 has also been implicated in reproduction. Both bile acids and *TGR5* were found in the testes, ovary and adrenals. Taurocholic acid (TCA), and CA were identified in all three tissues, while DCA was only found in the testis and ovary.[35] Most of the characterization of the impact of bile acids on reproduction has been done in males, specifically focusing on testicular effects. Males fed a diet supplemented with 0.5% cholic acid showed reduced fertility, characterized by rupture of the blood-testes barrier, increased spermatid apoptosis, germ cell sloughing, and reduced testosterone. These effects are likely due to bile acid activation of TGR5 signaling in the germ cells and farnesoid receptor 1 (FXR) signaling in the Leydig cells.[36,37] Similar symptoms were seen in male mice suffering from obesity and metabolic syndrome when treated with bile acids.[38] Finally, bile acids and their receptors have been implicated in puberty. Male rats who underwent a bile duct ligation prior to puberty had alterations in liver size and liver enzymes, but also had smaller testes and seminal vesicle size, indicating a defect in normal pubertal maturation.[39] Multiple other labs have shown alterations in the bile pool around puberty in both humans and mice.[40–42] In humans, females bile acid pools displayed greater changes than males during puberty.[42] Since the bile acid pool is responsive to hormonal changes during puberty, it seems logical that the pool may be affected by other changes in the hormonal milieu, like pregnancy.[43]

We investigated the function of the bile acid receptor TGR5 in the pituitary, and its role in reproduction, focusing on female mice. These studies show that TGR5

is not only expressed in the pituitary, but plays an active role in reproduction and when activated, is capable of inducing gonadotropin production. Loss of TGR5 causes minor reproductive deficits in female mice. These studies indicate that TGR5 is a functional link between reproduction and metabolism, and may be implicated in the link between delayed puberty and obesity or as a mechanism related to diabetic or obesity related infertility.

MATERIALS AND METHODS

ANIMALS

ERK1 null (*ERK1*$^{-/-}$), ERK2 floxed (*ERK2*$^{fl/fl}$) and GRIC mice have been described previously.[1,17,44] To create gonadotrope specific ERK1/2 knockout animals, they were crossed and designated ERKdko (*ERK1*$^{-/-}$, *ERK2*$^{fl/fl}$, *Cre*$^{+/-}$), or Control (*ERK1*$^{-/-}$, *ERK2*$^{fl/fl}$, *Cre*$^{-/-}$). TGR5 Knockout, *TGR5*$^{-/-}$, (TGR5ko) animals were the generous gift of the Dr. Kristina Schoonjans and the Auwerx lab and have been described previously.[27,45] Animals were handled in compliance with the Cornell University Institutional Animal Care and Use Committee.

Female TGR5ko and control animals were checked every 24 hours after weaning (day 21) for evidence of vaginal opening as previously described.[46] Inter-litter interval and litter size was collected from data from harem bred animals over an 8 month period. For all experiments, animals were humanely euthanized via CO2 asphyxiation, and blood, pituitaries, hypothalamus, ovaries, uterus, and liver were collected. Uterus and ovaries were carefully dissected from the surrounding fat, and weighed. Hypothalamus, pituitary, one ovary, half of the uterus, and a small amount of

liver were snap frozen. The remaining uterus and ovary were fixed overnight in 10 percent formalin.

GENOTYPING

Genomic DNA was isolated from tail snips (3 mm), or an equivalent quantity of other tissues as indicated, using a E-Z Tissue DNA Kit (Omega Biotek, Norcross, GA) per manufacturer's instructions. Routine PCR genotyping was performed on animals as previously described.[47] PCR confirmation of ERK1 knockout, ERK2 flox, GnRHR cre, and TGR5ko were performed, with primers as listed below.

Primer		**Sequence (5' to 3')**
TGR5 KO	TGR5 rec	GAT GGC TGA GAG GCG AAG
	TGR5 Common	AGA GCC AAG AGG GAC AAT CC
	TGR5 Wildtype	TGG GTG AGT GGA GTC TTC CT
ERK1	ERK1 Forward	AAG GTT AAC ATC CGG TCC AGC A
	ERK1 Reverse	AAG CAA GGC TAA GCC GTA CC
ERK2	ERK2 Forward	AGC CAA CAA TCC CAA CCC TG
	ERK2 Reverse	GGC TGC AAC CAT CTC ACA AT
GnRHR	GnRHR Forward	GAA CTA CAG CTG AAT CAG TC
	GnRHR Reverse	CTC TAA CAA ACT CTG TAC A
	GnRHR Homozygous	CGG AAT TCA TCG ATC ATA TCA GAT CC

RNA Sequencing Screen

To assess changes in the transcriptome regulated by GnRH and ERK signaling, adult female control and ERKdko mice were passively immunized against endogenous GnRH using a sheep anti-GnRH antiserum (generously provided by Dr. Terry Nett, Colorado State University) as previously reported.[48] Three days following passive immunization, animals received saline or dAla6-GnRH, an analog of GnRH not bound by the passive immunoneutralization. Animals were euthanized at time 0, 1, and 4

hours following dAla6-GnRH administration. Pituitaries were dissected free and pituitary RNA was isolated using Triazol (ThermoFischer, Waltham, MA) per the manufacturer's instructions. RNA samples (n = 3/genotype/treatment) were sent to the Weill Cornell Genomics and Epigenomics Core facility for library preparation and sequencing on a HiSeq 2000. The data were analyzed through a previously described computational pipeline and differentially expressed genes were analyzed using network analysis databases Reactome and KEGG.[49,50]

RNA ISOLATION AND QUANTITATIVE PCR

Tissues were collected and Trizol (ThermoFischer, Waltham, MA) extraction was performed per manufacturer's instructions. Reverse transcription in 1000ug reactions was performed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer directions. qRTPCR was performed using SYBRGREEN (ThermoFisher, Waltham, MA) and primers listed below. They were run on a BioRad CFX96 Touch Real-Time OCR Detection System (BioRad, Berkeley, CA).

Primer		**Sequence (5' to 3')**
Glyceraldehydes-3-phosphate dehydrogenase	*Gapdh* forward	ATGTTTGTGATGGGTGTGAA
	Gapdh reverse	ATGCCAAAGTTGTCATGGAT
Gonadotropin Releasing Hormone Receptor	*GnRHR* forward	TGCTCGGCCATCAACAACA
	GnRHR reverse	GGCAGTAGAGAGTAGGAAAAGGA
Luteinizing Hormone β-subunit	*LHβ* forward	CTGAGCCCAAGTGTGGTGTG
	LHβ reverse	GACCATGCTAGGACAGTAGCC

Follicle Stimulating Hormone β-subunit	*FSHβ* forward	GCCATAGCTGTGAATTGACCA
	FSHβ reverse	AGATCCCTAGTGTAGCAGTAGC
α-Glycoprotein Subunit	*α-GSU* forward	TCCAGGGCATATCCCACTCC
	α-GSU reverse	CATTTCCCATTACTGTGGCCTTA
TGR5	*TGR5* forward	GCTCCTGTCAGTCTTGGCCTAT
	TGR5 reverse	TTCCTCGAAGCACTCGTAGACA
Farnesoid receptor 1	*FXR* forward	CGA AGA CTC CCT CAC AGT TG
	FXR reverse	ACC TCT ACT TCA TCT CCT TCA CT

SUBCELLULAR MEMBRANE RAFT FRACTIONATION

Membrane raft fractions were isolated from αT3-1 cells and whole mouse pituitaries as described previously.[18,51,52] Briefly, αT3-1 cells and whole mouse pituitaries were lysed in a buffer containing low concentrations of non-ionic detergents and membranes were subjected to centrifugation in a discontinuous sucrose gradient. Low buoyant density membrane fractions were identified using the marker Flotillin 1 and compared to non-raft fractions of higher density. In some studies, membrane fractions (low and high density) were subjected to digestion with PNGaseF to cleave glycosylation moieties. The digested membrane samples were resolved by SDS PAGE and probed with antibodies directed against TGR5.

IMMUNOBLOTTING

Cells were homogenized in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 130 mM NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate, 0.5%

deoxycholate, 2mM EDTA, 5mM sodium vanadate, 0.2 mM phenylmethysulfonylfluoride, and 5 mM benzamidine. Protein concentrations were determined by Bradford assay. PNGase F treatment was performed according to manufacturer's instructions (New England Biolabs, Ipswitch, MA). Samples were boiled for 5 minutes in sodium dodecyl sulfate load buffer, resolved by SDS-PAGE, and transferred to polyvinylidine difluoride membranes by electroblotting. Membranes were blocked with 5% nonfat dry milk in TBST (10 mM Tris-HCl, pH 7.5; 150mM NaCl; 0.05% Tween 20) and then incubated with specified antisera (anti-TGR5, Abcam, Cambridge, UK; anti-CREB, anti-pCREB, Millipore, Billerica, MA; anti-Flotillin-1, BD, Franklin Lakes, NY; horseradish peroxidase-conjugated secondary antibodies from Bio-Rad, Hercules, CA).[1] Protein bands were visualized using enhanced chemiluminescence according to manufacturer's instructions (BioRad, Berkeley, CA) and imaged on ChemiDoc XRS (BioRad, Berkeley, CA) They were analyzed using Image Lab software (BioRad, Berkeley, CA).

CELL CULTURE

αT3–1 cells, an immortalized mouse gonadotrope cell line (generously provided by Dr Pamela Mellon, University of California, San Diego, CA), were cultured as described previously.[53,54] αT3–1 cells were maintained in DMEM containing 2mM glutamine, 100-U penicillin/mL, 100µg streptomycin/mL, 1x nonessential amino acids, 10% fetal bovine serum. Cells were grown in 5% CO2 in air at 37°C in a humidified environment. Buserelin (des-GLY10 [D-Ser(t-But)6]-LH-RH ethylamide; referred to as GnRHa) was obtained from Phoenix Pharmaceuticals Ltd.

All other chemicals were obtained from Sigma. In all experiments, GnRHa was used at 10nM. Cholic acid was dissolved in DMSO and used at 1, 10, and 100µM concentrations. INT-777 was used at 10uM, and GW4064 was used at 1, 10, and 100µM. Cells treated with U0126 (Promega), a MEK inhibitor, were incubated for 30 minutes prior to other treatment.

PITUITARY PRIMARY CULTURE

Primary pituitary culture was performed as previously described.[52] Pituitaries were subjected to collagenase digestion (1.5 mg/mL) with periodic trituration using a sterile Pasteur pipette. The dispersed cells were transferred to complete media, pelleted, and resuspended. They were aliquoted to 24-well plates pretreated with poly L-lysine at a density of approximately 750,000 cells/well. Primary pituitary cells were incubated in complete media at 37°C in a humidified atmosphere (5% CO2, 95% O2) overnight. The following day, the cells were gently washed in serum-free DMEM for 2 hours. The cells were treated with buserelin, cholic acid, INT777 or GW4064. The media was harvested 4 hours later and assayed for LH. The pituitary cells remaining in the wells were lysed to assay for total protein using the Bradford assay. Concentrations of LH were determined using a commercially available ELISA per the manufacturer's instructions (Genway Biotechnology), and LH concentration was standardized by protein content of the specific wells.

SUPEROVULATION

Females were injected with 5 IU of pregnant mare serum gonadotropin (PMSG) intraperitoneally. 46-48 hours later, they were injected with either 5IU human chorionic gonadotropin (hCG), 0.1ml of saline, or 30mg/kg of INT777 intraperitoneally. For assessing CL and follicle formation, they were humanely euthanized 72 hours later, and ovaries were collected for histological examination.

HISTOLOGY

For histological examination, tissues were fixed in 10% formalin, paraffin embedded, serially sectioned at 4µm, and stained with hematoxylin and eosin using standard histological techniques. For characterization of the ovarian follicular population, every third section was examined microscopically for identification of follicular and luteal tissue. For post-superovulation, ovaries were step-sectioned at 20µm and examined for identification of luteal tissue. Sections were scanned and digitized using an Aperio Scanscope (Vista, CA).

VAGINAL CYTOLOGY

The vaginal vault was swabbed, which was used to make a cytological smear. It was stained with Wright's Giemsa stain, and examined with light microscopy. Epithelial cells, parabasal cells and leukocytes were differentiated on the basis of morphology. An animal was deemed to be in estrus with >85% superficial epithelial cells. Estrus interval was calculated as the days from the onset of estrus until the onset of the subsequent estrus.

STATISTICS

Comparisons were made by Student's *t*-test. All data are expressed as means ± standard error of the mean. A p value of <0.05 was considered statistically significant.

RESULTS

Unbiased RNA sequencing screen revealed the presence of bile acid receptors as a target of GnRH action in the gonadotrope

GnRH action and signaling via ERKs 1 and 2 are required for fertility in female mice[1] (see Chapter 2 in this dissertation). In the absence of ERKs in the gonadotrope (ERKdko), female mice display hypogonadotropic hypogonadism and anovulatory infertility. To gain more mechanistic insight into how GnRH-inducible, ERK dependent signaling controls gonadotrope cell function, we performed an RNAseq screen in mice receiving carefully timed administration of GnRH. For these studies, control and ERKdko female mice were immunoneutralized against GnRH. They were injected with either vehicle or dAla6-GnRH, and euthanized after either 1 or 4 hours. Pituitaries were collected and submitted for RNA sequencing. Data sets were analyzed for differential mRNA expression. Differentially expressed genes were then subjected to KEGG and Reactome network analyses to determine interrelationships between groups of differentially expressed transcripts (Figure 1 and Tables 1 and 2). Network analyses revealed transcript changes within a cohort of GPCRs at the 1 and 4 hour time points. This cohort of genes suggested that several GPCRs were GnRH inducible and required ERK signaling for up-regulation at either 1

or 4 hours following dAla6-GnRH administration. Table 2 identified *Gpbar1* (G-protein membrane bile acid receptor)/*TGR5* as a GPCR robustly regulated by GnRH in an ERK signaling dependent manner.

Figure 1: Strategy for preparation of RNA sequencing samples. Animals were immunoneutralized against GnRH. We waited three days, then treated them with $dAla^6$ or saline, and were euthanized at 0,1, or 4 hours after treatment.

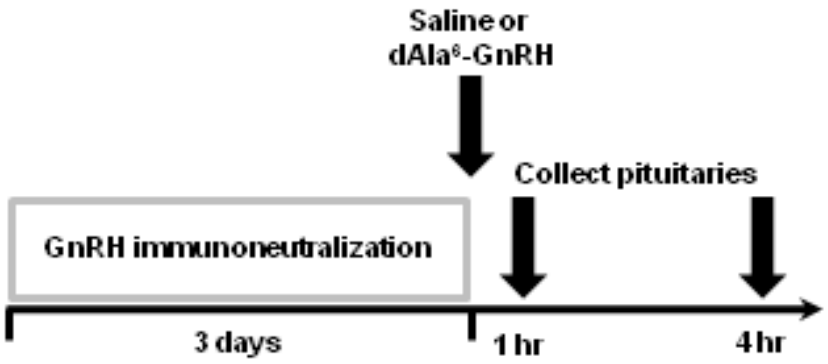

Saline or
dAla6-GnRH
Collect pituitaries
GnRH immunoneutralization
3 days
1 hr
4 hr

Table 1. Outcome from network analysis of RNAseq data set emphasizing signaling via GPCRs. (NS=non-significant)

Reactome gene networks	**ERKdko vs Control (p value @ T0)**	**ERKdko vs Control (p value @ 1 h)**	**ERKdko vs Control (p value @ 4 h)**
GPCR Downstream Signaling	NS	NS	0.00021
GPCR Ligand Binding	NS	0.097	0.00085
Signaling by GPCRs	NS	0.045	0.0016

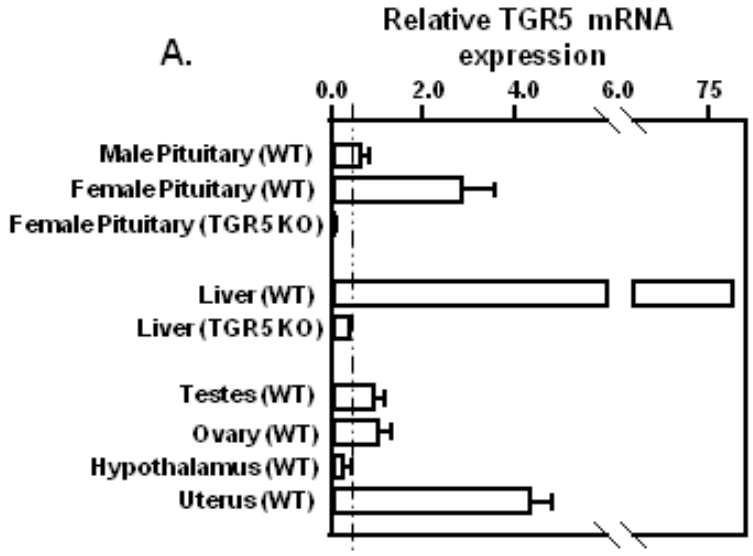
Table 2. Changes in Gpbar1/TGR5 mRNA levels following 1 and 4 hours of GnRH treatment comparing control and ERKdko female mice. (NS=non-significant)

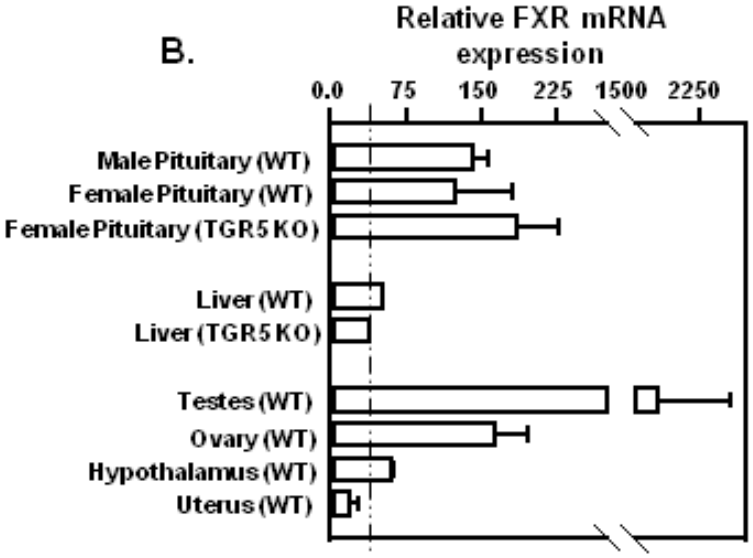
Transcript	**ERKdko vs Control (log fold @ 0 h)**	**ERKdko vs Control (log fold @ 1 h)**	**ERKdko vs Control (log fold @ 4 h)**
Gpbar1/ TGR5	NS	-5.11 (p = 0.027)	-7.51 (p = 0.001)

Bile acid receptors are expressed in multiple tissues in the hypothalamic-pituitary-gonadal axis

Given the surprising finding that *TGR5* was expressed and regulated within the gonadotrope, we next sought to determine the expression profile of *TGR5* and an additional bile acid receptor within the reproductive axis. To accomplished this, we obtained TGR5 null mice (a generous gift from Dr. Kristina Schoonjans, IFPL) to better confirm expression profiles. Pituitary, hypothalamus, uterus, ovaries, testes and liver were collected from wild type and TGR5ko animals and were assessed for *TGR5* and farnesoid X receptor (*FXR*) mRNA abundance (Figure 2). *TGR5* was found at highest levels in the liver of wildtype animals, but was also detected in male and female pituitaries, testes, uterus and ovaries. *TGR5* was below the detectable limits in tissues from TGR5ko animals and the preoptic area of the hypothalamus (Figure 2A). *FXR* was found at high levels in both male and female pituitaries, ovaries, and hypothalamus. Highest levels of *FXR* were found in the testes, and it was found at similar levels to *TGR5* in the liver (Figure 2B). These studies revealed the comprehensive expression of bile acid receptors within the reproductive axis. We next focused on potential mechanisms that bile acid signaling may influence within this endocrine axis initially focusing on the gonadotrope since this was the original tissue investigated in our RNA screen.

Figure 2: Identification of tissues expressing *TGR5* transcripts. Dotted line represents the lower detectable limit of the assay. **A)** *TGR5* is expressed in pituitaries of both male and female mice, as well as the testes, ovaries, uterus and liver. It was not expressed in the pituitary or liver of TGR5ko mice, or in the hypothalamus. **B)** *FXR* is expressed in the male and female pituitaries, in both wildtype and TGR5ko mice. It is also expressed in the testes in very high levels, as well as the ovary and hypothalamus. It is not expressed in the uterus.


A.
Relative TGR5 mRNA expression
0.0
2.0
4.0
6.0
75
Male Pituitary (WT)
Female Pituitary (WT)
Female Pituitary (TGR5 KO)
Liver (WT)
Liver (TGR5 KO)
Testes (WT)
Ovary (WT)
Hypothalamus (WT)
Uterus (WT)


B.
Relative FXR mRNA expression
0.0
75
150
225
1500
2250
Male Pituitary (WT)
Female Pituitary (WT)
Female Pituitary (TGR5 KO)
Liver (WT)
Liver (TGR5 KO)
Testes (WT)
Ovary (WT)
Hypothalamus (WT)
Uterus (WT)

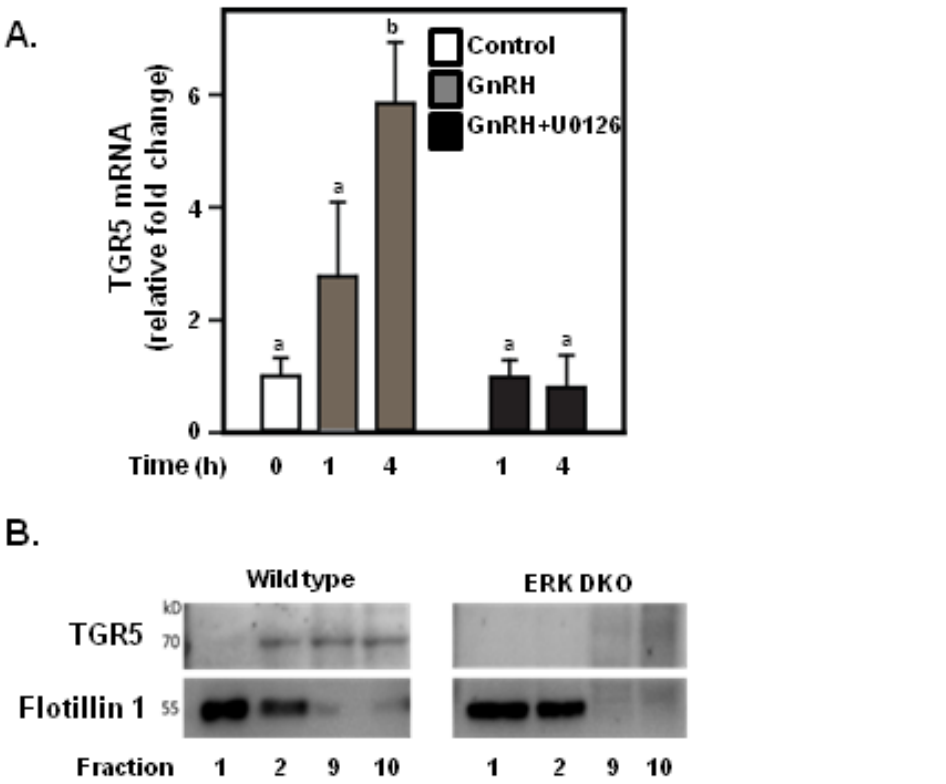
TGR5 is GnRH responsive and ERK1/2 dependent in pituitary gonadotropes

TGR5 mRNA was induced strongly at one and 4 hours following GnRH administration; this regulation was negated with deletion of ERK1/2 signaling in the pituitary gonadotropes (Table 2). Using the immortalized pituitary gonadotrope cell line, αT3-1 cells, these results were validated. GnRHa (buserelin) treatment for four hours caused significant upregulation of *TGR5* mRNA in these cells. This increase was completely ablated with the addition of U0126, a MEK inhibitor (Figure 3A). Confirmation that *TGR5* was GnRH responsive and ERK dependent *in vitro* caused us to investigate whether TGR5 protein was absent in pituitary in the ERKdko model *in vivo* (Chapter 2). Pituitaries were collected, and subjected to membrane raft fractionation to more clearly examine membrane localization of TGR5. Membrane fractions were subjected to immunoblotting for TGR5 in both high and low density fractions from discontinuous sucrose gradients. TGR5 was detected in the high and low density fraction of control animals, but was not seen in any membrane fractions of ERK1/2 knockout animals, confirming the importance of ERK signaling in TGR5 regulation (Figure 3B). Flotillin 1 was used as a marker of membrane raft fractionation.

The observation that TGR5 was localized to low buoyant density fractions in whole mouse pituitary prompted us to examine the membrane localization of this GPCR in more detail in αT3-1 cells (Figure 3A). Membrane raft fractions were resolved using SDS PAGE and immunoblotted with the TGR5 antibody. In some fractions, digestion with PNGaseF (a deglycosylase) was used to determine membrane localization of TGR5 as a function of TGR5 glycosylation state. In mock digested

fractions, TGR5 was detected as 95 kDa, 68 kDa and 35 kDa bands in high buoyant density membrane fractions, presumably reflecting variation in glycosylation state of the receptor. In low buoyant density raft fractions, the 68 kDa band was enriched while the 95 kDa and 35 kDa bands were reduced. PNGaseF treatment resulted in detection of the 35 kDa band in the raft fractions supporting speculation that in membrane rafts, only the glycosylated form of TGR5 was present (Figure 3C).

Figure 3: *In vitro* and *in vivo* verification of RNA sequencing results **A)** αT3-1 cells show significant increase in *TGR5* transcript levels after 4 hours of treatment with GnRHa. This increase was negated with pretreatment with the MEK inhibitor U0126. **B)** TGR5 western blot of membrane raft fractions from pituitaries of control and ERKdko mice. ERKdko mice lacked TGR5 expression in the pituitary. **C)** TGR5 expression in membrane raft fractions from αT3-1 cells. In low density fractions, TGR5 appears to be preferentially glycosylated, while in high density, non-raft fractions, TGR5 exists in both glycosylated and nonglycosylated states


A.
TGR5 mRNA (relative fold change)
Control
GnRH
GnRH+U0126
Time (h) 0 1 4 1 4
B.
Wild type
ERK DKO
TGR5
Flotillin 1
Fraction 1 2 9 10 1 2 9 10
C.
Low density raft fraction
High density non-raft fraction
Mock
PNGF
kD
95
72
55
43
34
26
Glycosylated TGR5
Non-glycosylated TGR5

TGR5 is functionally active in pituitary gonadotrope cells in vitro

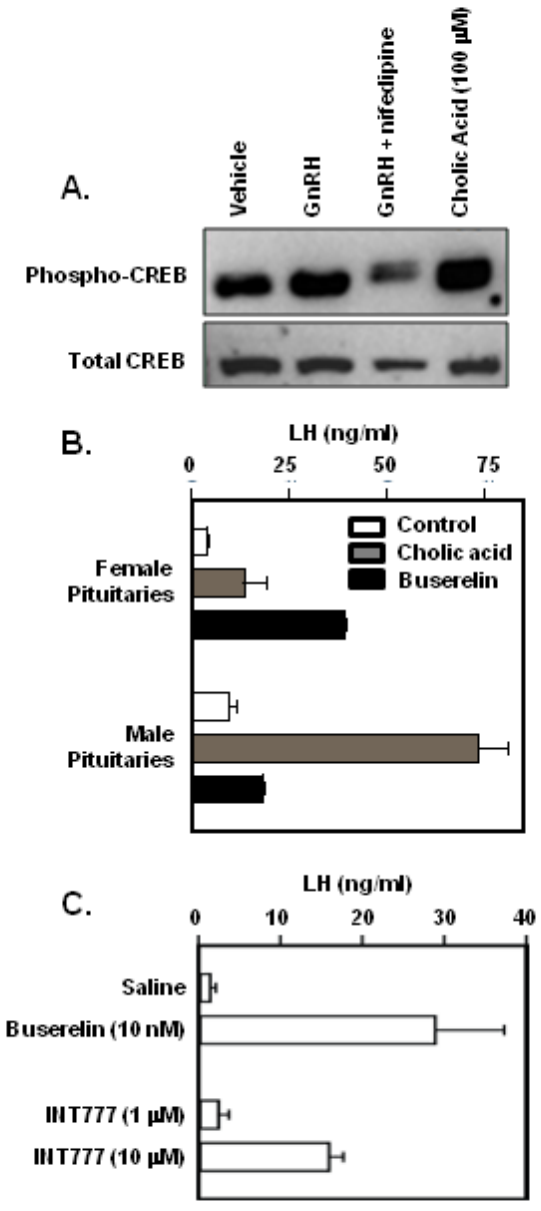
After confirming the presence and GnRH/ERK regulation of TGR5 in the pituitary, we sought to determine the functional activity of the receptor, initially utilizing αT3-1 cells. Cells were treated with vehicle, a TGR5 agonist, cholic acid , or a GnRH agonist, buserelin , for 15 minutes. Cells were collected and lysates were immunoblotted for cAMP response element binding protein (CREB), or phophorylated-CREB (pCREB). At 15 minutes of stimulation, both cholic acid and buserelin induced CREB phosphorylation consistent with the observations of others, indicating TGR5 is likely coupled to adenylyl cyclase (Figure 4A).[55,56] These studies supported the conclusion that TGR5 is functionally active in an *in vitro* gonadotrope cell model.

To confirm that TGR5 is functionally active in fully differentiated gonadotrope cells, male and female control mice were euthanized and pituitaries were collected. Pituitaries were dispersed into primary culture and plated overnight. The cells were then treated with either buserelin , cholic acid , the TGR5 agonist INT777 , or the FXR agonist, GW4064. Media was collected after 4 hours of treatment, and LH concentrations in the media were determined.

Primary culture from male animals showed LH secretion following GnRH stimulation, as well as cholic acid and INT777 stimulation. There was no LH production following Gw4064 stimulation (data not shown). Interestingly, females showed much lower LH response to Cholic acid and INT777 stimulation than males, but still showed no LH production from GW4064 (Figure 4B, C). Together, this data indicate that not only does TGR5 have a functional role in phosphorylation of CREB

in pituitary gonadotropes, it also plays a potentially important physiological role in the regulation of LH secretion. Stimulation of pituitary cells with TGR5 agonists causes LH secretion, though the threshold and response appears to vary between males and females. Interestingly, although FXR does appear to be present in the pituitary, it does not appear to be functional, as treatment with FXR agonist did not induce LH secretion.

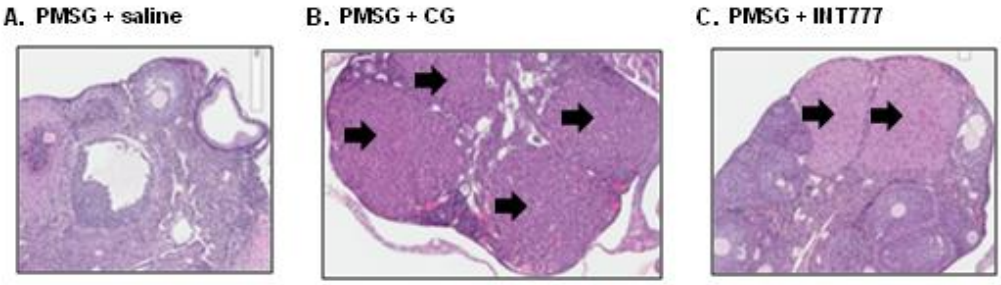
Figure 4: Pituitary TGR5 expression has functional significance A) Immunoblot showing CREB phosphorylation after treatment with GnRH and cholic acid. Total CREB shown as lane loading control. **B)** Primary pituitary culture treated with cholic acid (100µM) or GnRHa (10nM), buserelin, showed increased LH production compared with vehicle treated cells. **C)** Primary pituitary culture showed greater sensitivity to the specific TGR5 agonist INT-777 than cholic acid, secreting more LH in response to a lower concentration (10µM)


A.
Vehicle
GnRH
GnRH + nifedipine
Cholic Acid (100 µM)
Phospho-CREB
Total CREB
B.
LH (ng/ml)
0
25
50
75
Control
Cholic acid
Buserelin
Female Pituitaries
Male Pituitaries
C.
LH (ng/ml)
0
10
20
30
40
Saline
Buserelin (10 nM)
INT777 (1 µM)
INT777 (10 µM)

TGR5 alters gonadotropin transcript level and secretion

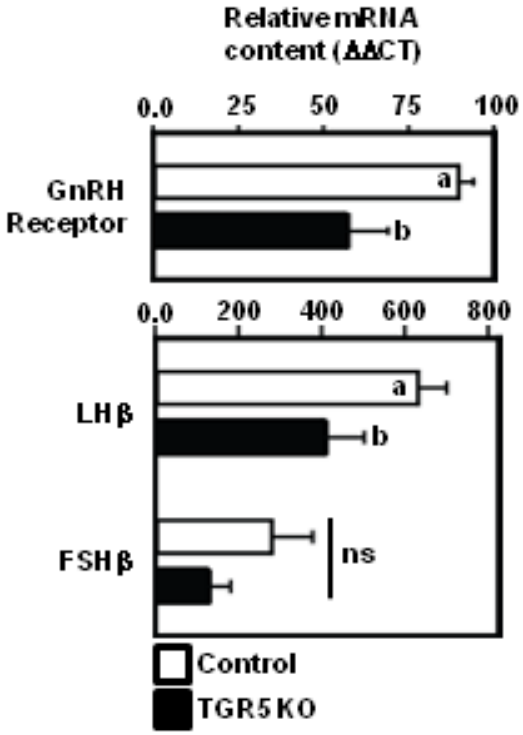
Following our observations that TGR5 agonists could incite gonadotropin secretion *in vitro*, we assessed the *in vivo* effects of bile acid stimulation on gonadotropin secretion. Wildtype C57/B6 animals were treated with pregnant mare serum gonadotropin (PMSG), followed by either saline, INT777, or human chorionic gonadotropin (hCG) 46 hours later. We speculated that since INT777 and cholic acid could induce LH secretion in vitro, this treatment might induce ovulation in PMSG-treated mice. Ovaries were collected 72 hours after the second treatment, serially sectioned and assessed for the presence of corpora lutea. Luteal tissue was observed in both ovaries from PMSG/hCG and PMSG/INT777 treated animals, but it was not seen in PMSG/saline treated animals. This indicates that treatment INT777, a TGR5 agonist, can initiate an LH surge capable of inducing ovulation and subsequent CL formation in female mice (Figure 5).

Figure 5: Treatment with INT-777 as part of a superovulation protocol induces ovulation. Control female mice were given PMSG (FSH agonist), then either saline (negative control), hCG (LH agonist) or INT-777 (TGR5 agonist) 48 hours later. Ovaries were collected 72 hours following the second injection, and assessed for follicular and luteal structures. **A)** Animals in the saline group showed no luteal formation, but displayed large antral follicles, an indication they did not ovulate, as expected. **B)** Animals in the hCG group showed multiple CL's in each ovary, indicating successful induction of ovulation. **C)** Animals treated with INT-777 showed a mix of CLs and antral follicles, indicating that INT-777 is capable of producing an LH surge commensurate with that needed for ovulation.


A. PMSG + saline
B. PMSG + CG
C. PMSG + INT777

Since a TGR5 agonist was capable of inducing gonadotropin production, we assessed baseline gonadotrope function in control and TGR5ko animals. Females of both genotypes were followed through the estrous cycle via vaginal cytology, and euthanized at proestrus. Pituitaries were extracted, and assessed for transcript levels of signature gonadotrope genes (*GnRHR*, *FSHβ*, and *LHβ*). *GnRHR* and *LHβ* were significantly reduced in TGR5ko animals compared to control animals at estrus ($p<0.05$). There was a trend towards *FSHβ* transcript reduction, ($p<0.1$), which was likely associated with small sample size and individual variation. (Figure 6)

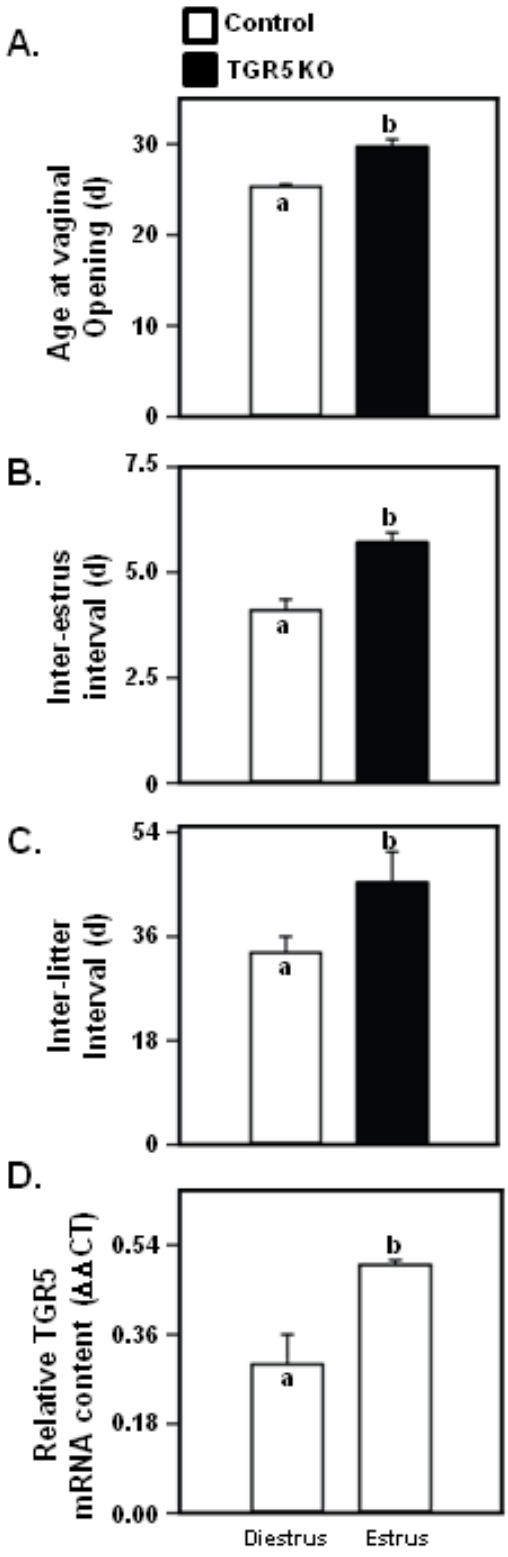
Figure 6: TGR5ko animals display blunted gonadotropin subunit transcript levels. TGR5ko females display about 20 percent lower levels of GnRHR, approximately a 30 percent decrease in Lhβ levels ($p<0.05$ for both). There was an approximately 50 percent reduction in FSHβ transcript levels, but this was not significant ($p<0.1$), due to high variability and small sample size.


Relative mRNA content (ΔΔCT)
0.0
25
50
75
100
GnRH Receptor
a
b
0.0
200
400
600
800
LHβ
a
b
FSHβ
ns
Control
TGR5 KO

TGR5ko mice show mild subfertility

Blunted gonadotrope transcript levels in TGR5ko animals lead us to assess them for a reproductive phenotype. While these animals are fertile, they display notable deficits in their reproductive capacity. Initially, TGR5ko females displayed a mildly prolonged inter-estrus interval, 4.1 days in controls compared to 6.7 days in TGR5ko animals (Figure 7B). The prolonged estrus interval may have contributed to a nearly 50 percent prolonged inter-litter interval in TGR5ko animals as well (Figure 7C). TGR5 transcript levels appeared to vary in the female pituitary throughout the estrous cycle; reaching a nadir at diestrus and a significantly higher level at estrus (Figure 7D). As TGR5 stimulation causes gonadotropin release (Figures 4, 5), loss of TGR5 in the pituitary during estrus could alter the hormonal milieu significantly enough to reduce the reproductive capacity of TGR5ko mice, as indicated above. There was no significant difference in body weights between TGR5ko and wildtype animals (data not shown).

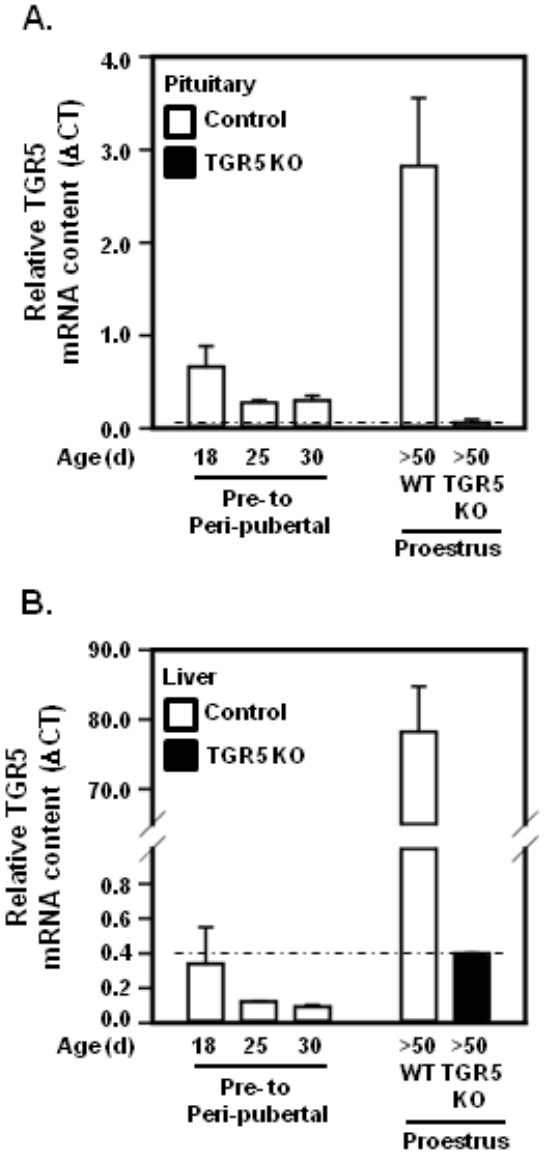
Figure 7: Female TGR5ko animals show reduced reproductive characteristics **A)** TGR5ko animals had an approximately 4 day delay in vaginal opening (day 25.2 versus day 28.8) **B)** TGR5ko animals had a significantly inter-estrus interval of 5.5 days, compared to 4.7 days in control animals. **C)** They also displayed a longer interval between litters, with an average of 45 days, while control animals had a litter on average every 33 days. **D)** TGR5 showed significantly higher expression of transcript levels during estrus than diestrus. For all panels, $p<0.05$.


A.
Control
TGR5 KO
Age at vaginal Opening (d)
30
20
10
0
a
b
B.
Inter-estrus interval (d)
7.5
5.0
2.5
0
a
b
C.
Inter-litter Interval (d)
54
36
18
0
a
b
D.
Relative TGR5 mRNA content (ΔΔCT)
0.54
0.36
0.18
0.00
a
b
Diestrus
Estrus

TGR5 transcript levels are altered with puberty

Bile acid pools have been shown to be altered during puberty, and conversely changes in bile acid metabolism can impact the onset of puberty and fertility in rats.[39–42] *TGR5* transcript levels were assessed in the pituitary and liver of control mice before, during and post-puberty. Liver *TGR5* transcript levels remained below detectable levels until after puberty. In the pituitary, *TGR5* mRNA levels decreased from pre-pubertal (post natal day 18) levels during puberty, but increased three fold above pn18 levels after puberty (Figure 8A, B). This indicates that changes in *TGR5* transcript levels during puberty are likely related to either changes in GnRH signaling or alterations in metabolism and bile acid pool that occur during puberty. Confirming physiologic importance of TGR5 in puberty onset, female TGR5ko mice displayed delayed puberty, by about 4 days, as assessed by day of vaginal opening (Figure 7A). This was independent of body weight (data not shown).

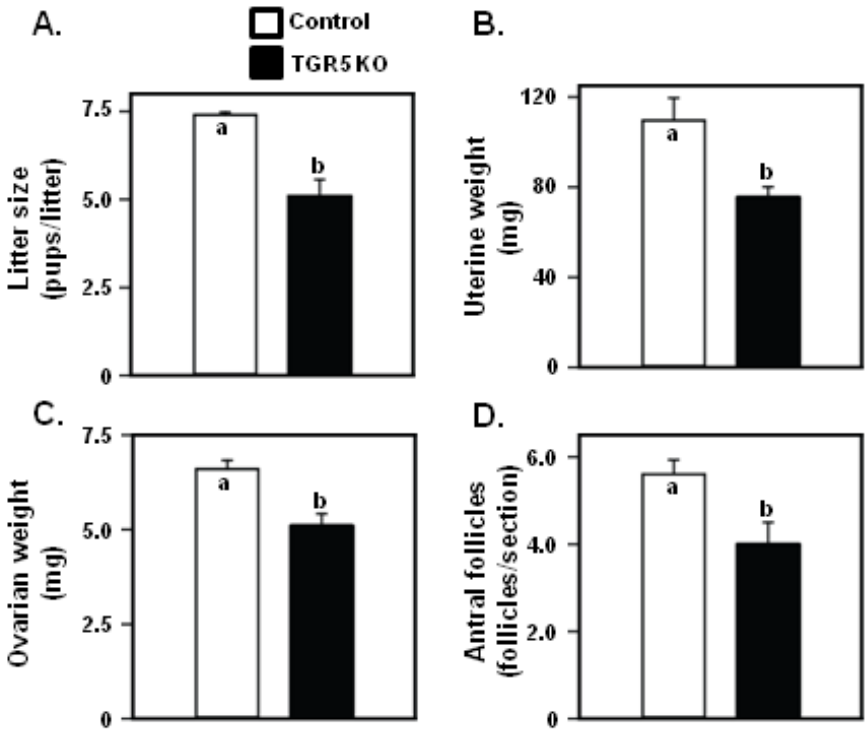
Figure 8: *TGR5* transcript expression varies throughout puberty A) *TGR5* transcript expression in the pituitary of control animals decreases from pn18 through puberty (pn25, 30) and increases significantly by adulthood. **B)** *TGR5* transcript levels in the liver remain under the detectable limit of the assay until pn30, as defined by the level found in tissues from TGR5ko animals. This level increases significantly after puberty (>pn50).


A.
Pituitary
Control
TGR5 KO
Relative TGR5 mRNA content (ΔCT)
4.0
3.0
2.0
1.0
0.0
Age (d)
18
25
30
>50
>50
WT
TGR5 KO
Pre- to Peri-pubertal
Proestrus
B.
Liver
Control
TGR5 KO
Relative TGR5 mRNA content (ΔCT)
90.0
80.0
70.0
0.8
0.6
0.4
0.2
0.0
Age (d)
18
25
30
>50
>50
WT
TGR5 KO
Pre- to Peri-pubertal
Proestrus

TGR5ko females show evidence of reduced HPG axis function

Coincident with changes previously characterized, female TGR5ko mice showed evidence of reduced gonadotropin and steroid hormone levels. They had a 30 percent reduction in uterine weight, and a nearly 50 percent reduction in ovarian weight, which can be associated with a decrease in estradiol levels (Figure 9B, C). The reduction in ovarian weight is likely due to loss of normal ovarian structures and activity; the TGR5ko females showed significantly fewer antral follicles (Figure 9D), which is consistent with the reduction of *FSHβ* transcript levels (Figure 6). The reduction in follicle count, in turn, appeared to lead to significantly smaller litters (Figure 9A).

Figure 9: TGR5ko females show evidence of reduced gonadotropin and steroid hormone function. **A)** TGR5ko females have an average of 5.1 pups per litter, while control animals have an average of 7.4, significantly more. **B)** They also an approximately 30 percent decrease in uterine wet weight (average of 72mg), compared to control animals (average 109mg). **C)** Ovarian weight in TGR5ko animals was also significantly reduced at 5.0mg, compared to 6.5mg in control animals. **D)** Finally, TGR5ko animals displayed fewer antral follicles (4.0) than control animals (5.6). For all data in this figure, $p<0.05$.


A.
Control
TGR5KO
Litter size (pups/litter)
7.5
5.0
2.5
0
a
b
B.
Uterine weight (mg)
120
80
40
0
a
b
C.
Ovarian weight (mg)
7.5
5.0
2.5
0
a
b
D.
Antral follicles (follicles/section)
6.0
4.0
2.0
0
a
b

DISCUSSION

TGR5, a GPCR bile acid receptor, is known to have roles in inflammation, liver and biliary disease, gastrointestinal physiology, and fertility.[21,57,58] An unbiased RNA sequencing screen found hypophyseal *TGR5* to be GnRH responsive in an *ERK* dependent manner. This was confirmed *in vitro* and *in vivo*. *TGR5* transcript expression was seen in the pituitary and the gonads. Activation of TGR5 by either the endogenous ligand, cholic acid, or a synthetic, specific TGR5 agonist, INT777, induced CREB phosphorylation in αT3-1 cells, and LH secretion in primary pituitary cells. It also induced ovulation in female mice as part of a superovulation protocol.

In vivo, TGR5ko females had reduced transcript levels of gonadotrope specific genes, *GnRHR*, *LHβ* and *FSHβ*. They also showed delayed puberty, prolonged estrus cycles, and had smaller litters at longer intervals. They displayed reduced ovarian and uterine weights, and had fewer antral follicles. Interestingly, *TGR5* levels in the pituitary seemed to vary throughout the estrus cycle; they were significantly higher in estrus than in diestrus, indicating hormonal regulation of TGR5. Corroborating this hypothesis of hormonal regulation of bile acid signaling, pituitary *TGR5* transcript levels fluctuate significantly throughout puberty. These findings add to our understanding of TGR5, and provide evidence it can act as a functional modulator of female reproduction.

To our knowledge, this is the first time a bile acid receptor has been implicated in gonadotropin production. Our studies show that bile acid stimulation causes LH secretion in pituitary primary culture, but bile acids can also cause a significant enough LH surge to induce ovulation and formation of corpora lutea in mice. The bile

acid pool changes with changes in steroid hormone milieu, such as pregnancy, indicating at least responsiveness to hormone concentrations. We know treatment with bile acids can induce LH production; so conversely, female TGR5ko animals may have reductions or alterations in LH synthesis and release. These changes could account for the reproductive deficits seen in those animals. Unfortunately, due to assay limitations, we do not know if bile acid treatment causes induction of FSH as well. Reduction of FSH would lead to the reduced antral follicles. Reduction in gonadotropin levels has been associated with reduction in litter size, estrus interval, ovarian and uterine weight (Chapter 2). Further evidence GnRH and gonadotropin signaling modulate TGR5 expression is demonstrated by the upregulation of TGR5 transcript levels during estrus. This could be due to induction by GnRH signaling. With an increase in TGR5, similar bile acid levels are likely to result in an increased signaling response and could contribute to the LH surge. In humans, the total amount of bile acids in enterohepatic circulation is known as the bile acid pool. The composition of this pool can vary, altering the percentage of the different types of bile acids.[59] Although the size of this pool doesn't alter throughout the menstrual cycle in humans, alterations in the composition could increase the activation of TGR5. Alternately, receptor upregulation could cause an increase in TGR5 signaling, without a change in the pool size. [43]

Supplementing male mice with cholic acid caused infertility, germ cell apoptosis and decreases in testosterone. These changes appear to be mediated through both TGR5 and FXR, via CAR, SHP, and Dax1 signaling.[38,60] TGR5ko males did not show these phenotypes when supplemented with CA.[36] Contrarily, our data indicates

that loss of TGR5 in females causes subfertility. Our only bile acid supplementation was an acute exposure to CA, while the male animals were exposed over time to a 0.5% supplementation. There also could be differences in male and female metabolism of bile acid. In our studies, female pituitary primary culture was less responsive to CA and INT777 than male pituitary primary culture. Additionally, males did not show changes in bile acid saturation, or the levels of cholesterol within the bile acid pool with puberty, although females did.[42] Similarly, in rats, females had a larger bile acid pool than males during puberty, though it was similar to males at other time points.[41] These indicate there is sex specific bile acid regulation, which could explain the differences noted between our studies and those of the Volle group.[36–38,60]

Due to the multitude of studies displaying alterations in bile signaling through puberty and the direct impact of bile duct ligation on reproductive function, we chose to investigate the relationship of TGR5 transcript level and puberty.[39–42,61] Interestingly, TGR5ko animals showed a delay in puberty, and *TGR5* transcript levels fluctuated before and after puberty: they increased 3fold from pre-pubertal levels post-puberty The alterations during puberty could be part of the link between nutrition, metabolism and reproduction, since changes in body mass and body composition are known to regulate onset of puberty.[62–65]. Signaling in the HPG axis alters during puberty as well. GnRH pulsatility increases and estradiol changes from inhibition of the pituitary permissive of gonadotropin production.[66,67] While kisspeptin and KNDY neurons have been implicated of the pubertal transition, it is possible that TGR5 and bile acid signaling may also contribute.[68]

Our data identifies a novel regulator of gonadotropin production in the pituitary, bile acid receptor TGR5. Reduced reproductive capacity of TGR5ko females indicates that TGR5 and bile acids could be used as a modulator of reproductive function. This has potential implications for management and treatment of obesity and diabetes related infertility. Additionally, TGR5 and bile acid metabolism may be implicated in alteration of the onset of puberty.[69–73] Further studies should focus on the mechanisms and clinical implications, along with long term effects of bile acid supplementation and the effects on female reproduction.

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CHAPTER 5

Conclusions and Future Directions

Introduction

Gonadotropin-releasing hormone (GnRH), released from the median eminence of the hypothalamus, is transported directly to the pituitary through the hypophyseal portal system.[1] There, it binds to the GnRH receptor (GnRHR), activating pituitary gonadotropes and stimulating the secretion of gonadotropin hormones, follicle stimulating hormone (FSH) and luteinizing hormone (LH). These peptide hormones are heterodimers, composed of unique β-subunits (LHβ and FSHβ), and a common glycoprotein hormone α-subunit (αGSU). [2] The gonadotropins act upon the gonads, promoting gametogenesis and steroidogenesis.[3]

GnRHR is a G-protein coupled receptor (GPCR) that lacks the intracellular C-terminal tail associated with GPCRs, which renders it resistant to down regulation and desensitization.[4–9] Ligand (GnRH) binding activates a signaling cascade beginning with Gαq, and phospholipase C β (PLC). The signaling cascade results in intracellular calcium release and extracellular calcium influx.[10–13] This increase in intracellular calcium initiates mitogen activated protein kinase (MAPK) cascade activation.[14–16]

The MAPK cascade involves serial phosphorylation of subsequent serine-threonine kinases.[17–20] While there are multiple MAPK cascades, the studies in this dissertation focus on understanding the role of ERK1/2 signaling in reproduction, using transgenic mice. ERK1 knockout (ko) mice have been shown to be viable and fertile, but ERK2ko is embryonically lethal.[21–24] To circumvent this difficulty, we

utilized multiple Cre drivers, most notably GRIC (GnRH Receptor IRES Cre), which causes Cre expression specifically in cells expressing GnRHR.[25] Utilizing this mouse model, we worked to understand the role of ERK1/2 signaling in the reproductive axis, specifically in cells expressing GnRHR. This led us to investigate the role of GnRHR and ERK in aging, pregnancy and aspects of metabolism.

ERKs, GnRHR, and gonadotropins in aging

The first set of studies described in this thesis focused on understanding and describing the deleterious effects of chronic hypogonadotropism on gonadal aging, reproductive characteristics and body weight. Our lab, among others, has shown the importance of ERK in gonadotropin production and signaling.[26–28] Utilizing the GRIC mouse, we excised ERK2 in pituitary gonadotropes. Our studies revealed a phenotype similar to that described by Bliss et al, and displayed the absolute requirement for ERK signaling in gonadotropin production and secretion. (Table 1) [26] Briefly, at 6 months of age, the females were infertile, anovulatory, and acyclic, while the males displayed mild subfertility.

Table 1: Summary of reproductive characterization of ERK deletion using the αGSU and GRIC Cre mice

	αGSU cre	**Gric Cre**
Female		
Estrous cycle	Anestrous	Prolonged cycle
Female fertility	Infertile	Infertile
Ovarian histology	No CLs	No CLs
LH serum levels	NS from control	Reduced from controls
FSH serum levels	NS from control	Reduced from controls
Castration response	Blunted LH response, normal FSH response	Blunted LH/FSH response
Response to exogenous gonadotropin	Ovulation, not paired	Ovulation followed by pregnancy
Spontaneous copulatory plug	None	33%
Male		
Male fertility	Normal	Subfertile
Castration response	NS from control	Blunted LH/FSH response
LH serum levels	NS from control	NS from control
FSH serum levels	NS from control	Trend lower, NS from control
Testes histology	Normal	Reduced tubule size

*NS: No significant changes

These mice, with Cre excision of GnRHR associated ERK signaling, are a potential model for idiopathic hypogonadotropic-hypogonadism (IHH). To this end, we studied changes in the HPG axis in the ERK double knockout (ERKdko) mice over time. Reduction of pituitary gonadotrope function resulted in premature gonadal aging and profoundly reduced reproductive capacity as they aged. Despite similar gonadotrope related transcript levels, at 12 months old, ERKdko females displayed altered ovarian histology, including areas of tissue disorganization and acellularity. The ERKdko animals were also significantly heavier than control animals, as they aged indicating a link between metabolism and loss of reproductive potential. Aged (18 month old) ERKdko males displayed evidence of testicular degeneration, including deteriorating or degraded tubules, tubule calcification, and giant spermatid cells. These animals also had marked reduction in sperm count, and testicular and seminal vesicle weight, and the males showed increased body weight at 9 and 12 months compared to control animals.

One of the hypotheses for these phenotypes was alterations in serum peptide and steroid hormones. Though we assayed male serum for FSH and testosterone levels, serum LH assays are underway. For females, quantifying the steroid hormone levels in serum from young and aged females, and peptide hormones in aged animals would give us a better understanding of the impact of gonadotrope ERK loss on steroidogenesis and whether gonadotropin secretion decreases with age in these animals.

Additionally, characterizing females at more advanced ages would provide information on the effects of long term hypogonadotropism, as the phenotype

progresses with age in males. . Testes from ERKdko animals did not show a histological phenotype until 18 months, but ovaries showed changes at 12 months. Ovaries from 18 month old animals may show a more severe phenotype, which would elucidate requirements for gonadotropin secretion for maintenance of the HPG axis. This effect appears specific to hypogonadotropism, due to the lack of similar phenotype in age matched Stra8 ERKdko males.

Loss of ERK signaling in the placenta results in delayed parturition and fetal mortality

Even more striking than the gonadal degeneration phenotype seen in these animals was their inability to maintain a pregnancy and produce viable pups, indicating the requirement for ERK signaling throughout pregnancy. Again using the GRIC model, we assessed female ERKdko animals' response to exogenous gonadotropin administration. Though they formed CLs in response to a superovulation protocol, only one third of treated females became pregnant. Along with poor conception rates, ERK signaling seems to be of paramount importance for pregnancy termination as well. These animals not only suffered from prolonged gestation and a dystocia, but they had absolute fetal mortality.

The data reported in this dissertation supports the conclusion that this dramatic neonatal mortality phenotype may be due to loss of ERK signaling in GnRHR expressing cells in the placenta. The placentas from ERKdko animals showed abnormal cystic areas in the decidua and junctional zone, along with other histological abnormalities, including decreased vascularization and peri-parturient decidual

apoptosis. Placental ERK signaling in decidual cells and in later gestation in trophoblasts regulates placental growth and its loss abrogates the normal cascade to initiate parturition. ERK signaling is required for normal placentation, and total loss of ERK2 signaling results in catastrophic placental failure and embryonic lethality.[29] Our model indicates that even partial loss of ERK signaling can have drastic consequences.

Although the studies in this dissertation served to characterize the pregnancy and placental phenotype seen in the GRIC mouse, there are several areas for further study. Understanding the respective maternal and fetal contributions to the phenotype observed in Chapter 3 is a prime area of interest. Recall, in the current studies on ERKdko females all the pups died before PN day 3 and the causality of this fetal loss remains a gap in our knowledge. Do these neonates die due to ERK loss or insufficient maternal care? Performing terminal cesarean sections on ERKdko dams at term, and cross-fostering the pups to a surrogate dam would allow us to assess pup viability in relation to ERK status in the pups. Staining the placentas from these pups for TUNEL would show whether the loss of apoptosis resulted from a delay in parturition initiation or loss of signal to initiate placental separation. A more elegant approach would be to use embryo transplantation, taking embryos from ERKdko dams and transplanting them into Control females, and conversely, embryos from Control dams into ERKdko females. We would expect the ERKdko fetuses to grow normally and not exhibit this placental phenotype. When performing the reverse experiment (control embryos transferred into ERKdko dams), we would expect to recapitulate the prolonged gestation phenotype. These studies would allow us to understand the basis of the changes in pregnancy, parturition and placentation. We could then use this model

system to examine various pharmacological approaches to initiation of parturition to rescue the delayed parturition phenotype. For example, supplementing the ERKdko animals at e20 with pharmacologic agents known to be involved in parturition, such as calcium, oxytocin, dexamethasone, or PGF2α, could help elucidate the mechanism behind the dystocia phenotype. These types of studies could have important implications regarding how IHH patients may be treated under similar circumstances. While these experiments would explain the clinical effects and rescue the phenotype, unbiased screening (RNA seq, or proteomics) of the ERKdko and Control animals would provide insight into the molecular basis of the phenotype. Comparing these results with the RNA seq (Chapter 4) and proteomics previously performed in our lab could illuminate additional ERK targets.[30]

An additional set of experiments would investigate the endogenous levels and effects of hormone supplementation throughout pregnancy. Anecdotally, many of the ERKdko mice seem to undergo abortions during the course of gestation. Measuring changes in estradiol, progesterone, LH and FSH throughout pregnancy could aid in understanding the cause behind these losses. Blunted progesterone upregulation throughout pregnancy could contribute to embryonic losses, and is an important differential diagnosis for spontaneous abortions. Monitoring the changes in gonadotropins throughout gestation in ERKdko animals and comparing them to control levels might help identify key time periods of required gonadotropin signaling. If these hormones are found to be at deficient levels, supplementation throughout pregnancy in ERKdko animals may help rescue the phenotype observed. We analyzed placental development, histology, and vascularization at e18.5 and GnRHR

expression at e12.5 and e18.5, but performing serial assessments throughout the course of gestation would help elucidate the origin of the histological changes seen at e18.5, and the changes in GnRHR localization between e12.5 and e18.5. Assessing fetal development (weight, crown-rump length, fetal resorption sites) and placentation (histology, GnRHR expression, ERK loss) in ERKdko dams at additional time points would provide increased clarity on the mechanisms underlying the phenotype. Identifying the day of Cre activation in the placenta could help elucidate specific cell types affected and identify the functional role of ERK and GnRHR in placental development. Historically, it was believed women with IHH suffer from infertility due to hormonal abnormalities.[31] This model elucidates another possible avenue to consider, especially in patients with GnRHR mutations.

Pituitary bile acid receptor, TGR5, modulates reproduction

Although our previous studies had illuminated novel roles of ERK signaling in aging and placental formation and function, they focused on the clinical outcomes and physiologic importance of the intact signaling cascade. In contrast, our third set of studies focused on a known location and role of ERK signaling in pituitary gonadotropes and identification of different gene transcripts regulated by GnRH and ERK.

Again, using the GRIC mice, we conducted unbiased screens (RNA sequencing) to identify ERK-dependent targets downstream of GnRH signaling in the pituitary. These screens revealed a multitude of targets, including the GPCR bile acid receptor, TGR5. We confirmed TGR5's presence in the pituitary, along with

upregulation in response to GnRH and negation of this effect in the absence of ERK. After confirming both the localization of TGR5 and its response to GnRH, we treated primary pituitary culture cells with a TGR5 agonist. Amazingly, this treatment resulted in LH secretion. Subsequently, female mice treated with PMSG and a TGR5 agonist resulted in ovulation and CL formation in a manner consistent with a superovulation paradigm. TGR5ko females were found to be subfertile and displayed delayed onset of puberty. Together, these data indicate TGR5 plays a functional role in the HPG axis, modulating fertility and onset of puberty.

One caveat of the studies described above is the loss of TGR5 is not tissue-specific, and the phenotypes observed could be due to TGR5 expression and signaling in multiple parts of the HPG axis. To this end, we have procured a TGR5floxed ($TGR5^{f/f}$) mouse, and plan to cross these animals with several Cre lines already established in our lab to provide tissue-specific knockouts. These include GRIC Cre (pituitary gonadotropes), Kisspeptin Cre (hypothalamus), Stra8 Cre (testis) and ZP3 Cre (ovary). Characterization of the progeny of these crosses will allow analyses of the isolated effect of loss of TGR5/ERK signaling in various tissues within the reproductive axis and more carefully dissect the role of bile acid signaling on reproductive potential.

Delay of puberty by 4 days in TGR5ko animals was a significant finding. Hypothesizing that bile acids and TGR5 signaling helps modulate the timing of puberty, we plan to feed prepubertal control females a TGR5-specific agonist INT777 supplemented diet. Since this would constitutively activate TGR5 signaling in various tissues including within the reproductive axis, we hope it may cause puberty to occur

earlier with TGR5ko females as a negative control. Measuring LH and FSH after INT777 supplementation would allow quantification of changes in gonadotropin production and secretion by TGR5. Additionally, adding more time points to the study assessing TGR5 transcript levels during the pubertal transition might help elucidated the precise changes in timing of bile acid and TGR5 signaling during puberty.

Despite the plethora of data indicating TGR5 is functional in the gonadotrope, we have not yet localized this receptor within the pituitary. We are collaborating with the IHC core at Cornell to develop IF conditions for colocalization of TGR5 and LH in pituitary gonadotropes and potential other endocrine cell lineages.

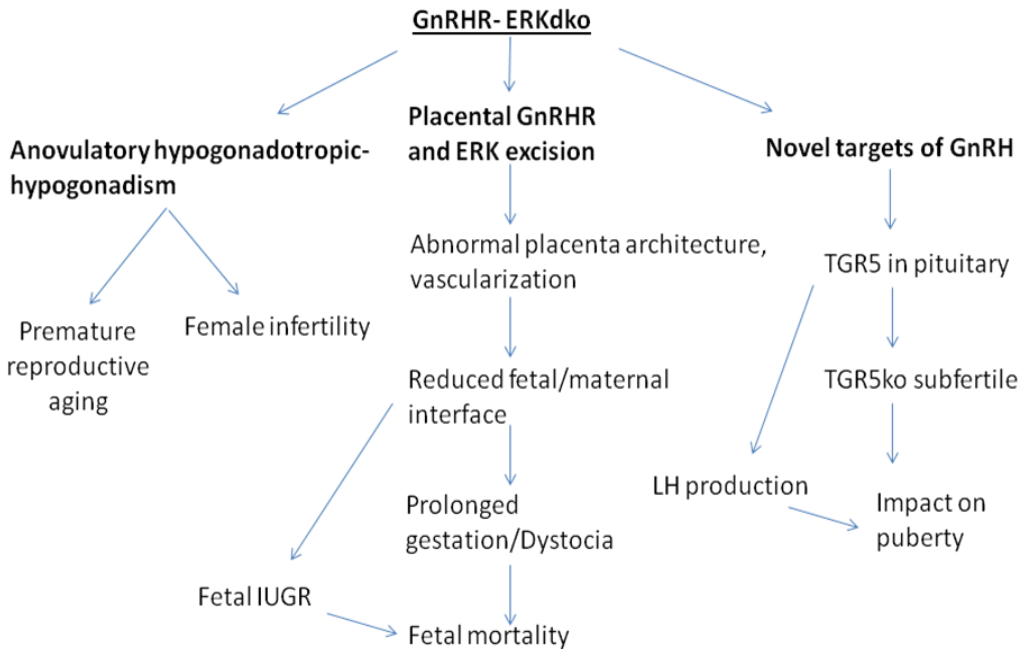
TGR5 is preferentially activated by hydrophobic bile acids, compared to hydrophillic. Treating either αT-31 cells or primary pituitary cells with bile acids of different hydrophobicities and assessing TGR5 activation through CREB phosphorylation or LH secretion would further our understanding of the mechanisms behind this phenomenon. If TGR5 agonist-induced gonadotropin secretion occurs independently from GnRHR signaling, cholic acid or other TGR5 agonists could be an integral part of a fertility treatment regimen. To assess this, we would treat primary cells with antide, a GNRH antagonist, and a TGR5 agonist, to understand if it is independent of GnRHR signaling.

The three data chapters in this dissertation describe three exciting and novel roles for ERK2 signaling in the context of GnRH signaling. These roles confirm the diversity of ERK expression and the requirement for ERK signaling in routine function and normal tissue proliferation. Investigating the role of ERK signaling in a variety of tissues and cell types the HPG axis provides insight into GnRH signaling,

the role of gonadotropins in maintaining gonadal health over time, and GnRHR's role in the placenta. Additionally, we have identified a novel target of GnRH signaling, TGR5, which shows promise to be a link between nutrition, metabolism, reproduction and puberty.

Although the field of reproduction has been well studied in many aspects, the studies included in this dissertation show that ERK signaling has far wider implications than those already described in the literature. All three manuscripts describe novel roles of GnRH associated ERK signaling with important clinical implications. Understanding the role of hypogonadotropism in gonadal aging and the role of ERK in placentation could help improve fertility for those with IHH, Kallmanns syndrome, or other GnRHR associated mutations. Identification of TGR5 as a key regulator of reproduction opens new avenues into understanding the interconnected processes of reproduction and metabolism, especially in the context of puberty. It is possible that the alterations in body weight in the ERKdko animals as the aged (Chapter 2), could be related to reduced TGR5 expression and function in the pituitary gonadotropes (Chapter 4). Identifying the mechanisms such as these, which control the basic regulation of reproduction, hold promise for improving clinical fertility and contraceptive treatments.

Figure 1: Loss of ERK1/2 signaling in the reproductive axis has wide ranging and disastrous consequences for reproductive potential


GnRHR- ERKdko
Anovulatory hypogonadotropic-hypogonadism
Placental GnRHR and ERK excision
Novel targets of GnRH
Abnormal placenta architecture, vascularization
TGR5 in pituitary
Premature reproductive aging
Female infertility
Reduced fetal/maternal interface
TGR5ko subfertile
LH production
Prolonged gestation/Dystocia
Impact on puberty
Fetal IUGR
Fetal mortality

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