IN VIVO CONSEQUENCES OF THE EXPRESSION OF CONSTITUTIVELY ACTIVE LUTEINIZING HORMONE RECEPTORS

by

THOMAS PAUL MEEHAN

(Under the Direction of Prema Narayan and J. David Puett)

ABSTRACT

Luteinizing hormone (LH) is critical for reproduction in mammals. Its activity is delineated through the signaling of a member of the G-protein coupled receptor superfamily, the luteinizing hormone receptor (LHR), which LH shares with the very closely related hormone, human chorionic gonadotropin (hCG). Constitutively activating mutations in LHR occur in cases of sporadic and familial male-limited precocious puberty, a disorder characterized by prepubertal testosterone synthesis and Leydig cell hyperplasia. Additionally, a somatic mutation in LHR, D578H, has been described in the Leydig cell adenomas of boys presenting with precocious puberty. In order to establish an *in vivo* model for chronic LHR activation, transgenic mice were generated utilizing two constitutively active receptors, a D556H rat LHR (equivalent to D578H in human LHR) and a yoked hormone receptor complex (YHR), in which the heterodimeric hormone hCG is fused and then covalently attached to the N-terminus of rat LHR. These receptors were cloned under the control of the mouse inhibin α-subunit promoter to target expression to the gonads of transgenic mice. D556H rLHR founder mice were infertile while several lines were established from YHR founders. In male mice, expression of YHR causes premature testosterone production and seminal vesicle development, as well as impaired testicular development. Female mice undergo precocious puberty resulting from an increase in steroid hormone production and increased folliculogenesis, as well as premature ovarian aging characterized by follicular cysts and interstitial cell hyperplasia and luteinization. In addition to the transgenic mouse model, a tetracycline-regulated system was established to control the expression of YHR, which was shown to be under tight control for the dose and time of doxycycline exposure.

INDEX WORDS: Luteinizing hormone, Chorionic gonadotropin, Luteinizing hormone receptor, Precocious puberty, Constitutive activity, Transgenic mice, Tetracycline-regulated

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B.S., The University of Georgia, 1995

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2003

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August 2003

DEDICATION

I dedicate this to my mother and father who have supported me through the lean years of graduate school.

ACKNOWLEDGEMENTS

First of all, I would like to thank my mentors Drs. Prema Narayan and David Puett who have taught more about science than I ever thought I could know. I know I wasn't always focused on the right things, but thank you for being patient until I realized what was most important. And thanks to my committee, Drs. Mary Bedell, Mark Brown, Kojo Mensa-Wilmot, and Michael Pierce, who have been helpful both with their suggestions, as well as with their time.

I would also like to thank the entire Puett lab, past and present. It is so nice to work in an environment where everyone is truly concerned with each other both personally and in their research. I would especially like to thank Judy Gray for her countless hours spent genotyping the mice she feared so much and for her excellent organization of the lab. Thanks also to Krassi, Lisa, Geneva, Greg, Rebecca, Yongsheng, and to my undergrads over the years Derrick, Matt, Megan, and Walt.

A heartfelt thanks to my great friends Sean and Jason whose friendship I will always cherish. I would not have been able to make it through the last few years without them. Also, to my friends in graduate school, Jason, Jon, Tim, Mike, and Peter, who have always been there to help me relax.

And last but certainly not least, I would like to recognize my family who has always encouraged me to keep going towards my goals in spite of any setbacks. I don't think they realized just how long it would take to get this done, but they understood that I was working hard and that is all you can ask.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Reproduction in mammals is dependent on the actions of glycoprotein hormones secreted in an endocrine manner. Signaling by these hormones to the glycoprotein hormone receptors in the gonads causes increased steroidogenesis and is required for the maintenance of fertility. Recent identification of naturally occurring mutations in gonadotropins and their receptors, as well as the generation of mouse models, have provided new insight into the mechanism of actions of these hormones and receptors. The study in this dissertation details, for the first time, the expression of constitutively active luteinizing hormone receptors in transgenic mice.

Structure of glycoprotein hormones

The family of gonadotropin hormones is comprised of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), secreted by the pituitary of all mammals, and chorionic gonadotropin (CG), secreted from the syncytiotrophoblast cells in the placenta of primates and equids (1). These glycoprotein hormones, along with the pituitary-derived thyroid-stimulating hormone (TSH), are heterodimers that share a common α subunit non-covalently associated with a hormone-specific β subunit (Fig. 1.1) (2). The β subunits of human (h) LH and CG have high amino acid sequence homology with the major difference being a 30 amino acid extension of hCG known as the C-terminal peptide (CTP) (2), which contains four O-linked oligosaccharides and is involved in

increasing the circulatory half-life of the hormone (3). The crystal structure of hCG (4, 5), as well as that of hFSH (6), reveals an elongated structure containing a cystine-knot motif found in several growth factors.

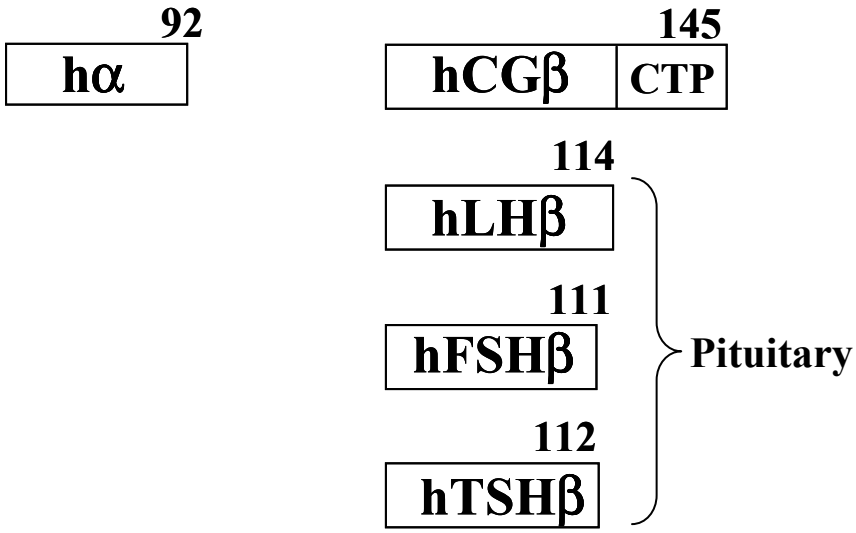

Fig. 1.1. Schematic of the glycoprotein hormone subunits. Each heterodimeric hormone shares a common α-subunit with a hormone-specific β-subunit. LH, FSH, and TSH are secreted from the pituitary, and hCG is secreted from the placenta. The CTP of hCGβ consists of the C-terminal 30 amino acid extension. The number of residues for each subunit is indicated.

Reproductive functions of gonadotropins

Normal reproductive function in males and females is dependent on the actions of both LH and FSH secreted from the pituitary in response to gonadotropin releasing hormone (GnRH) from the hypothalamus (Fig. 1.2) (7). LH signals through receptors expressed in Leydig cells to stimulate steroidogenesis, i.e. the conversion of cholesterol to

testosterone (8). Testosterone and FSH, acting together on Sertoli cells, are required for the maintenance of spermatogenesis (7). LH receptor (LHR) expression in the Leydig cells occurs during fetal life, postnatally, at puberty, and throughout adult life (8).

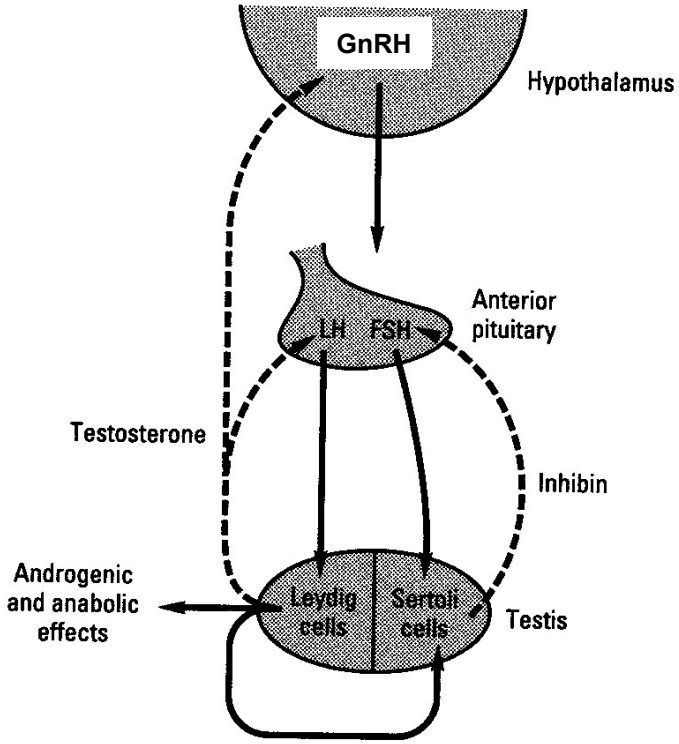

Fig. 1.2. Physiological actions of LH and FSH in males. Solid arrows indicate stimulatory effects; dashed arrows indicate inhibitory effects. Adapted from Ganong (7).

Testosterone produced by the Leydig cells during prenatal development is required for male sexual differentiation. In humans, this steroid hormone production is thought to be dependent on the actions of hCG from the placenta on LHR in the fetal Leydig cells (9). Rodents, however, do not produce a CG-like molecule, and the production of testosterone is stimulated by paracrine factors in the testes (10). LH

secretion from the pituitary and stimulation of LHR at the onset of puberty promote testosterone synthesis, which acts to manifest the secondary sexual characteristics of puberty (9).

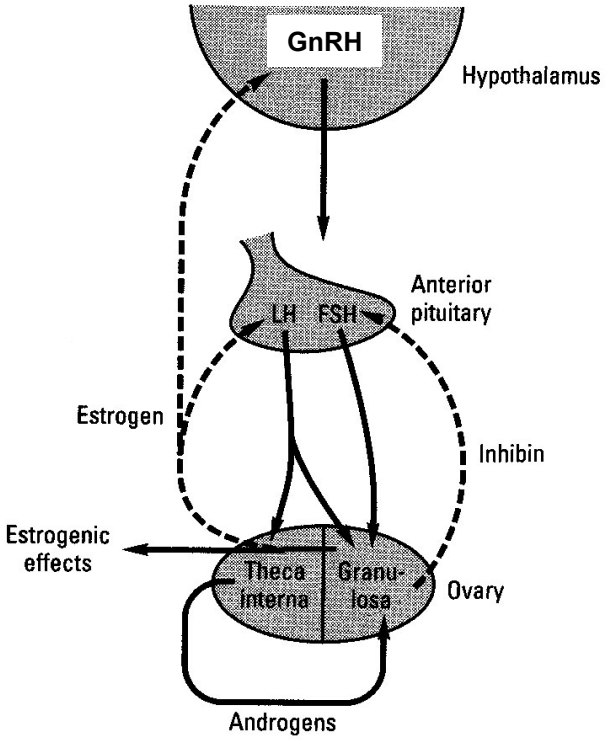

Fig. 1.3. Physiological actions of LH and FSH in females. Solid arrows indicate stimulatory effects; dashed arrows indicate inhibitory effects. Adapted from Ganong (7).

During follicular development in females, LH and FSH are responsible for steroidogenesis and growth. Stimulation of LHR expressed in theca cells causes increases in androgen biosynthesis, while stimulation of FSH receptor (FSHR) expressed in granulosa cells promotes the conversion of androgens to estrogens (Fig. 1.3) (11). Early in follicular growth, LHR is only expressed in the theca cells, but later LHR

expression occurs in granulosa cells in response to FSHR signaling (12). A surge of LH acting on LHR is responsible for ovulation and luteinization of the granulosa cells to form the corpus luteum, which secretes progesterone to prepare the uterus for implantation. The corpus luteum will regress if no fertilization occurs due to a decrease in LH levels, but if fertilization and implantation occurs, hCG, secreted from the placenta, signals through LHR to rescue the corpus luteum, which continues the secretion of progesterone to maintain the pregnancy during the first trimester (1).

The hypothalamic-pituitary-gonadal axis (HPG) controls the levels of the gonadotropins through feedback inhibition (Fig. 1.2, 1.3). Steroid hormones secreted from the gonads negatively regulate the hypothalamus and pituitary to control the circulating levels of LH and FSH. Sertoli cells in the testis and granulosa cells in the ovary also secrete inhibins and activins that can directly modulate the levels of FSH. In humans, the HPG axis is active briefly after birth and then quiescent until the beginning of puberty (13). In rodents, however, the HPG axis remains active from birth throughout life (14).

Structure and function of gonadotropin receptors

The gonadotropin hormones bind to and signal through unique members of the G-protein coupled receptor (GPCR) superfamily, receptors that contain seven transmembrane spanning regions and signal through the actions of heterotrimeric G-proteins. LH and hCG signal through a common receptor, the luteinizing hormone/chorionic gonadotropin receptor (LHR) (Fig. 1.4) (8). Similar to the other glycoprotein hormone receptors, LHR

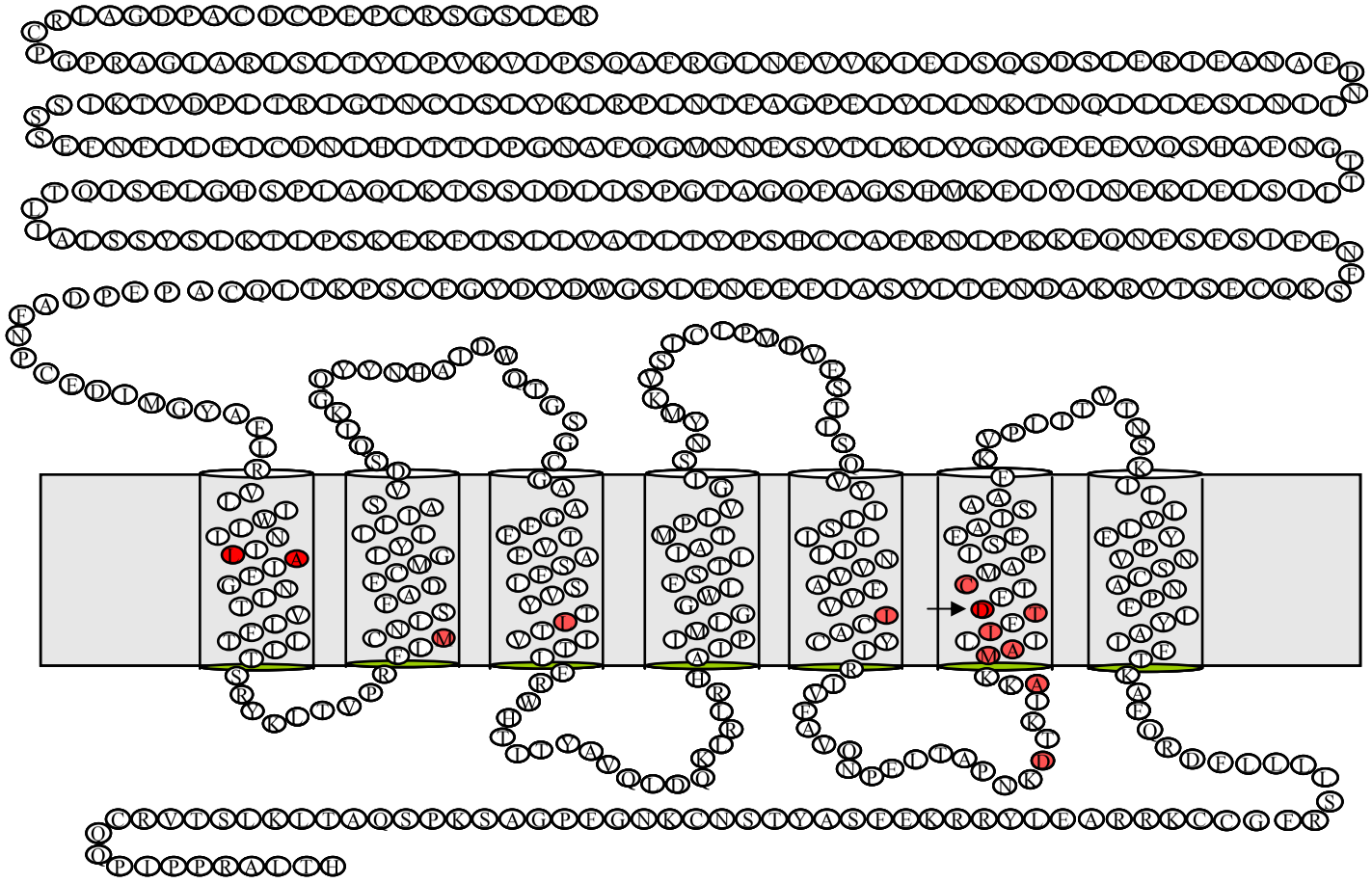

Fig. 1.4. Schematic diagram of the rat LH/CG receptor. Mutations equivalent to ones found in human LHR in patients with familial male-limited precocious puberty are shaded. The arrow indicates the aspartic acid residue that is most commonly mutated in FMPP.

has a large extracellular domain (341 amino acids) that contains the ligand binding site (8). The gene for the receptor contains 11 exons, with exons 1-10 and part of exon 11 encoding the extracellular domain of the receptor. Exon 11 encodes the transmembrane domains, the intracellular and extracellular loops, and the C-terminal region of the receptor (15).

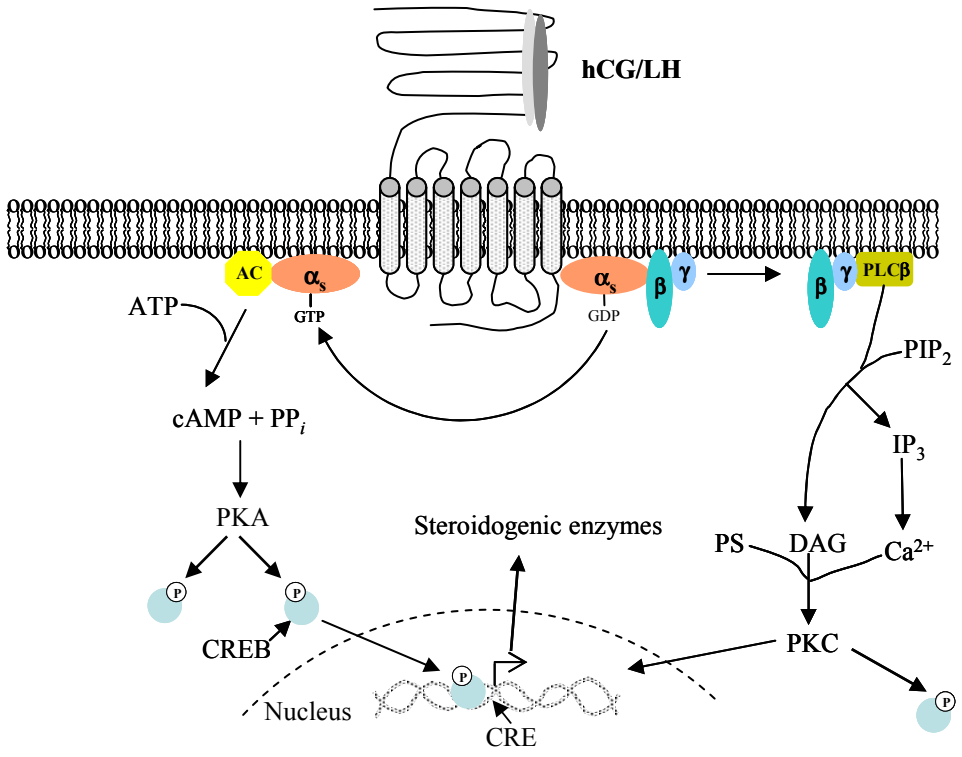

Fig. 1.5. Representation of LHR signaling pathways. Adapted from Narayan et al. (16).

Binding of hormone to the extracellular domain of LHR is thought to result in a conformational change in the transmembrane segments that leads to the exchange of guanidine triphosphate (GTP) for guanidine diphosphate (GDP) on the α-subunit of the stimulatory G-protein (G_s) (Fig. 1.5) (8). The α subunit separates from the βγ-subunits

and increases the activity of adenylate cyclase (AC), which converts adenosine triphosphate (ATP) into 3′, 5′ cyclic adenosine monophosphate (cAMP). Binding of LH or CG to LHR stimulates an increase in cAMP leading to activation of the protein kinase A (PKA), which phosphorylates proteins in the cytoplasm including the cAMP-responsive transcription factor, CREB. Phosphorylated CREB enters the nucleus and binds to cAMP-responsive elements (CREs) found in the promoter regions of many genes, including those for the steroidogenic enzymes, resulting in an increase in transcription (17). In the target tissues for LH action, the increase in transcription of the steroidogenic enzymes is the main physiological result (8). To a lesser extent, LHR can also couple to the phospholipase Cβ (PLCβ) signaling pathway (8, 18, 19), however, the mechanism and significance of signaling to this pathway are poorly understood.

Disorders involving mutations in LHR

Mutations in LHR have been detected in clinically relevant phenotypes. Inactivating mutations lead to primary amenorrhea in females and Leydig cell hypoplasia and pseudohermaphroditism in males (9). Activating mutations are found in patients with sporadic and familial male-limited precocious puberty (FMPP), which is characterized by increased testosterone levels and Leydig cell hyperplasia (20). Many different mutations of LHR have been found in patients with FMPP, although most are clustered in the sixth transmembrane domain (Fig. 1.4). The most frequently observed amino acid change is the D578G transition in the sixth transmembrane helix, and three other naturally occurring mutations (D578E (21), D578Y (22), and D578H (23, 24)) have also been

found at that residue. All of the other mutations that have currently been identified in FMPP occur in the transmembrane regions or in the third intracellular loop (9).

When expressed in cell culture, LHRs with activating mutations have an agonist-independent increase in basal cAMP production (20). This result correlates with the increased testosterone production found in FMPP patients. The age of onset of FMPP varies with the receptor mutant. In patients with the D578G mutation, signs of puberty are found at 3-4 years of age, while in patients with the D578Y mutation, puberty occurs as early as 1 year of age (22, 25). *In vitro* studies of these mutations demonstrate that the D578Y mutation has a higher level of agonist-independent activation of the receptor than the D578G mutation (18). This difference in the extent of constitutive activation of the receptor is the most likely explanation for the altered timing in the appearance of FMPP.

Once puberty is achieved in FMPP patients, reproductive function continues normally and the patients remain fertile; however, the long term effects of LHR constitutive activation have not been well established. A 35-year-old patient that had been diagnosed with FMPP associated with the D578G mutation was later reported to have developed a testicular seminoma (26), but this case report does not clearly demonstrate the cause of the seminoma.

In addition to FMPP, a constitutively active LHR mutant (D578H) has been described in Leydig cell adenomas in boys (23, 24). This mutation was found as a somatic mutation in the cells of the adenoma but not in the normal tissue surrounding it.

Though the phenotype for a constitutively active LHR is clear in males, no ovarian phenotype has been described in females. One hypothesis for the lack of precocious puberty in female carriers of LHR activating mutations is that the ovary does

not express LHR before puberty (8); although, to date, only a few female carriers of activating mutations have been analyzed. These analyses did not demonstrate any reproductive alterations, however the mutated receptors studied have lower levels of constitutive activity and are associated with the less severe cases of FMPP (27-29). None of the more severe mutations have been found in females and are usually transferred paternally or occur sporadically (9).

Mouse models to elucidate gonadotropin function

Genetically altered mice have been utilized extensively to study the roles of individual gonadotropins and their receptors. These models have reaffirmed current paradigms, as well as shed light on some of the variation between mouse and human reproductive functions.

The female phenotypes associated with the deletion of the gonadotropin hormone and receptor genes in mice have been as expected. Deleting FSH (30) or FSHR (31, 32) genes results in arrested follicular development at the preantral stage demonstrating the previously determined role of FSH signaling in early follicle development. The knock-out of the LHR gene (33, 34) also gives the expected result of anovulation with folliculogenesis blocked at the antral stage, which corresponds to the timing of LHR expression in granulosa cells reaffirming the role for LHR in late follicular development and ovulation.

On the other hand, the phenotypes in male mice with disrupted expression of FSH or FSHR are different than would be anticipated. In humans, absence of FSH signaling causes infertility from a lack of spermatogenesis (35, 36) while patients with mutations in

FSHR are fertile despite impaired spermatogenesis (37). Mice with deletion of either FSHβ (30) or FSHR (31, 32) genes are still fertile in the presence of reduced testicular size, thus, demonstrating a difference in the necessity of FSH signaling between human and mice.

The most significant difference in phenotype between humans and mice occurs in the case of inactivating LHR. In humans, lack of LHR signaling leads to XY pseudohermaphroditism from a lack of testosterone synthesis during development (9) while mice differentiate normally and have defects only in postnatal reproductive development (33, 34). The mouse knockout of the common glycoprotein hormone α-subunit also demonstrates normal differentiation in the absence of glycoprotein hormones (38). These models illustrate that testosterone synthesis *in utero* in mice occurs via a LH and LHR-independent mechanism while hCG-mediated signaling by LHR is required for testosterone production in humans (9).

Mice overexpressing bovine LHβ (39) and hCG (40) have been produced to model the role of increased levels of LH found in humans with polycystic ovarian syndrome (PCOS) (41) and the effects of aberrant expression of hCG from tumors (42). LHβ fused to the CTP of hCGβ was expressed under the control of the bovine glycoprotein α-subunit to target the expression to the pituitary. These mice undergo precocious puberty with infertility and polycystic ovaries (39, 43). In a strain-dependent fashion, mice overexpressing LHβ also develop tumors of granulosa cell origin (44).

Transgenics for hCGβ were generated with expression of the transgene under the control of the ubiquitin C promoter to express hCG in multiple tissues in an attempt to analyze the effects of prolonged exposure (40). These animals have reproductive defects

including precocious puberty, infertility, and increased ovarian steroidogenesis. They also develop pituitary adenomas and mammary gland tumors by 12 months of age. The presence of these tumors is dependent on ovarian function as ovariectomy eliminates their growth. The more severe phenotype in the hCGβ overexpression model is likely due to the lack of feedback inhibition of the ubiquitin C promoter which resulted in a 40-fold increase in serum hCG over LH levels (40), as compared to only a 5- to 10-fold increase in LHβ transgenic mice (39).

Yoked hormones and receptors

Several laboratories have utilized genetically engineered glycoprotein hormones in order to study structural and functional aspects of hormone signaling. Two independent labs produced the first tethered or yoked (Y) glycoprotein hormones in which the α and β subunits of hCG are fused in a single polypeptide chain (45, 46). The initial construct, termed yoked hCG1 (YhCG1) (Fig. 1.6) by Narayan et al., contains the N-terminus of the α-subunit combined to the C-terminus of the β-subunit with the CTP acting as a natural flexible linker region and the signal sequence of hCGβ driving the secretion. YhCG1 binds to LHR with equal affinity as the native heterodimer.

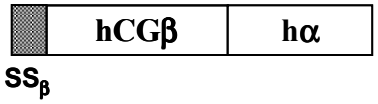

Fig. 1.6 Schematic diagram of YhCG1. The β-subunit of hCG with its signal sequence (SS_β) is covalently attached to the human glycoprotein hormone α-subunit.

Subsequent studies have varied the order of the subunits in the construct and have found that when the α-subunit is placed first, the binding affinity of the hormone is reduced but the signaling is unchanged (47-49). In addition to these single hormone constructs, Boime et al. have produced trimers and tetramers in which two or more β-subunits of different glycoprotein hormones are combined with a single α-subunit (50-52). These tethered hormones are capable of activating multiple glycoprotein hormone receptors, thereby, demonstrating the permissiveness of the glycoprotein hormone structure in activation of their receptors.

YhCG1 has been covalently linked to the rat LHR (rLHR) by an additional CTP and a factor Xa cleavage site between the C-terminus of the α-subunit and the N-terminus of rLHR (Fig. 1.7). This receptor has been termed the yoked hormone receptor (YHR), and, when expressed in COS-7 and HEK 293 cells, it leads to an increase in basal cAMP compared to the wild type LHR and mock transfected cells (53). The increase in cAMP production is 20-fold higher than the normal receptor and is not further stimulated by exogenous hormone. This significant increase in activity is higher than most of the naturally occurring mutations in LHR. These yoked hormone receptors provide useful tools to dissect further the *in vivo* and *in vitro* functions of LHR.

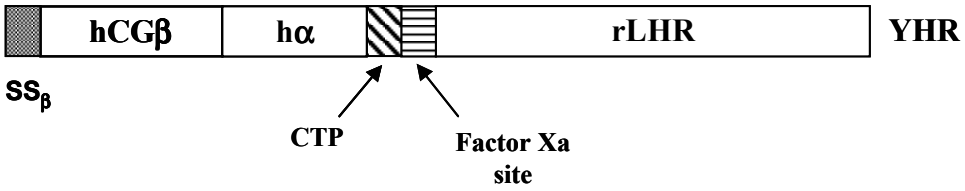

Fig. 1.7 Schematic diagram of YHR. YhCG1 is covalently attached to rat (r) LHR via a CTP and a factor Xa cleavage site.

Objectives of this study

In order to develop an *in vivo* model of constitutively active LHR expression, this study details the generation of transgenic mice for two activated LHRs. Mutations leading to constitutively active LHR have been described in cases of familial and sporadic male-limited precocious puberty, as well as in patients with Leydig cell adenomas. Both sets of patients present with gonadotropin-independent precocious puberty caused by premature testosterone synthesis. In female carriers of these mutations, no phenotype has been found. The hypothesis of this research is that the constitutively active LHR will cause alterations in reproductive development in the mouse. For the purpose of determining the *in vivo* functions of constitutively active LHR, the following objectives were proposed:

1. Generate transgenic mice expressing constitutively active LHR and establish lines for study (Chapter 2). Toward this goal, YHR and D556H rLHR (equivalent to the D578H mutation in hLHR found in Leydig cell adenomas) were cloned under the control of the mouse inhibin α-subunit promoter, which has been utilized previously to target the expression of transgenes to the gonads of mice (54, 55).
2. Characterize male mice for abnormalities in reproductive development (Chapter 2). Compare the phenotype of the mice to that of FMPP patients including steroid and gonadotropin hormone levels, as well as development of the testes and seminal vesicles.
3. Determine if female mice expressing constitutively active LHR possess any alteration in reproductive development (Chapter 3). Characterize these mice for

precocious puberty and the effects of transgene expression on ovarian and uterine histology.

4. Establish a regulated system for YHR expression in cell culture to determine the feasibility of controlling YHR expression (Chapter 4). The development of this system will allow for future controlled expression of YHR in transgenic mice, as well as a more detailed investigation of the signaling properties of YHR.

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CHAPTER 2

PREPUBERTAL INCREASE IN TESTOSTERONE IN MALE TRANSGENIC MICE EXPRESSING A CONSTITUTIVELY ACTIVE LUTEINIZING HORMONE RECEPTOR[1]

[1] Meehan, T.P., M.O. Overcast, D. Puett, and P. Narayan. Submitted to *Endocrinology*, 4/30/2003.

Abstract

Constitutively active LH receptors (LHR) have been described clinically. In addition to the well established role of mutant LHR in precocious puberty, a mutation leading to a replacement of aspartic acid 578 with histidine has recently been described in boys with Leydig cell adenomas. Both cases presented with gonadotropin-independent precocious puberty due to aberrant testosterone production. In order to create an *in vivo* model for constitutively active LHR, two transgenic mice were generated, one that expresses D556H rat LHR (equivalent to D578H human LHR) and a second with a genetically engineered yoked hormone receptor (YHR), in which an engineered single chain heterodimeric human chorionic gonadotropin was covalently attached to LHR. The constitutively active LHRs were cloned under the control of the mouse inhibin α-subunit promoter to target transgene expression to the gonads. Mice expressing D556H LHR were infertile. Several lines of mice were established from YHR founders (YHR^+). These animals exhibited prepubertal increases in testosterone levels and seminal vesicle weights and decreases in both serum FSH and LH. YHR^+ mice also had a significant decrease in testes weight, apparently resulting from a reduction in the size of the seminiferous tubules. These data indicate that the single chain YHR is functional *in vivo* and demonstrate that YHR^+ mice provide a novel system to understand further the reproductive consequences of premature LHR activation.

Introduction

Human chorionic gonadotropin (hCG), LH, and FSH comprise the family of gonadotropin hormones which, together with TSH, are heterodimeric glycoproteins

consisting of a common α-subunit and a hormone specific β-subunit (1). The β-subunits of LH and hCG have high sequence identity differing mainly in the 30 amino acid C-terminal peptide of hCG that is absent in LH. These hormones signal through a common member of the glycoprotein hormone receptor superfamily of G-protein coupled receptors, the LH receptor (LHR), characterized by seven transmembrane domains and a large extracellular domain which confers hormone binding specificity (2). LHR signals primarily through the cAMP pathway, although high levels of hormone can mediate signal transduction via the inositol phosphate pathway. LHR, which is essential for reproduction in mammals, is expressed primarily in the Leydig cells of the testes and in the theca and granulosa cells of developing follicles, as well as luteal cells, in the ovaries. In Leydig cells, LHR expression occurs during fetal life, postnatally, at puberty, and throughout adult life with gonadotropin required for maintenance of steroidogenesis (3). LH secretion from the pituitary and stimulation of LHR at the onset of puberty promotes testosterone synthesis in the testes, which acts to manifest the secondary sexual characteristics of puberty in males. Prenatally, binding of hCG, secreted from the placenta of primates, to LHR is responsible for the synthesis of testosterone which is required for male sexual differentiation (3).

Naturally occurring mutations in LHR have been identified in humans. In males, inactivating mutations lead to pseudohermaphroditism, while constitutively activating mutations cause gonadotropin-independent precocious puberty, a disorder characterized by prepubertal increases in testosterone synthesis and Leydig cell hyperplasia (3, 4). The activating mutations in the LHR gene occur both in a familial and sporadic fashion and are dominant to the wild-type LHR gene. Most of these mutations arise in the

transmembrane domains of LHR with the most common occurrence in the sixth transmembrane domain at aspartic acid residue 578 (3, 4). Also, a somatic mutation, D578H, occurs in some boys that have testosterone secreting Leydig cell adenomas leading to precocious puberty (5, 6). Female carriers of constitutively active LHR mutations are normal.

Genetically altered mice have been utilized to determine the functions of LH and LHR in reproduction (7-9). These models have, in many cases, reaffirmed current paradigms, as well as, illustrated the differences between humans and mice in sexual development. For instance, in humans with an inactive LHR, the male phenotype is a lack of masculinization of the reproductive tract (3). However, in the LHR knockout mouse, development proceeds normally, suggesting that LHR is not required for sexual differentiation in mice (8, 9). Prepubertal testosterone is instead produced by paracrine factors in the testes. To date, no model has been created for constitutively active LHR in mice. A related model of LH overexpression (7) and the recently described hCG overexpression (10) transgenic model have focused on the female phenotype.

In order to establish an *in vivo* model to examine the physiological and potential pathophysiological effects of chronic LHR activation, we have generated transgenic mice expressing two constitutively active LHRs. The first is the D556H rat LHR (homolog of naturally occurring D578H mutation in human LHR) and the second is a genetically engineered yoked hormone-receptor complex (YHR) previously described from our laboratory (11). The latter was constructed by covalently linking the single chain or yoked hCG (YhCG) (12, 13), containing the two subunits of the heterodimeric hCG, with rat LHR in a single polypeptide chain. When expressed in HEK 293 and COS-7 cells,

YHR exhibits signaling characteristics of a constitutively active receptor with increases in basal levels of cAMP approximately 20-fold over that of LHR and is not further stimulated by the addition of exogenous hCG (11).

D556H LHR and YHR were cloned under the control of the mouse inhibin α-subunit promoter because of its documented success in targeting expression of transgenes to the gonads of mice (14, 15), particularly to the Leydig/Sertoli cells of the testes and the theca/granulosa cells of the ovary. To our surprise, founder mice expressing D556H LHR were unable to reproduce, whereas those expressing YHR were fertile. In this study, we describe, for the first time, establishment and characterization of transgenic mouse lines expressing a constitutively active LHR (YHR^+). Our initial analysis of YHR^+ male mice indicates that gonadal expression of YHR results in prepubertal increases in testosterone and impairs testicular development. Thus, the transgenic YHR^+ lines will provide a novel model to further investigate the developmental consequences of constitutive LHR activation.

Materials and Methods

Inositol phosphate (IP) assay

Stable lines expressing myc-rLHR, D556Y myc-rLHR (cDNA kindly provided by Dr. Mario Ascoli, University of Iowa), D556H myc-rLHR, and YHR were generated in HEK 293 cells following transfection by Lipofectamine (Invitrogen, Carlsbad, CA) and selection with 700 μg/ml Geneticin (Invitrogen). Cells from each line were plated at a density of 7.5×10^4 cells/well in maintenance media (DMEM containing 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 100

μg/ml Geneticin). Cells were labeled overnight with 2.5 μCi/ml ^{3}H-myo-inositol (Perkin Elmer, Boston, MA) in inositol free media then preincubated in Waymouth media containing 0.1% BSA and 10 mM LiCl for 15 min at 37 C, followed by incubation with (1 μg/ml) or without hCG (basal) for 30 min at 37 C. Perchloric acid was added to lyse the cells and extracts were neutralized prior to the purification of inositol phosphates by chromatography using the AG1-X8 resin (Biorad, Hercules, CA) (16). Total IP was measured by scintillation counting and expressed as fold over LHR basal.

Generation of transgenic mice

YHR and D556H rLHR-myc (rat homolog of human D578H mutation) were cloned under the control of a 6 kb fragment containing the mouse inhibin α-subunit promoter (Fig. 2.1A). PCR products were generated from YHR in pcDNA3 (3317 bp) and D556H rLHR-myc in pcDNA3 (2617 bp) containing the coding sequence for the receptors and the bovine growth hormone polyadenylation signal (bGHpA) with *Nhe* I restriction sites added to each end. A partial digest was performed on an inhibin-α/*bcl-2* in pBluescript-SK (-) construct (kindly provided by Dr. Aaron Hsueh at Stanford University) with *Xba* I to remove the *bcl-2* coding sequence. The PCR products were digested with *Nhe* I and inserted into the *Xba* I site of the vector. Following a *Cla* I digest and gel purification to remove the prokaryotic sequences, the transgene was microinjected into (C57BL/6xSJL) F2 fertilized eggs. Potential founders born following implantation into pseudopregnant females were genotyped by PCR and Southern blot analysis of DNA extracted from tail biopsies. Forward PCR primers were designed to the anneal to the β-subunit of hCG in

YHR and to the myc tag in D556H rLHR-myc so as not to amplify the endogenous LHR. Both reverse primers anneal to the extracellular domain of rLHR.

Establishment of transgenic lines

Microinjection of the transgenes generated 8 transgenic founder mice each for YHR (5 male, 3 female) and D556H LHR (3 male, 5 female). Founder mice were bred to C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) and first generation progeny were tested for the transmission of the transgene by PCR. Tissue specific expression of the transgene was determined by RT-PCR. Transgenic lines could not be established from D556H LHR founder mice because the mice either did not reproduce (n=4), transmit the transgene (n=2), or express the receptor (n=2). YHR founder mice were all fertile with 5 of 8 transmitting the transgene, and 4 of 8 expressing the receptor in the gonads and adrenals of F1 progeny. RNA samples from the testes and adrenals of YHR expressing lines were subjected to semi-quantitative RT-PCR in order to identify lines of mice for further study. Two lines were chosen which had high and similar levels of YHR expression in the gonads and low levels of adrenal expression. The two lines presented with similar phenotypes and the data presented are from fifth through eighth generations in the C57BL/6J background of one of these lines. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Georgia.

RNA isolation, RT-PCR, and semi-quantitative RT-PCR

Total RNA was collected from each tissue following homogenization with a Kontes homogenizer in RNA Stat-60 (Tel-test, Inc., Friendswood, TX). Following treatment

with DNase I (DNA free, Ambion, Austin, TX) for 30 min at 37 C, 2 μg of each RNA sample was subjected to reverse transcription (RT) utilizing the Retroscript kit (Ambion). PCR was performed on 1/4 of the RT reaction for 30 cycles (30 s at 95 C, 1 min at 60 C, 1 min at 72 C) using primers for YHR, D556H, or mouse glyceraldehyde phosphate dehydrogenase (mGAPDH). To ensure specificity of the PCR, reactions without reverse transcriptase and without template were performed. PCR products were resolved on a 1.5% agarose gel in Tris-borate-EDTA (TBE) buffer, stained with ethidium bromide, and visualized by UV.

For semi-quantitative RT-PCR, the PCR reactions were carried out in the presence of α-^{32}P-dCTP (Perkin Elmer, Boston, MA) utilizing the QuantumRNA™ Classic II 18S internal standard kit (Ambion). Reactions were multiplexed with primers for YHR and 18S rRNA. Pilot experiments were performed to determine the cycles at which a linear range of amplification is achieved and to determine the ratio of 18S rRNA Competimers™ to use to attenuate the 18S rRNA signal to the level of the YHR signal such that each would be in the linear range of amplification at the same cycle. PCR products were visualized utilizing a phosphorimager following resolution on a 6% acrylamide gel in TBE buffer. Relative expression levels are given as a ratio of YHR signal to 18S rRNA signal and normalized to 1 for the lowest level.

Real-time RT-PCR was performed on an I-Cycler (Biorad, Hercules, CA) utilizing a Sybr® green PCR master mix (Applied Biosystems, Foster City, CA). Primers were designed to amplify a 73 bp and a 51 bp fragment for YHR and 18S rRNA, respectively. YHR primers were designed to anneal to the α-subunit. Standard curves were made by a dilution series of a reference sample and expression levels were

determined relative to the dilution utilized. A ratio of YHR/18S rRNA was calculated and normalized to the lowest level to get relative expression levels. Melting curve analysis was performed to ensure that no primer-dimer amplification was occurring.

Measurements of hormone levels

Blood was collected by cardiac puncture following asphyxiation of mice with CO_2, and serum was collected by centrifugation. Testosterone concentrations were measured by coat-a-count RIA (Diagnostics Products Corporation, Los Angeles, CA) with a sensitivity of 0.2 ng/ml. Testicular testosterone was measured by RIA (Pantex, Santa Monica, CA) following extraction of testicular homogenates with diethyl ether. One (8 and 12 weeks) or two (3 and 5 weeks) testes were first homogenized in 5 ml PBS using a polytron homogenizer and then extracted 3x with 5 ml diethyl ether. Testicular testosterone levels are expressed as the total amount in both testes. Rat LH and FSH were iodinated by iodogen and serum LH and FSH levels were measured utilizing the standards and antiserum purchased from Dr. A. Parlow of the NIDDK National Hormone and Peptide Program. The sensitivity of the LH and FSH assays were 0.1 and 0.8 ng/ml, respectively.

Histology and immunohistochemistry

Histology of the testes was determined following fixation in 10% buffered formalin. Tissues were embedded in paraffin and 5 μm serial sections were stained with hematoxylin and eosin prior to histological examination. Staining of Leydig cells was accomplished by immunohistochemistry with an antibody to 3βHSDI (kindly provided by Dr. Anita Payne, Stanford University). A 1/800 dilution of the antibody was used for

staining utilizing Histostain Plus kit with DAB chromogen (Zymed Laboratories, Inc., South San Franscisco, CA). Counterstaining of nuclei was performed with hematoxylin.

Statistical analysis

Statistical analysis comparing age-matched controls and transgenic animals was performed by the unpaired T-test utilizing the Prism software (GraphPad Software, Inc., San Diego, CA). $P<0.05$ was considered significant.

Results

In vitro bioactivity of constitutively active LHR

Previous studies have demonstrated that cells expressing YHR (11, 17), rat D556H LHR (D556H LHR) (18), and rat D556Y LHR (D556Y LHR) (19) exhibit an elevation in the basal levels of cAMP. Of these three constitutively active receptors, only D556Y LHR is further stimulated by addition of exogenous hCG. The corresponding mutations in the human receptor, D578H and D578Y, also cause an elevation in the basal levels of intracellular inositol phosphate (IP) in COS-7 (5, 16) and MA-10 cells (20). To determine if YHR, rat D556H LHR, and rat D556Y LHR also exhibited constitutive activation of the IP pathway, stable HEK 293 cell lines expressing these receptors, as well as wild type rat LHR, were generated. ^{125}I-hCG binding assays and cAMP measurements confirmed the receptor expression in these cell lines (data not shown). Total IP production was measured for each receptor in the presence and absence of 1 μg/ml hCG. YHR and D556H LHR were shown to stimulate basal levels of IP production (YHR, 1.8-fold; D556H LHR, 2.2-fold) over basal LHR (Fig. 2.1B). All

receptors except YHR were shown to further stimulate IP production in the presence of exogenous hCG.

D556H transgenic mice are infertile

Transgenic lines of D556H LHR could not be established because the 8 founder mice either did not reproduce, transmit the transgene to their progeny, or express receptor. Only one (613) of three male D556H LHR founder mice produced offspring, and examination of the male founder mouse (Fig. 2.2A) and its first generation progeny (data not shown) showed that the receptor was not expressed, as demonstrated by RT-PCR. The other two male founder mice (604 and 621), which were infertile, were dissected at 8 months of age and shown to express the receptor in the testes and adrenals by RT-PCR (Fig. 2.2A). Histological analyses of the testes from the two transgenic founder mice did not reveal any apparent morphological differences compared to non-transgenic controls that would account for the infertility (data not shown).

YHR^{+} mice are fertile and display gonadal expression

In contrast to the D556H LHR mice, all of the YHR founder mice produced offspring. Three of the founders did not transmit the transgene, while the remaining five transmitted the transgene and four lines expressed the receptor in the testes and ovaries of first generation progeny as determined by RT-PCR (data not shown). YHR expression was also determined in multiple tissues by RT-PCR (Fig. 2.2B). In addition to the expected expression in the testes and ovaries, mRNA was also detected at high levels in the brain as well as at lower levels in the heart and adrenal consistent with previous reports of

inhibin-α expression in these tissues (21). There was no amplification of endogenous mouse LHR sequence as evidenced by lack of a specific band in RNA samples from WT mice. Controls without reverse transcriptase also did not reveal contaminating DNA (data not shown). Semi-quantitative RT-PCR was performed on first generation progeny from 3 lines to identify those for further study (Fig. 2.2C). Later, real-time RT-PCR was also completed on the ovaries from 2 lines to obtain relative expression levels (Fig. 2.2D). All three lines demonstrate good expression of YHR in the gonads, while the 553 line also shows higher adrenal expression. The two lines with relatively lower adrenal expression (537 and 571) were chosen for further study of the effects of YHR expression in the gonads on reproductive function and development. Southern blot analysis on F1 progeny from 537 and 571 determined a single integration site of the transgene and copy numbers of 4 and 10, respectively (data not shown). Both of these lines were characterized and male mice exhibit similar testicular phenotypes and hormone levels. The remainder of the data presented is from male mice of the 571 line.

Prepubertal elevation in testosterone levels in YHR$^+$ mice

In order to determine if YHR expression affects testosterone production, levels of testosterone were measured in serum and in testicular extracts. Serum testosterone levels increased as early as 3 and 5 weeks of age in YHR$^+$ mice (Fig. 2.3A), with 5-week-old values significantly higher than control mice. At 3 weeks of age, all of the YHR samples had detectable levels of serum testosterone while the WT did not. Thus, it is clear that the transgenic mice had higher levels of serum testosterone than wild type mice, despite that fact that statistical analysis cannot validate a comparison when one of the values is

zero. At older ages (8 and 12 weeks of age), the serum testosterone levels were not significantly different. Testicular testosterone levels (Fig. 2.3B) exhibited the same pattern as the serum levels with significant increases at 3 and 5 weeks of age over non-transgenic littermates. At 8 and 12 weeks of age, there was no difference in testicular testosterone levels of WT and YHR^+ animals. The similarities in testosterone levels between the serum and testicular extracts illustrates that the increase in testosterone in prepubertal mice is most likely due to YHR expression in the testes and not in other steroidogenic tissues.

Consistent with the increase in testosterone levels, the size of the seminal vesicles (Fig. 2.4A, B) was dramatically increased in prepubertal YHR^+ mice. Most significantly, increases in seminal vesicle weight occur at 3 (1.9-fold, n=9-10), 4 (2.2-fold, n=4-5), and 5 weeks of age (1.9-fold, n=10). These results parallel the increase in testosterone synthesis that occurs at prepubertal ages. There are no significant differences in seminal vesicle weight at 6 and 8 weeks of age, however, by 12 weeks of age, seminal vesicle weight of YHR^+ mice is significantly decreased in weight (1.3-fold reduction, n=6-8).

YHR expression suppresses serum gonadotropin levels

As might be expected with an increase in steroidogenesis, gonadotropin hormone levels were decreased in YHR^+ mice. Serum LH levels were reduced in 3-, 4-, and 5-week-old mice (Fig. 2.5A), but most of these values from transgenic mice were below the standard curve of the assay and statistical analyses could not be performed. The levels for most of the control mice at these ages were measurable and, therefore, demonstrate the reduction in transgenic mice. Eight week old YHR^+ mice showed no difference in serum LH levels

verses controls, while levels from 12 week old animals were reduced, however, not significantly. In contrast to LH levels, serum FSH levels were significantly decreased in YHR^+ mice at all ages (Fig. 2.5B). At 3 and 5 weeks of age, FSH levels were reduced approximately 19-fold (n=5-6) and 12-fold (n=6), respectively. At older ages, the decrease in FSH was not as severe with an approximate 2-fold reduction at 8 (n=6-8) and 12 (n=6) weeks of age.

YHR expression affects testicular development

YHR^+ mice exhibited a significant decrease in testes size at 3-12 weeks of age (Fig. 2.6A, B). The decrease in weight is most severe at 6 weeks of age (54% reduced, n=10), but is present by 3 weeks of age (35% reduced, n=9-10). At later ages, the effects on testes development have been reduced, however, the testicular weight in YHR^+ mice is still significantly reduced at 8 weeks (40% reduced, n=9-10) and 12 weeks (22% reduced, n=6-8).

Histological analysis of the testes demonstrated that the reduction in testicular weight of YHR^+ mice is manifested by a reduction in the tubule diameter that is readily evident at 5 weeks of age (Fig. 2.7A vs. 2.7B). A change in tubule size can also be observed at 8 weeks of age (not shown) but is not apparent at 12 weeks of age (Fig. 2.7C vs. 2.7D), consistent with the finding that the testes size at 12 weeks is not as drastically reduced. Other than the overall size of the tubules, there are no clear differences in the histology of the tubules at the light microscope level. Also, these animals are fertile, therefore, no major defect in sperm production exists. A qualitative analysis of hematoxylin and eosin stained sections also did not reveal any changes in the volume of

the interstitium or Leydig cell morphology. Immunohistochemistry with 3βHSDI antiserum to specifically stain the Leydig cells further confirmed this observation (Fig. 2.7E vs. 2.7F). However, a more quantitative comparison of Leydig and Sertoli cells in YHR^+ and WT mice by detailed stereological analysis is warranted.

Discussion

The results presented herein describe for the first time the effects of expression of constitutively active LH receptors in transgenic mice. Male mice expressing YHR have prepubertal increases in testosterone synthesis and seminal vesicle weight with related decreases in serum LH and FSH levels. Expression of YHR results in a decrease in testicular weight, which is apparent in testicular histology as a decrease in the diameter of the seminiferous tubules.

Mutations in LHR that cause constitutive activation have been described in patients with sporadic and familial male-limited precocious puberty (FMPP), a disorder characterized by elevated testosterone and Leydig cell hyperplasia. The mouse model described in this study has both similarities and differences with the phenotype of FMPP.

The increase in prepubertal testosterone is consistent with FMPP patients (3) and is indicative of the expression of a constitutively active LHR in the testes. Testicular testosterone measurements support the pattern of the serum levels and indicate that the increase in synthesis most likely occurs in the testes and is not due to expression of YHR in other steroidogenic tissues, e.g. adrenal glands. The dramatic differences in testosterone levels between WT and YHR^+ mice observed at prepubertal ages (3 and 5 weeks) are not apparent at adult ages (8 and 12 weeks) when normal mice would be

producing high levels of testosterone. Correspondingly, testosterone levels in adult FMPP patients are similar to normal males (3). Consistent with the increase in testosterone in YHR^+ mice, an increase in the weights of the seminal vesicles was seen in transgenic mice at the prepubertal ages of 3-5 weeks of age. This increase is reflective of the well established role of testosterone on seminal vesicle growth and differentiation (22, 23).

The expression of YHR in prepubertal transgenic mice results in a decrease in serum gonadotropin levels concomitant with as increase in testosterone synthesis. This effect is most likely due to the feedback inhibition of testosterone on the synthesis and secretion of LH from the pituitary and suggests that the hypothalamic-pituitary-gonadal (HPG) axis is functional in mice at these early ages (24). In contrast to LH, FSH levels are significantly decreased at all ages assayed, which demonstrates a difference in the regulation of the synthesis and secretion of LH and FSH in YHR^+ mice. One possibility is that YHR signaling elevates inhibin, which negatively regulates secretion of FSH (25). Inhibin α-subunit synthesis has been shown to be regulated by an increase in cAMP in cultured Sertoli cells (26, 27).

This decrease in LH and FSH is in contrast to the phenotype observed in humans with FMPP wherein basal levels of LH and FSH remain at prepubertal levels and a normal prepubertal response is presented after GnRH stimulation (28). However, one patient with very high testosterone levels had a decreased response to GnRH stimulation and failed to develop central puberty indicating a feedback effect (28). One likely explanation for the difference between FMPP and YHR^+ mice is the age at which the HPG axis is activated in humans verses rodents. Unlike humans, pituitary gonadotropin

secretion and feedback regulation are functional at birth in male rats (24), and our data suggest that this is also the case in mice.

The decrease in testicular size and seminiferous tubules in YHR^+ mice is interesting considering that patients with FMPP typically have an increase in testicular volume at prepubertal ages. Interestingly, mice overexpressing LHβ also demonstrate a decrease in testicular size, however, the male phenotype of these animals was not further investigated (7). In FMPP patients, serum FSH is below detectable levels and is, therefore, considered prepubertal (3, 28). In YHR^+ mice, the levels of serum FSH, which are measurable at early ages in mice, are decreased and may be responsible for the reduction in testes size consistent with the smaller testes found in the FSH β and FSH receptor knockout mice (29, 30). Additionally, expression of FSH or a constitutively active FSH receptor on the gonadotropin-deficient hypogonadal (*hpg*) mouse background, which have reduced testis size, results in an increase in testicular weight (31, 32) demonstrating a direct role for FSH in testicular development. Furthermore, FSH is believed to have a mitogenic role on Sertoli cell proliferation, and FSH expression during the perinatal period has been shown to be important in this process (33-35). The decrease in serum FSH levels in the first few weeks of postnatal life in YHR^+ mice could therefore decrease Sertoli cell numbers and consequently reduce the tubule size in these mice.

Except for the reduction in seminiferous tubule diameter, the histology of the testes in YHR^+ mice is not qualitatively different from controls. This is in contrast to the testes of FMPP patients which exhibit Leydig cell hyperplasia (3). It has been suggested that Leydig cell proliferation and differentiation is regulated by Sertoli cells, and FSHR mediated signaling is an important component of this regulation (36). Since FSHR is

expressed exclusively in Sertoli cells, a decrease in their cell numbers in YHR mice could potentially result in a decrease in trophic factors required for Leydig cell proliferation. Alternatively, the factors required for Leydig cell development in mice could be different from humans.

One surprising result in this study is that the D556H LHR transgenic founder mice do not reproduce while YHR founder mice produce progeny, even though both D556H LHR and YHR activate cAMP and IP signaling (Fig. 2.1B) (5, 11, 18). However, since intracellular signaling is complex and can be context dependent, it is possible that the transgenes have disparate fertility phenotypes because their downstream signaling features are not precisely the same. It should be noted that, although mutations at D578 in humans are present in patients with FMPP (3), the D578H mutation has only been identified as a somatic mutation in Leydig cell adenomas (5). This suggests that the D578H mutation may not be compatible with germline transmission.

In summary, we have demonstrated the effects of expression of a constitutively active LH receptor in transgenic mice. Further investigation of the phenotype of these mice both at prepubertal and adult ages will be informative in determining the importance of LHR mediated signaling in Leydig and Sertoli cell growth and differentiation.

Acknowledgments

We especially want to thank Dr. Sally Camper and the University of Michigan Transgenic Animal Model Core Facility for the generation of the transgenic founder mice. We also wish to thank Dr. Mary Bedell and Ms. Aparna Mahakali Zama for their assistance with mouse procedures, Dr. Carlos A. Alvarez for establishment of stable HEK

293 cell lines, Dr. Barry G. Harmon for his assistance with the histological analysis, and Dr. Joe Crim for his assistance with the radioiodination of LH and FSH. We gratefully acknowledge Ms. Judy Gray for assistance with the mouse genotyping.

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Figure legends

Fig. 2.1. Transgenic constructs and production of IP by constitutively active LH receptors. A. YHR and D556H myc-rLHR were cloned under the control of a 6 kb fragment containing the mouse inhibin α-subunit promoter in pBS-SK (-) with a bovine growth hormone polyadenylation signal (bGHpA). YHR consists of the hCG β-subunit with its signal sequence linked to the human α-subunit via its C-terminal peptide (CTP), which in turn is linked to the extracellular domain of the LH receptor by an additional CTP and a Factor Xa recognition sequence. D556H myc-rLHR is preceded by the signal sequence for LHR and a 10 amino acid human c-myc tag. B. IP production in HEK 293 cells expressing wild-type myc-rLHR, D556Y myc-rLHR, D556H myc-rLHR, and YHR. Cells were incubated in the absence (basal) or presence (1 μg/ml) of hCG. Data shown are the mean ± ranges of two independent assays of the same cell line.

Fig. 2.2. Tissue-specific expression of constitutively active LHR in transgenic mice. A. Expression of D556H in the testes and adrenals of founder mice at 8 months of age as determined by RT-PCR. GAPDH primers were utilized to ensure the amplification of the RNA sample. Control testes and adrenals are from aged-matched C57BL/6J breeder mice. Controls without reverse transcriptase (not shown) did not reveal any amplification from contaminating DNA. B. YHR expression in multiple tissues of YHR^{+} and WT control mice. O, ovary; A, adrenal; K, kidney; Li, liver; Lu, lung; H, heart; B, brain; and T, testes, (-), template-free control. C. Relative expression in F1 progeny from multiple YHR lines as determined by semi-quantitative RT-PCR. YHR expression in the testes and adrenals is given as a ratio to 18S rRNA signal as an internal standard and

normalized to the lowest expression level. D. Real-time RT-PCR on ovary samples from 537 and 571 line. YHR expression is given as a ratio to 18S rRNA and normalized to the lowest expression level.

Fig. 2.3. Testosterone levels in transgenic mice. Testosterone was determined in serum (A) and in testicular extracts (B) by RIA at 3, 5, 8, and 12 weeks of age. Most of the serum testosterone levels at 3 weeks of age in the WT animals were below the standard curve and were assigned the value of the lowest standard (0.2 ng/ml) for graphing purposes. Statistical analyses could not be performed on these samples. Testicular testosterone (B) is the total amount in ether extracts from both testes. For 8 and 12 weeks, 1 testes was used and the amount corrected for the weight of both testes. n=6-11; ***, $p<0.001$.

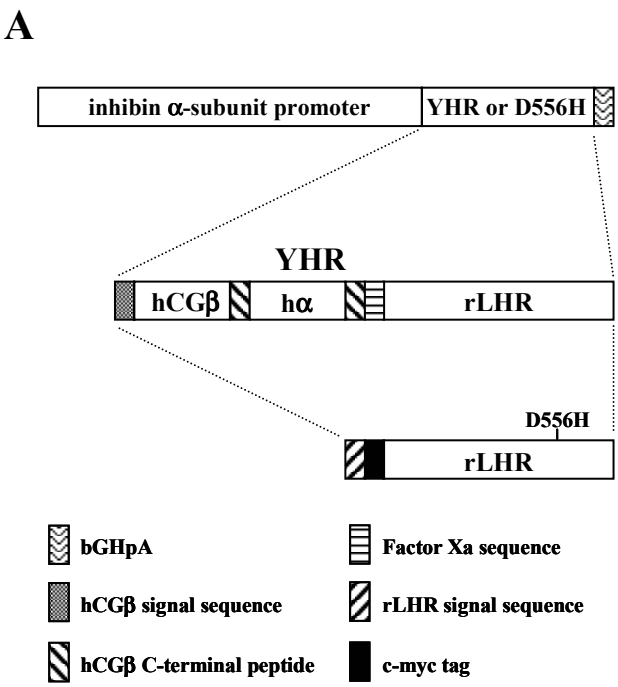
Fig. 2.4. Seminal vesicles in YHR^+ mice. A. Seminal vesicles of YHR^+ and WT mice at 5 weeks of age. B. Seminal vesicle weights in animals 3-12 weeks of age. n=4-11; *, $p<0.05$; ***, $p<0.001$.

Fig. 2.5. Serum LH and FSH levels. LH (A) and FSH (B) levels as determined by RIA on serum samples from mice at 3, 5, 8, and 12 weeks of age. Some LH values at 3 and 5 weeks of age were below the standard curve and were assigned the value of the lowest standard (0.2 ng/ml) for graphing purposes. Statistical analyses could only be performed at 8 and 12 weeks for LH and no significant differences were found. Serum FSH levels (B) were significantly reduced at all ages. n=4-10; ***, $p<0.001$.

Fig. 2.6. Effects of YHR^+ expression on testicular development. A. Testes in 5-week-old YHR^+ mice compared with a non-transgenic littermate (WT). B. Testes weights in animals from 3-12 weeks of age. n=4-10; **, $p<0.01$; ***, $p<0.001$.

Fig. 2.7. Histology of testes from mice. Hematoxylin and eosin stained sections from 5-week-old (A, B) and 12-week-old (C, D) testes from control (A, C) and YHR^+ mice (B, D). Note that the YHR^+ testes shows little or no disruption of spermatogenesis at both ages but has decreased seminiferous tubule volume at 5 weeks of age. Immunohistochemistry was performed on 5-week-old testes (E vs. F) with antiserum to 3βHSDI to specifically stain Leydig cells. No difference in Leydig cell number or size is apparent. panels A-D (400X); panels E, F (200X).

Fig. 2.1


A
inhibin α-subunit promoter
YHR or D556H
YHR
hCGβ
hα
rLHR
D556H
rLHR
bGHpA
hCGβ signal sequence
hCGβ C-terminal peptide
Factor Xa sequence
rLHR signal sequence
c-myc tag

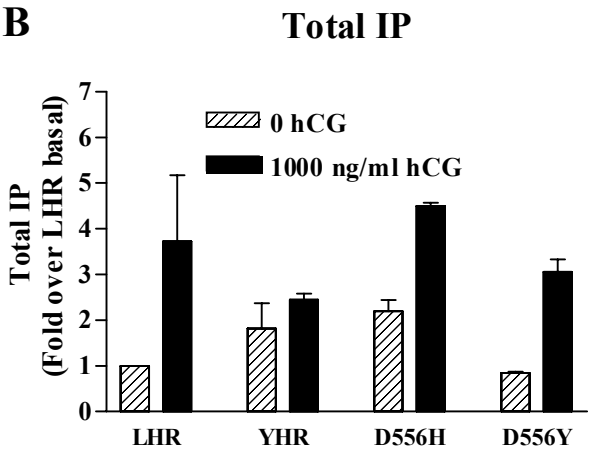

B
Total IP
Total IP
(Fold over LHR basal)
0 hCG
1000 ng/ml hCG
0
1
2
3
4
5
6
7
LHR
YHR
D556H
D556Y

Fig. 2.2

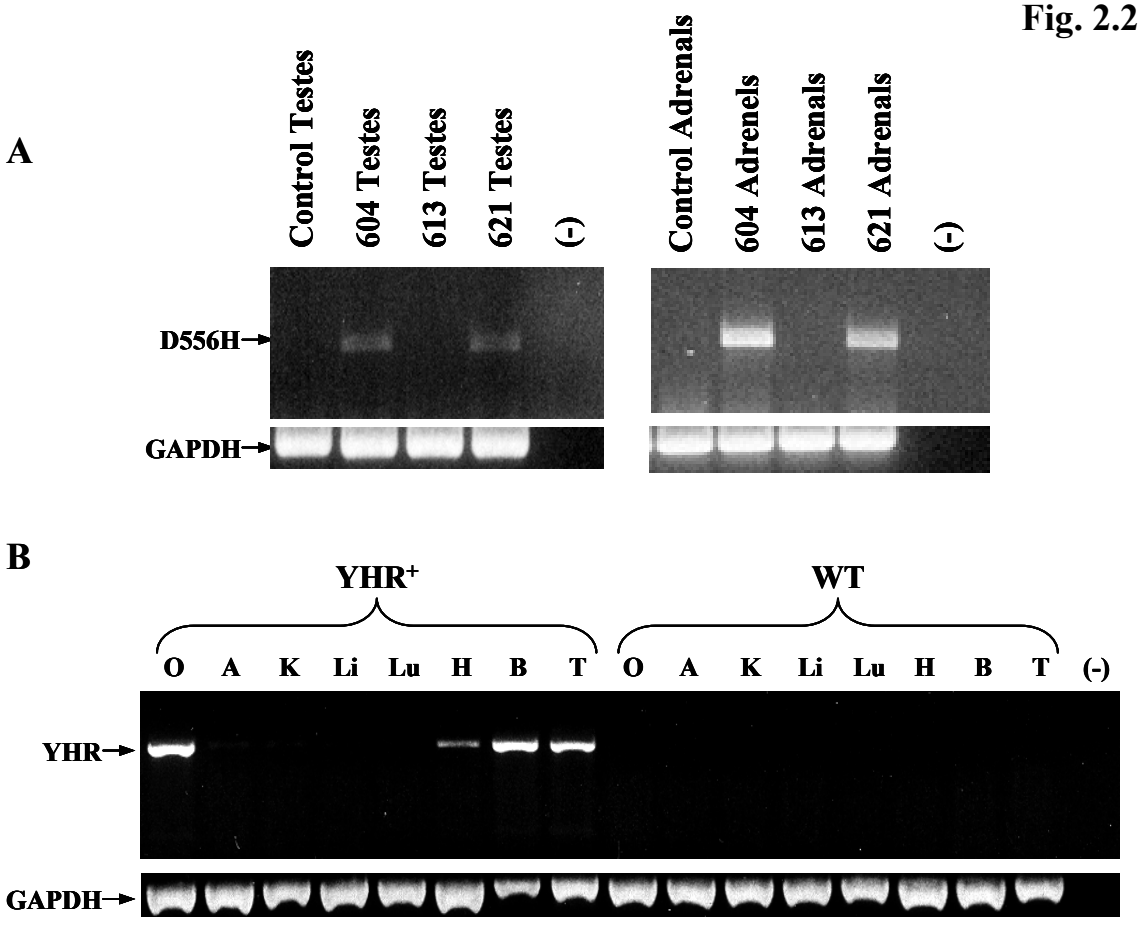

A
Control Testes
604 Testes
613 Testes
621 Testes
(-)
Control Adrenals
604 Adrenels
613 Adrenals
621 Adrenals
(-)
D556H
GAPDH
B
YHR+
WT
O A K Li Lu H B T
O A K Li Lu H B T (-)
YHR
GAPDH
C
Relative expression (arbitrary units)
10
5
0
Testes
Adrenals
537
553
571
D
Relative expression (arbitrary units)
3
2
1
0
537 Ovary
571 Ovary

Fig. 2.3

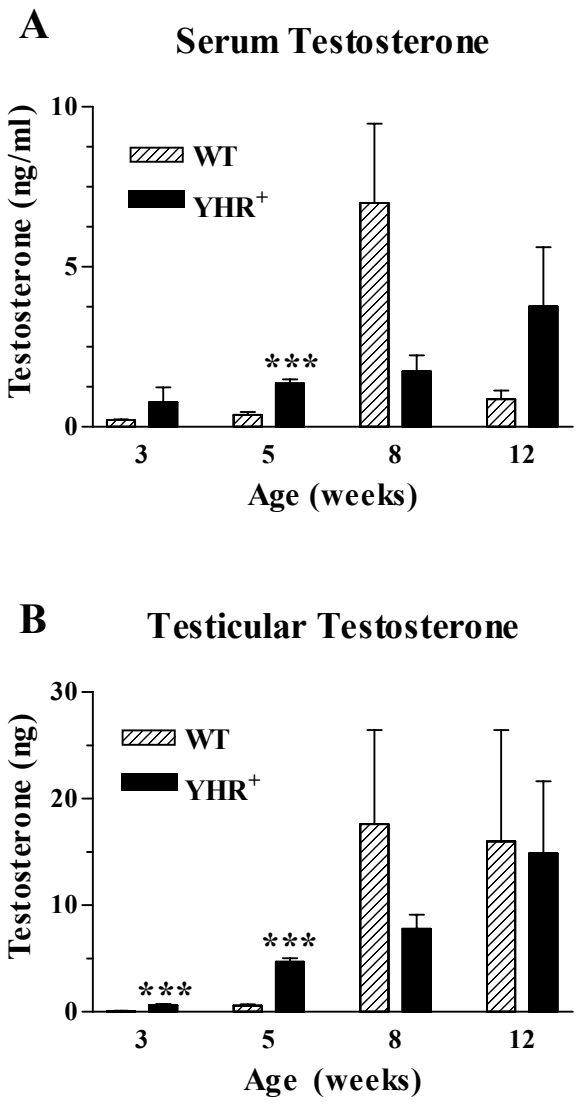

A
Serum Testosterone
Testosterone (ng/ml)
10
5
0
WT
YHR+
***
3
5
8
12
Age (weeks)
B
Testicular Testosterone
Testosterone (ng)
30
20
10
0
WT
YHR+
***
***
3
5
8
12
Age (weeks)

Fig. 2.4

A


WT
YHR+

B

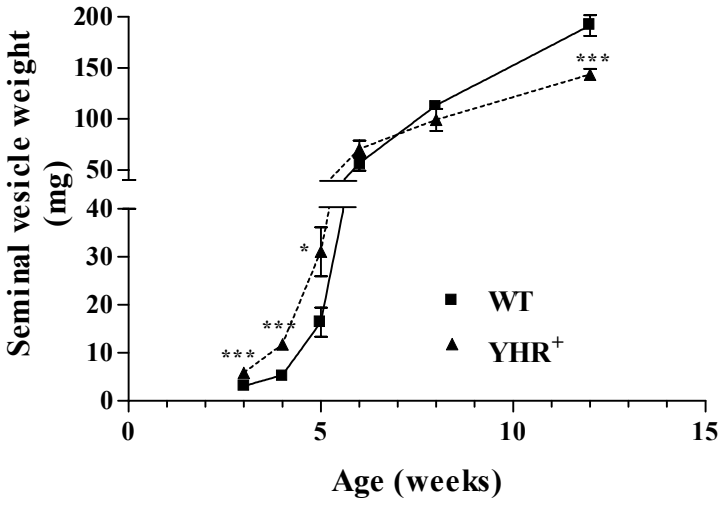

Seminal vesicle weight (mg)
200
150
100
50
40
30
20
10
0
0
5
10
15
Age (weeks)
WT
YHR+
***
***
*
***

Fig. 2.5

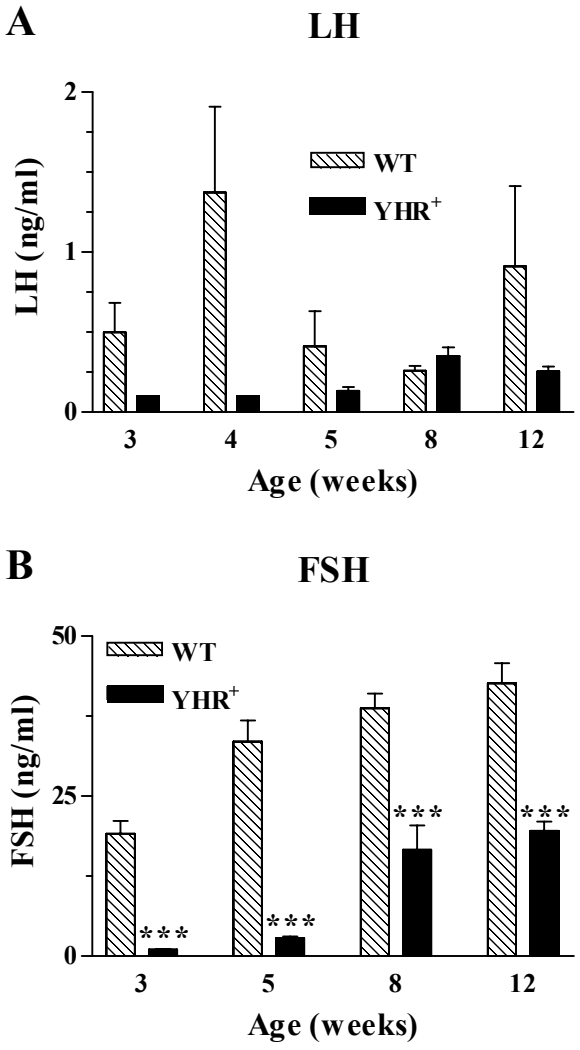

A
LH
LH (ng/ml)
2
1
0
WT
YHR+
3
4
5
8
12
Age (weeks)
B
FSH
FSH (ng/ml)
50
25
0
WT
YHR+
***
***
***
***
3
5
8
12
Age (weeks)

Fig. 2.6

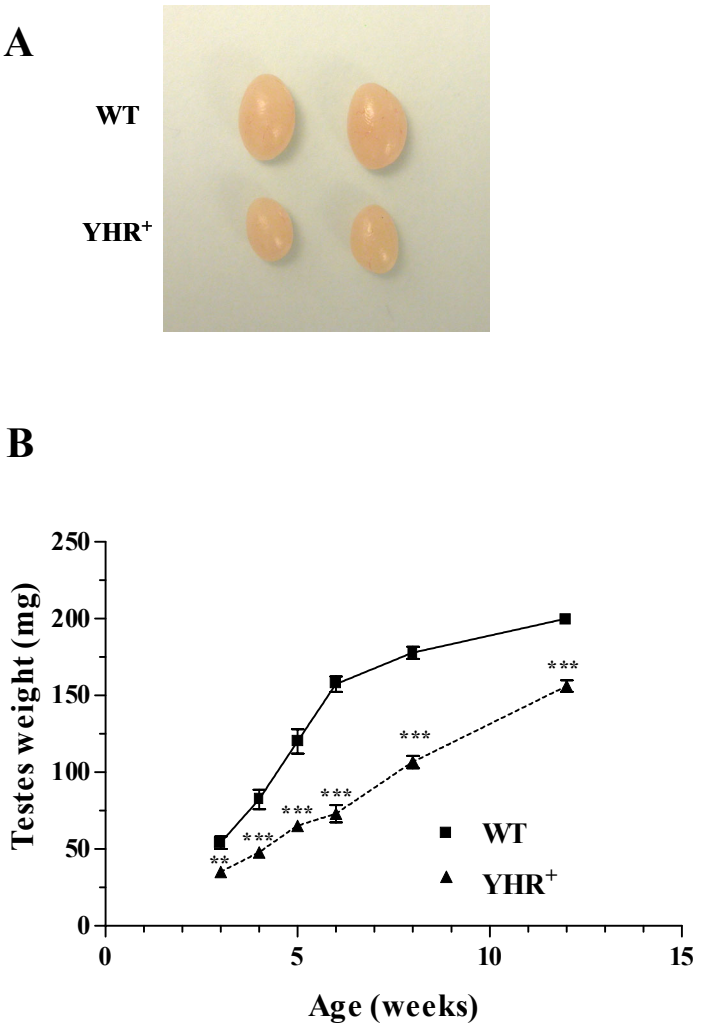
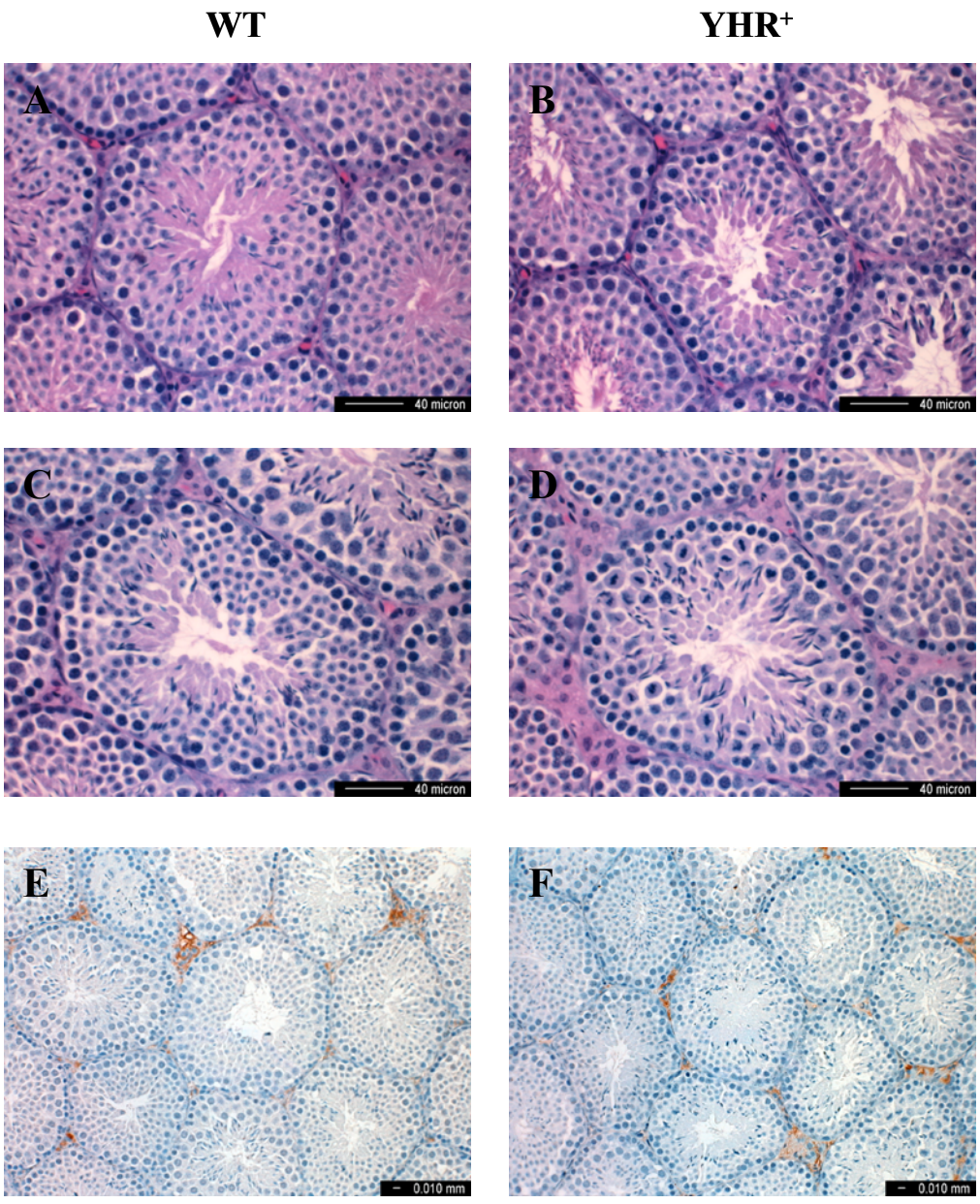

A
WT
YHR+
B
Testes weight (mg)
250
200
150
100
50
0
0
5
10
15
Age (weeks)
WT
YHR+
**
***
***
***
***
***

Fig. 2.7


WT
YHR+
A
B
C
D
E
F
40 micron
40 micron
40 micron
40 micron
0.010 mm
0.010 mm

CHAPTER 3

PRECOCIOUS PUBERTY AND OVARIAN CYSTS IN FEMALE MICE EXPRESSING A CONSTITUTIVELY ACTIVE LUTEINIZING HORMONE RECEPTOR[1]

[1] Meehan, T.P., B.G. Harmon, and P. Narayan. Submitted to *Biology of Reproduction*, 5/16/2003.

Abstract

We have recently reported the generation of transgenic mice expressing constitutively active luteinizing hormone receptors (LHR) under the control of the mouse inhibin α-subunit promoter. Towards this end, two constitutively active LHRs were utilized, a D556H mutation that is found in Leydig cell adenomas of boys and a genetically engineered yoked hormone receptor complex (YHR), in which a fusion protein of hCG is covalently linked to the N-terminus of rat LHR. Both male and female founder mice expressing D556H LHR failed to reproduce. In this study, we have investigated the effects of constitutively active LHR expression on female reproduction. YHR-expressing mice (YHR^+) were analyzed at 5 and 12 weeks of age for alterations in uterine and ovarian histology, as well as steroid and gonadotropin hormone levels. YHR^+ mice undergo precocious puberty with early vaginal opening and accelerated uterine development, in addition to an enhanced level of follicular development including the presence of corpora lutea. Increases in ovarian estradiol and serum progesterone are consistent with these results. At 12 weeks of age, the ovary exhibits a relative increase in the number of degenerating follicles and in the amount of interstitial tissue, comprised of cells that are hypertrophic and luteinized. Additionally, hemorrhagic cysts develop in approximately 25% of the transgenic mice. These degenerative changes are consistent with an aging ovary. These results suggest that chronic LHR activation in female mice leads to precocious sexual development, ovarian lesions, and premature ovarian aging.

Introduction

Reproduction in females is dependent on the pituitary hormones LH and FSH which function in the maturation of follicles and ovulation. These hormones are heterodimeric glycoproteins comprised of a common α-subunit and a hormone specific β-subunit, that are secreted from the pituitary [1]. In equids and primates, an additional hormone, chorionic gonadotropin (CG), is secreted from the placenta during pregnancy to maintain the corpus luteum. CG, which shares the α-subunit with LH and FSH, has high sequence similarity in its β-subunit with LH, differing mainly in the C-terminal peptide extension present only in CG [1].

The glycoprotein hormones signal through the actions of heptahelical G-protein coupled receptors of a unique family containing a large ectodomain which confers the hormone binding specificity. LH and CG act via a common member of this receptor family, the LH receptor (LHR), which signals through the actions of heterotrimeric G-proteins to primarily increase intracellular cAMP [2]. LHR is expressed in the theca cells of all follicles and in the granulosa cells of developing follicles, where FSH receptor signaling is required for LHR expression [3]. Signaling through LHR in theca cells leads to an increase in androgen synthesis, followed by conversion to estrogens in granulosa cells. An LH surge from the pituitary triggers ovulation and luteinization of the granulosa cells to form the corpus luteum [4].

Female carriers of inactivating mutations in LHR present with amenorrhea and infertility from a lack of ovulation, illustrating the important role for LHR in female reproduction [5]. These inactivating mutations have been modeled in mice by knocking out the endogenous LHR [6, 7]. In males, activating mutations of LHR cause familial

and sporadic precocious puberty [5, 8], as well as Leydig cell adenomas [9, 10]. In female carriers of activating mutations, however, no clear phenotype is evident.

We have previously described the generation of transgenic mice expressing constitutively active LHR and the characterization of male phenotype [11]. For this study, D556H myc-rat LHR (D556H LHR), equivalent to the D578H human LHR found in Leydig cell adenomas [9], and the yoked hormone receptor (YHR), a genetically engineered receptor with the subunits of the heterodimeric hCG fused and then covalently attached to the ecto domain of LHR [12], were utilized. These two constitutively activated receptors were cloned under the control of the mouse inhibin α-subunit promoter to target expression to the gonads [13]. Male mice expressing YHR were found to exhibit prepubertal increases in testicular and serum testosterone and seminal vesicle weight, consistent with expression of constitutively active LHR. In addition, testicular development was impaired in these mice [11].

Herein, we describe the female phenotype of YHR and D556H LHR expressing mice. Female founder mice expressing D556H LHR failed to reproduce; those expressing YHR exhibited precocious puberty, accelerated folliculogenesis, and enhanced ovarian steroidogenesis. In adult mice, extensive interstitial cell hypertrophy, follicular atresia, and follicular cysts were evident. These results indicate that, unlike humans, chronic LHR activation results in significant ovarian abnormalities in mice.

Material and methods

Generation of transgenic mice

Establishment of transgenic mice expressing YHR (YHR^+) and D556H LHR have been previously described [11]. Briefly, YHR and D556H LHR were cloned under the control of the mouse inhibin α-subunit promoter with a bovine growth hormone polyadenylation signal (Fig. 3.1). The transgenes were microinjected into (C57BL/6xSJL) F2 fertilized eggs. Potential founder mice were genotyped by PCR and Southern blot analysis and bred to C57BL/6J mice to establish lines. D556H LHR founder mice failed to reproduce with no lines being established, while several lines were established from YHR founder mice. These lines were characterized for their expression levels of the receptors, and the two lines that were chosen for further analysis exhibited similar phenotypes [11].

For this study, animals from the F5-F7 generations of YHR^+ mice in the C57BL/6J background from the 571 founder line were utilized. Female mice were subfertile and male YHR^+ mice were bred to maintain the line. The onset of puberty was determined as the first day of vaginal opening as investigated daily beginning at day 21. Cycling studies were performed by analyzing daily vaginal smears for a period of 3 weeks. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Georgia.

RNA isolation and RT-PCR

Total RNA was isolated from ovaries and adrenals of D556H LHR founder mice at 8 months of age utilizing RNA Stat-60 solution (Tel test, Inc., Friendswood, TX). RT-PCR was performed on 0.5 μg of each sample with the Titan one tube RT-PCR kit (Roche,

Indianapolis, Indiana) using primers for D556H LHR and glyceraldehyde phosphate dehydrogenase (GAPDH). To ensure specificity of the PCR, reactions without reverse transcriptase and without template were performed. PCR products were resolved on a 1.5% agarose gel in Tris-borate-EDTA buffer, stained with ethidium bromide, and visualized by UV.

Measurement of hormone levels

Estradiol and testosterone were measured following extraction of the ovaries with diethyl ether. Both ovaries were homogenized in 0.5 ml PBS and extracted 3 times with 2 ml diethyl ether. Estradiol and testosterone concentrations were measured utilizing ELISA kits from Neogen (Lexington, KY) following manufacturer instructions. The sensitivities of the assays were 20 pg/ml and 2 pg/ml for estradiol and testosterone, respectively.

Serum levels of progesterone, LH, and FSH were determined by RIA. The progesterone assay (Diagnostics Products Corporation, Los Angeles, CA) has a sensitivity limit of 0.1 ng/ml. Rat LH and FSH were iodinated by iodogen, and serum LH and FSH levels were measured with standards and antisera purchased from Dr. A. Parlow of the NIDDK National Hormone and Peptide Program. The sensitivity of the LH and FSH assays were 0.1 ng/ml and 0.8 ng/ml, respectively. Measurement of hormone levels from 12-week-old mice were from randomly cycling animals.

Histology of ovaries and uteri

Tissues were fixed in 10% buffered formalin and embedded in paraffin. Serial sections (5 μm) were stained with hematoxylin and eosin prior to histological examination by light microscopy.

Statistical analysis

Unpaired t-tests utilizing the Prism software (GraphPad Software, Inc., San Diego, CA) were performed to compare the WT and YHR^{+} mice. $P<0.05$ was considered significant.

Results

D556H LHR mice are infertile

As previously found for the male founder mice [11], female founder mice transgenic for D556H LHR failed to reproduce. Of the 5 female founder mice, only mice 615 and 618 produced litters (Table 3.1); however, the transgene was not transmitted to the progeny. Following numerous breeding attempts with several different C57BL/6J males, cycling studies were performed at 8 months of age on transgenic founder mice and age-matched, non-transgenic mice as controls. Daily vaginal smears for 3 weeks revealed that D556H LHR founder mice were in diestrus the entire time and, therefore, were not cycling. Controls animals exhibited normal cyclicity with a duration of about 4-5 days per cycle. Cycling for founder mouse 598 could not be determined due to an imperforate vagina.

D556H LHR female founder mice were dissected at 8 months of age, and one ovary was utilized for RNA preparation and the other was sectioned for histological examination. RT-PCR revealed expression of D556H LHR mRNA in ovaries and

adrenals (Fig. 3.2A) of founder mice. Two mice (598 and 614) had expression in both the ovary and adrenal, while mouse 618 had only ovarian expression, and mouse 603 only showed adrenal expression. D556H LHR expression in the ovary of mouse 603 could not be determined because the RNA could not be amplified as shown by the lack of band in the GAPDH primer control. It is likely that mouse 603 expresses D556H LHR mRNA in the ovaries based on expression in the adrenals and the similarity in the ovarian histology between founder mice 603 and 614 (data not shown). Histological examination of the ovaries of founder mice revealed several common features that are illustrated by comparing ovarian sections from WT and 614 founder mice (Fig. 3.2, B vs C). With the exception of mouse 615, there is a clear increase in the number of corpora lutea (CL) and a decrease in the number of developing follicles in the D556H LHR ovary. In addition, interstitial cell hypertrophy was evident in the ovaries of all the founder mice and hemorrhagic cysts were present in the ovaries of mouse 603. The histology of the ovary from mouse 615, which does not express D556H LHR, was similar to that from a WT mouse indicating that transgene expression correlates with the pathological changes (data not shown).

YHR$^+$ mice undergo precocious puberty

YHR$^+$ mice exhibited precocious puberty as evidenced by the premature vaginal opening that occurred at 23.5 days (n=8) as compared to WT controls at 31.3 days (n=9) ($p<0.001$). In addition, there was a 10-fold increase in the size of the uterus at 5 weeks of age (Fig. 3.3A, Table 3.2). The non-transgenic mice had thin, atrophic uteri, compared to the enlarged YHR uteri. This precocious development is readily visualized in the uterine

sections which show a marked dilation of the lumen (Fig. 3.3, B vs C). The endometrial cells lining the lumen are hypertrophic (Fig. 3.3, D vs E) and the stroma is edematous. These changes in the YHR^+ uterus are consistent with the trophic effects of estrogen.

The ovaries of 5-week-old YHR^+ mice exhibit increased folliculogeneis with a larger number of follicles at later stages of development, including the presence of CLs which are absent in WT controls at this age (Fig. 3.4, A vs. B). Many of these CLs in YHR^+ mice retain the oocyte (Fig. 3.4C), indicating luteinization of the granulosa cells in the absence of ovulation. It is important to note, however, that the theca and granulosa cells of developing follicles appear unchanged.

Enhanced steroidogenesis in YHR^+ mice

To determine the effect of premature LHR activation on ovarian steroidogenesis, hormone levels were measured (Table 3.2). Ovarian estradiol (E2) and serum progesterone are both elevated compared with control animals, consistent with the enhanced uterine growth and luteinization in the ovaries of YHR^+ mice. Although ovarian testosterone (T) was not affected in YHR^+ mice, the T/E2 ratio was significantly decreased, demonstrating a higher level of aromatization of androgens in these mice. Serum LH and FSH were also decreased in 5-week-old mice indicating a functional hypothalamic-pituitary-gonadal (HPG) axis.

Adult YHR^+ mice exhibit degenerative changes in the ovary

YHR^+ female mice were subfertile and produced litters sporadically. At 12 weeks of age, YHR^+ mice showed an increase in the amount of intersititial tissue that appears

disorganized and luteinized (Fig. 3.5, A vs B). Although follicles at various stages of growth were still evident in these ovaries, they were mostly at the periphery. The theca and granulosa cells of the follicles appear normal. YHR^+ mice contain many small follicles with degenerative ova interspersed in the interstitium, and there is an overall increase in the number of atretic follicles (Fig. 3.5, C vs D). Extensive interstitial cell hypertrophy characterized by vacuolated cytoplasm was evident. About 25% of YHR^+ mice developed hemorrhagic cysts (Fig. 3.5B) which can be observed as early as 5 weeks of age. Some of the cysts contain what appears to be normal granulosa cells, while others are surrounded by luteinized cells. In contrast, ovaries from age-matched controls contain all stages of follicle development, including CLs. Many of the morphological changes seen in the ovaries of 12-week-old mice are similar to those found in mice over one year of age.

At 12 weeks of age, steroid hormone and gonadotropin levels of randomly cycling mice were not significantly altered between WT and YHR^+ mice, and their average uterine weights were similar (Table 3.2). Histological analysis of the uterus revealed no major differences in the uterus from WT and YHR^+ mice (Fig. 3.6, A vs B) or in the morphology of the endometrial cells and endometrial stroma (Fig. 3.6, C vs D). The development of the endometrial glands was similar. Occasionally, a grossly enlarged uterus, with as much as a 10-fold increase in weight, was observed in YHR^+ mice (Fig. 3.6E). These uteri have a markedly distended fluid-filled lumen (hydrometria) with attenuated myometrium and endometrial stroma containing few endometrial glands (Fig. 3.6F). The ovaries of these mice had multiple large hemorrhagic cysts that are readily apparent on gross dissection. These individual mice exhibit periods of prolonged estrus

and their ovarian estradiol and testosterone levels (data not shown) were elevated compared to YHR^+ mice with a normal uterus.

Discussion

Expression of a constitutively active LHR exerts many reproductive alterations in female mice. D556H LHR mice are infertile and there is massive luteinization in the ovary. YHR^+ mice exhibit precocious puberty with increases in steroid hormone synthesis leading to accelerated ovarian and uterine development. Adult transgenic mice exhibit ovarian interstitial cell disorganization and hypertrophy with increased follicular degeneration, and about 25% of YHR^+ mice develop follicular or luteal cysts.

Constitutively active LHRs have been described in humans with familial and sporadic male-limited precocious puberty (FMPP) [8]. These male patients have increases in prepubertal testosterone production and Leydig cell hyperplasia. Female carriers of these mutations fail to develop a distinct phenotype even though LHR is critical for female reproduction [5]. However, current analysis of the female phenotype has been fairly limited and the families investigated have mutations involving some of the milder FMPP phenotypes [14-16]. The relative increases in basal cAMP production produced by most activating mutations found in FMPP [8] are smaller than those resulting from YHR, which has basal cAMP production approximately 25-fold higher than the wild-type receptor [12]. Naturally occurring mutations in LHR that result in higher levels of constitutive activation have only been found in sporadic cases of precocious puberty [17, 18] and in Leydig cell adenomas [9, 10], with no reports in females. It is also possible that the prepubertal human ovary expresses little or no LHR,

and, therefore, no aberrant signaling occurs to produce steroid hormones and cause precocious puberty. Also, many of these activated LHR can still respond to the high doses of LH required for ovulation [5].

In contrast to human females, mice expressing constitutively active LHR have a distinct ovarian phenotype. The prepubertal morphological and histological changes in the transgenic mice can be readily explained by the hormonal alterations that are induced by YHR expression in the ovaries. The early increase in estrogen levels is manifested by an early vaginal opening, e.g. 8 days prior to the WT control, and by the appearance of a mature, enlarged uterus at 5 weeks of age. The suppression in the levels of LH and FSH suggests that the estrogen-mediated feedback of the HPG axis is fully functional [19] and YHR expression is sufficient for follicular development and maturation. The presence of CLs, some with oocytes, and elevated levels of serum progesterone in the context of suppressed LH levels suggests that luteinization occurs in the absence of ovulation. Interestingly, the level of intraovarian testosterone is not elevated in YHR mice, suggesting that the conversion to estrogen is more efficient in YHR^+ ovaries. This implies that aromatase must be upregulated in these mice even though FSH levels are suppressed, perhaps due to expression of YHR in the granulosa cells. This result is in contrast with the model of the LHβ-overexpressing mice, wherein hyperandrogenemia has been implicated in the development of precocious puberty, accelerated follicular development and cyst formation [20, 21]. The similarity in the ovarian phenotype between this model and the YHR^+ mice suggests that small increases in estrogen levels may be equally important in the development of ovarian pathology. Unlike LHβ-overexpressing mice, YHR^+ mice do not develop granulosa cell tumors; however, Keri et

al. have determined the tumor formation to be a strain-dependent event not occurring in the C57BL/6J mice utilized in this study [22].

In addition to the LHβ-overexpressing mice, a recent model of hCGβ overexpression exhibits reproductive disturbances similar to YHR^+ mice, including precocious puberty, infertility, massive luteinization and follicular cysts accompanied by prepubertal increases in estrogen, testosterone and progesterone [23]. Unlike in YHR^+ mice, these hormonal imbalances persist into adulthood leading to a more severe phenotype including pituitary adenomas and mammary gland tumors. These differences may be due to receptor desensitization or negative regulation of the inhibin α-subunit promoter resulting in decreased YHR signaling. In contrast, mice overexpressing hCGβ under the control of the ubiquitous and constitutively active ubiquitin C promoter produce circulating levels of hCG over 40-fold higher than normal LH levels and these mice continue to secrete high levels of steroid hormones for as long as one year [23]. Nonetheless, the ovaries of YHR^+ mice develop interstitial cell luteinization and hypertrophy, increased follicular degeneration, and cysts suggesting that the early prepubertal increases in steroidogenesis may be sufficient for the development of early degenerative changes in the ovary.

Even though there is a difference in their reproductive ability, D556H LHR founder mice and YHR^+ mice have a similar ovarian phenotype including interstitial cell hypertrophy and follicular cysts. The presence of many CLs in the D556H LHR founder ovaries is indicative of a pseudopregnant state and may explain the lack of reproduction. It is also important to note that the YHR lines utilized in this study were generated from a male founder mouse and through breeding of transgenic males. Additionally, female

YHR^+ mice were subfertile and produced litters sporadically with some females never producing litters. Therefore, the phenotypes from these two constitutively active receptors are not significantly different.

In summary, these studies show that chronic LHR activation gives rise to a distinct phenotype in the female mouse. The combination of accelerated follicular development at early ages and increased degeneration in adult mice suggests that chronic LHR activation will lead to early reproductive senescence in the female. In support of this hypothesis is the observation that the morphological changes of the 12-week-old ovary resemble that of an ovary from a 1-year-old mouse. Although the phenotype of precocious puberty has not been observed in women with a constitutively active LHR, it will be of interest to determine if these women exhibit premature ovarian failure. Furthermore, this YHR^+ transgenic mouse model provides an opportunity to examine the molecular basis of early reproductive senescence.

Acknowledgements

We are deeply grateful to Dr. David Puett for his support and encouragement, helpful suggestions, and for critically reading the manuscript. We wish to thank Dr. Mary Bedell and Ms. Aparna Mahakali-Zama for their assistance with mouse procedures, Dr. Joe Crim for his help with the radioiodination of mLH and mFSH, Ms. Judy Gray for the genotyping of the mice utilized in this study, and Dr. Mark Farmer for helping with the photography of the histological sections.

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Figure legends

Fig. 3.1. Schematic of DNA constructs utilized for transgenic mouse production. The constitutively active LHRs, D556H LHR or YHR, were cloned under the control of the mouse inhibin α-subunit promoter with a bovine growth hormone polyadenylation signal (bGHpA). YHR consists of hCGβ with its signal sequence (SS_β) linked to human α-subunit through the C-terminal peptide (CTP) of hCGβ. This yoked hormone, in turn, is covalently attached to the extracellular domain of LHR via an additional CTP and a factor Xa cleavage sequence. The D556H transgenic construct (not shown) contains a myc-tagged rLHR with the signal sequence for rLHR.

Fig. 3.2. Characterization of the D556H LHR transgenic founder mice. A. RT-PCR was utilized to determine expression of D556H LHR in the ovaries and adrenals of founder mice at 8 months of age. GAPDH primers were utilized as a positive control for RNA quality. Control ovary and adrenal were from an age-matched non-transgenic mouse. Reactions without reverse transcriptase or template were performed to ensure the specificity of the amplification (not shown). B and C. Sections of control ovary and D556H LHR founder (614) ovary at 8 months of age. *, corpus luteum. Scale bars represent 100 μm (B, C).

Fig. 3.3. Uterus from YHR^+ mice at 5 weeks of age. A. Gross morphology of the uterus and ovaries of 5-week-old mice demonstrating precocious uterine development. B and C. Photomicrographs of a cross-section of the uterine horn of a WT and YHR^+ mouse. Note the large increase in size and a dilated lumen in the YHR^+ mouse. D. Higher magnification of the WT uterus showing the endometrial cells (EC) and endometrial stroma (ES). E. Higher magnification of the YHR^+ uterus showing the hypertrophy of the EC and edema of the ES, consistent with the trophic effects of estrogen. Scale bars represent 0.5 mm (B, C) and 50 μm (D, E).

Fig. 3.4. Light micrographs of the ovary at 5 weeks of age. A. WT ovary containing only limited follicular development with no follicles larger than the early antral stage and no corpora lutea. B. YHR^+ ovary revealing increased follicular development including the presence of corpora lutea (*) with retained oocyte (arrow). C. Higher magnification of the YHR^+ ovary. Note the presence of the oocyte in a corpus luteum. Scale bars represent 250 μm (A, B) and 100 μm (C).

Fig. 3.5. Photomicrograph of the ovary of 12-week-old mice. A. WT ovary containing follicles at all stages of development, including the presence of a corpus luteum. B. YHR^+ ovary demonstrating the presence of large atretic follicles (AF), follicular cysts (*), and interstitial cell luteinization. C. Higher magnification of WT ovary showing the interstitial tissue. D. Higher magnification of the YHR^+ ovary showing interstitial cell

(IC) hypertrophy containing small lipid-filled vacuoles and many follicles with degenerating ova (arrowhead). Scale bars represent 250 μm (A, B) and 50 μm (C, D).

Fig. 3.6. Histological pictures of the uterus at 12 weeks of age. A and B. WT and YHR^+ uterus demonstrating normal uterine development. C and D. Higher magnification of the WT and YHR^+ uterus showing normal stromal and endometrial cell development. E. YHR^+ uterus from an animal with a cystic ovary revealing a markedly dilated lumen filled with a clear fluid. F. Higher magnification of YHR^+ uterus shown in Panel E. Note the attenuation of the endometrial stroma and myometrium due to uterine distension. Scale bars represent 0.5 mm (A, B, E) and 50 μm (C, D, F).

TABLE 3.1. Summary of studies with D556H LHR founder mice.

Founder	Litters (#)	Progeny (#)	Transgenic progeny (#)	Cycling	Gonadal expression	Adrenal expression
598	0	0	0	ND	Yes	Yes
603	0	0	0	No	ND	Yes
614	0	0	0	No	Yes	Yes
615	2	13	1	No	No	No
618	1	9	0	No	Yes	No

ND, not determined.

TABLE 3.2. Summary of steroid and gonadotropin hormone levels in transgenic mice

	Estradiol (pg)[1]	Testosterone (pg)[1]	T/E2 Ratio	Progesterone (ng/ml)[2]	LH (ng/ml)[2]	FSH (ng/ml)[2]	Uterus (mg)
5 week-old							
WT	32 ± 4 (8)	90 ± 13 (8)	2.8 ± 0.2 (8)	0.36 ± 0.02 (5)	0.18 ± 0.03 (6)	9.35 ± 0.78 (6)	20 ± 4 (9)
YHR^+	85 ± 29 (7)	91 ± 19 (7)	1.5 ± 0.4 (7)[a]	1.47 ± 0.32 (5)[a]	< 0.10 (6)[*]	6.54± 0.97 (6)[b]	192 ± 68 (10)[b]
12 week-old							
WT	74 ± 27 (7)	39 ± 14 (7)	0.6 ± 0.1 (7)	1.26 ± 0.57 (5)	0.43 ± 0.15 (6)	8.27 ± 2.90 (6)	81 ± 7 (7)
YHR^+	134 ± 70 (7)	105 ± 51 (7)	1.1 ± 0.3 (7)	1.96 ± 0.48 (5)	0.23 ± 0.03 (6)	12.43 ± 4.63 (6)	373 ± 207 (9)

Data are mean ± SEM (n).
[1] Estradiol and testosterone levels were measured in ovarian extracts.
[2] Progesterone, LH, and FSH were measured in serum.
[*] LH levels are below the sensitivity limits of the assay.
[a] denotes significantly different from WT controls, $p<0.01$.
[b] denotes significantly different from WT controls, $p<0.05$.

Fig. 3.1

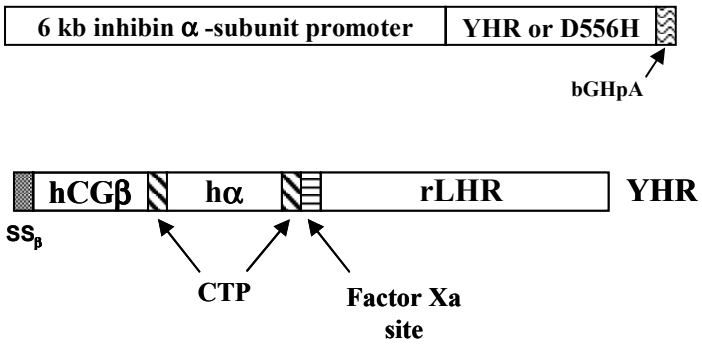
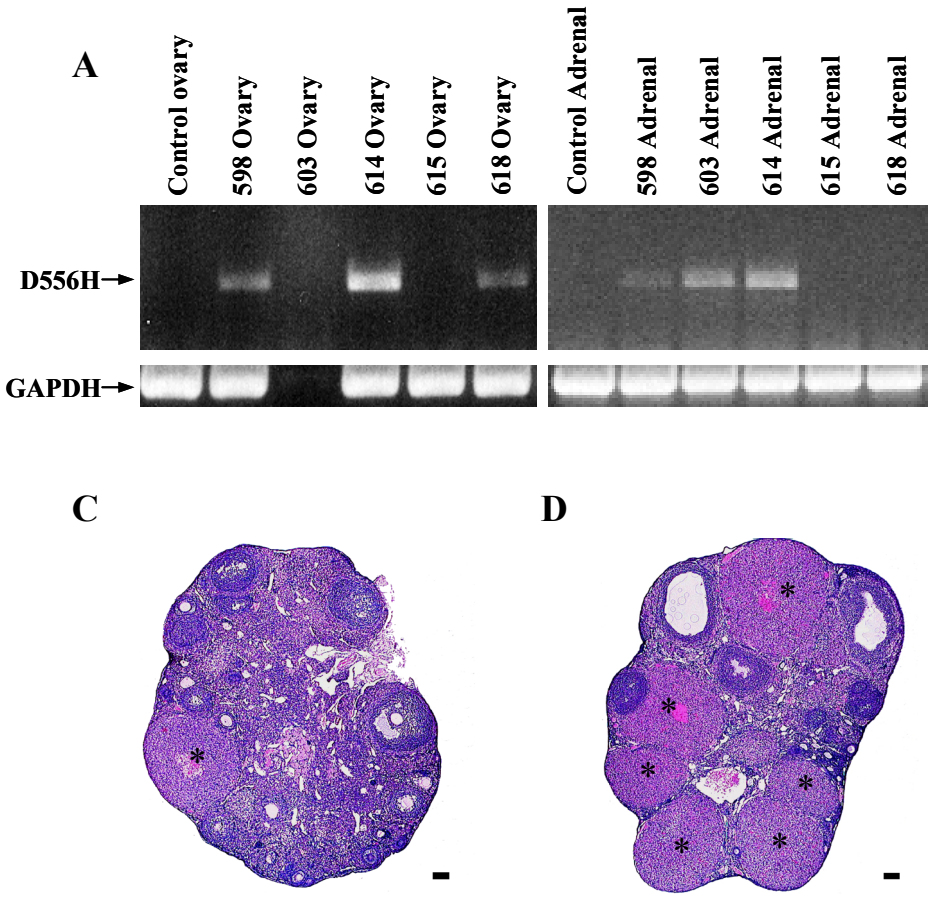

6 kb inhibin α -subunit promoter
YHR or D556H
bGHpA
hCGβ
hα
rLHR
YHR
SSβ
CTP
Factor Xa site

Fig. 3.2


A
Control ovary
598 Ovary
603 Ovary
614 Ovary
615 Ovary
618 Ovary
Control Adrenal
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614 Adrenal
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618 Adrenal
D556H
GAPDH
C
D

Fig. 3.3

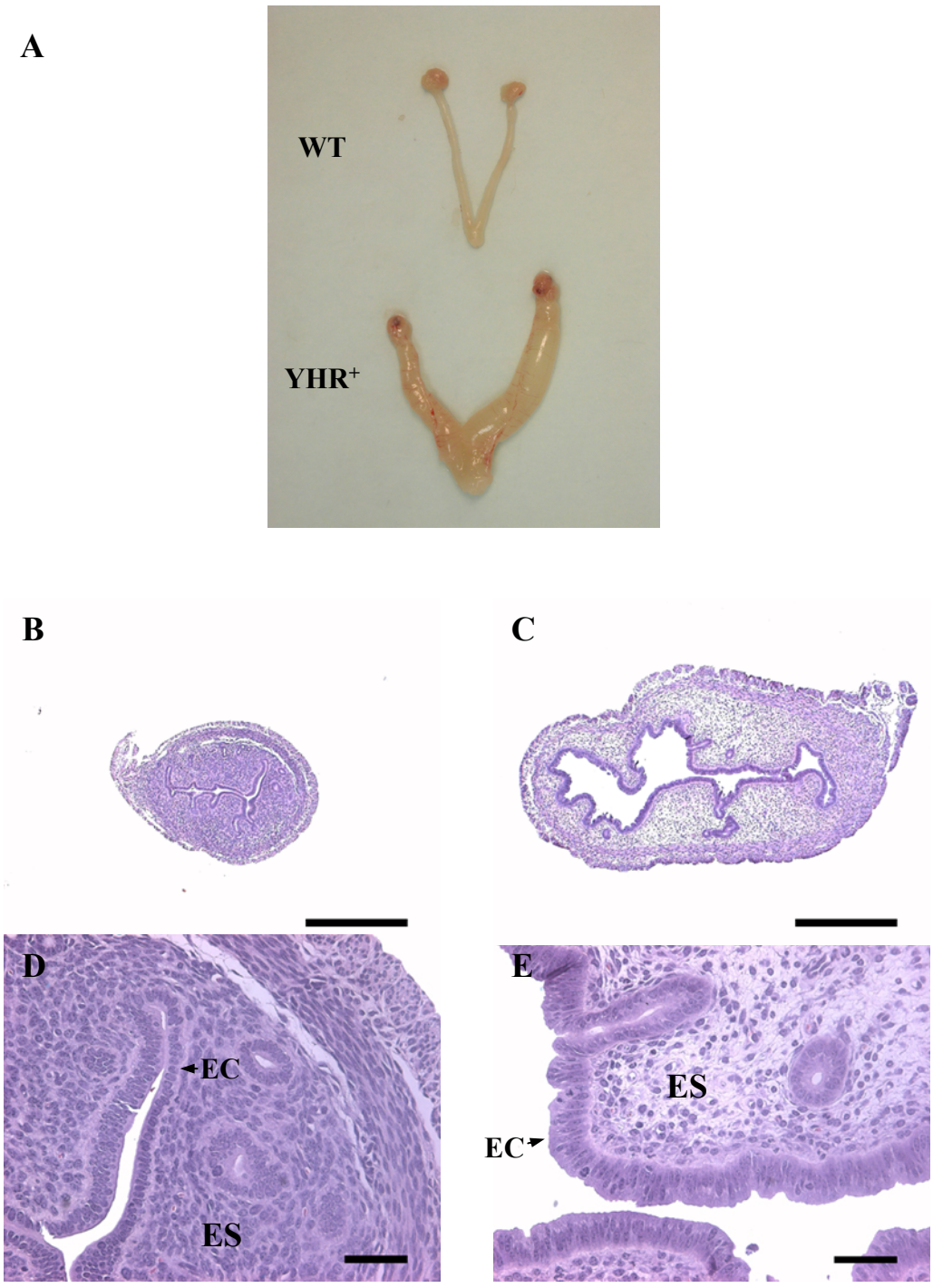


Fig. 3.4

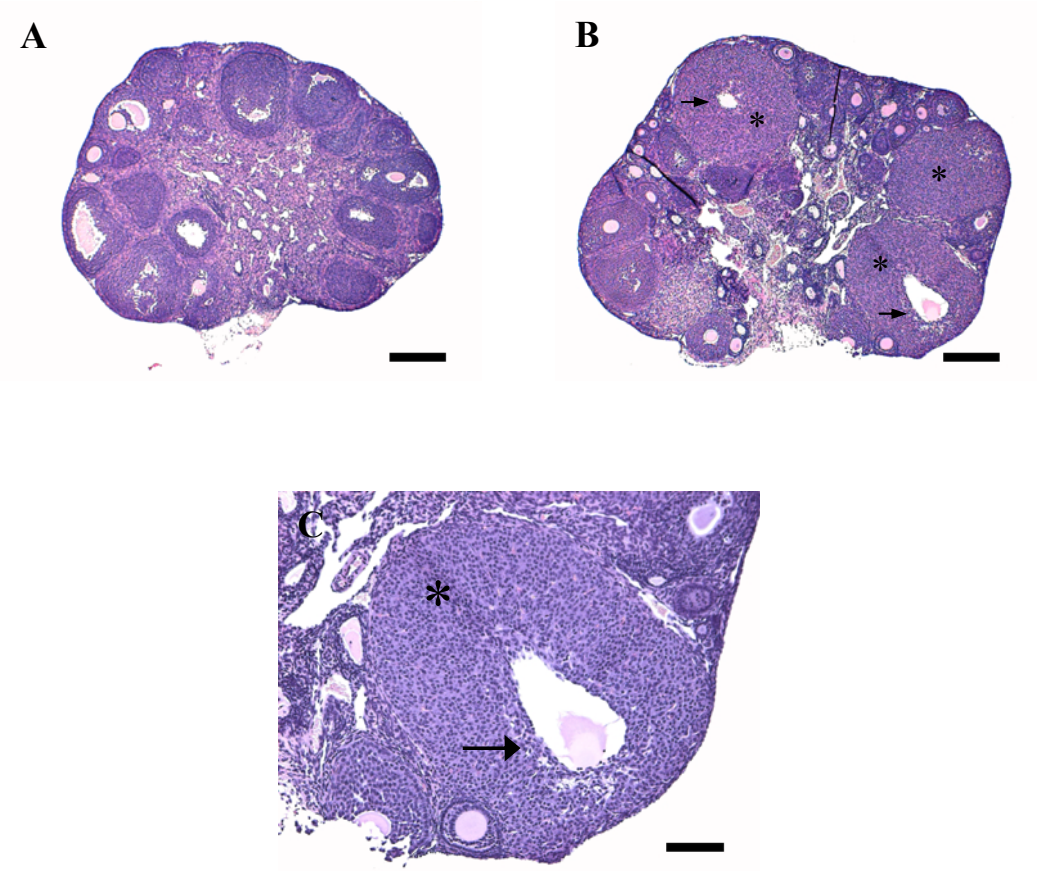


Fig. 3.5

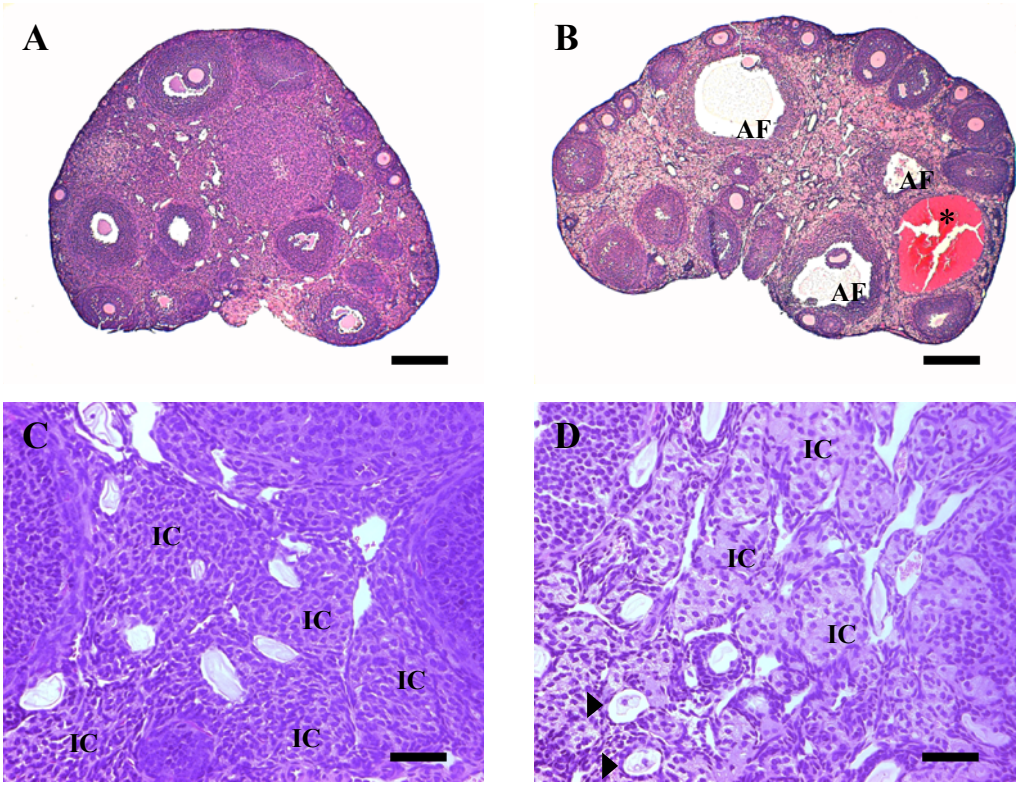
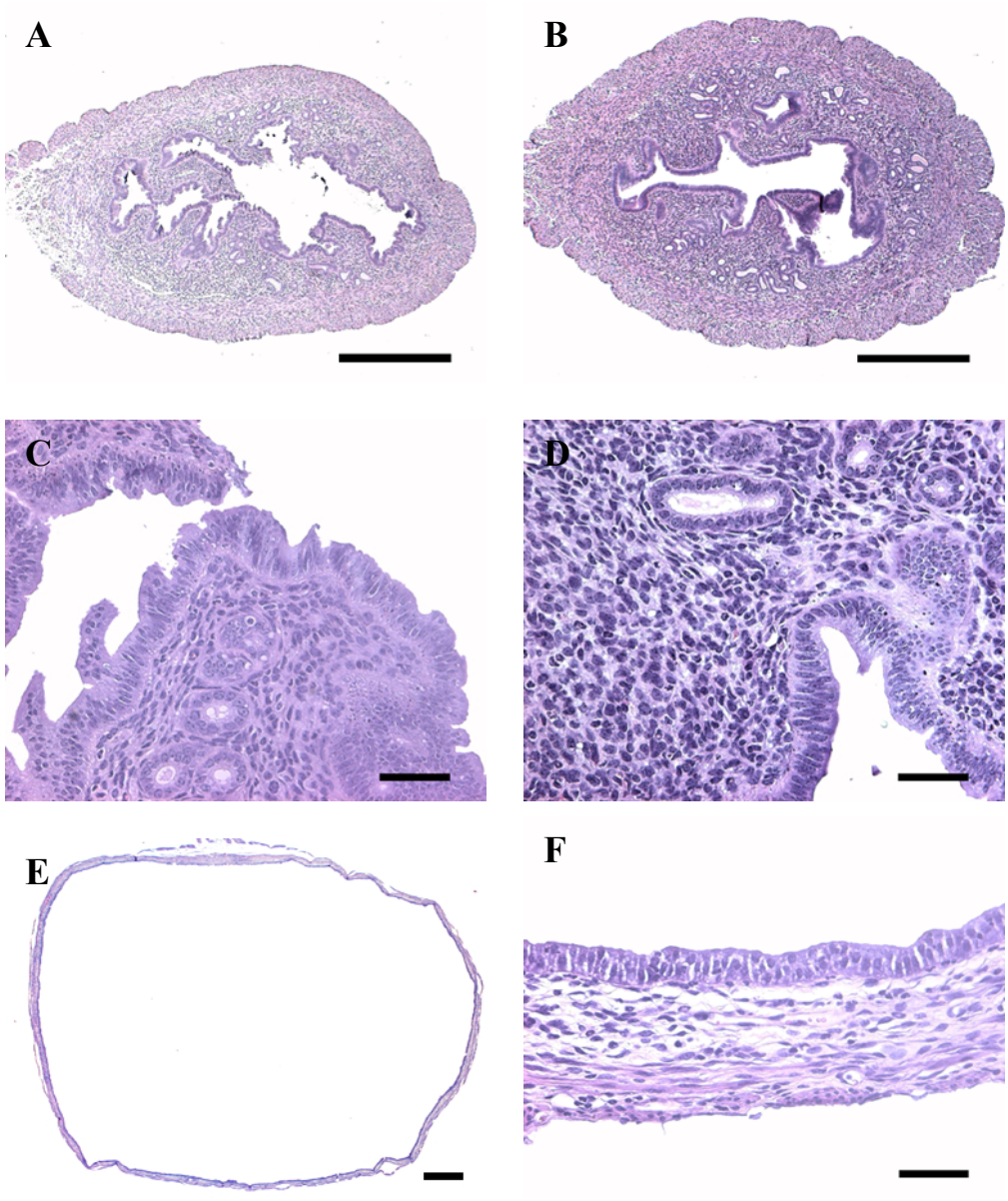


Fig. 3.6



CHAPTER 4

TIGHTLY REGULATED AND INDUCIBLE EXPRESSION OF A YOKED HORMONE-RECEPTOR COMPLEX IN HEK 293 CELLS[1]

[1] Meehan, T.P., D. Puett, and P. Narayan. Submitted to *Journal of Molecular Endocrinology*, 5/7/2003.

Abstract

We have previously reported the construction of a constitutively active luteinizing hormone receptor (LHR) by covalently linking a fused heterodimeric hormone to the extracellular domain of the G protein-coupled receptor. This yoked hormone-receptor complex (YHR) was found to produce high levels of cAMP in the absence of exogenous hormone. Stable lines expressing YHR were generated in HEK 293 cells to obtain lines with different expression levels; however, in a relatively short time of continued passage, it was found that YHR expression was greatly reduced. Herein, we describe the development of clonal lines of HEK 293 cells in which the expression of YHR is under the control of a tetracycline-regulated system. Characterization of clonal lines revealed tight control of YHR expression both by dose and time of incubation with doxycycline. These experiments demonstrated a good correlation between expression levels of the receptor and basal cAMP production. Moreover, the reduction in receptor expression following doxycycline removal revealed that YHR mRNA and protein decayed at similar rates, again suggesting a strong linkage between mRNA and protein levels. The controlled expression of YHR in this cell system will allow for a more detailed analysis of the signaling properties associated with constitutive receptor activation and may prove to be advantageous in developmental studies with transgenic animals.

Introduction

The luteinizing hormone receptor (LHR) is necessary for reproduction in mammals. Binding and signaling through LHR by luteinizing hormone (LH) or chorionic gonadotropin (CG) primarily leads to an increase in the intracellular levels of cAMP.

LHR activation is responsible for gonadal steroidogenesis, ovulation, and male sexual differentiation (Ascoli *et al.* 2002).

We have previously reported several studies that have utilized a fusion protein of the gonadotropin hormone, hCG, with its receptor, LHR, to examine various structure-function aspects of receptor activation in cell culture (Wu *et al.* 1996; Narayan *et al.* 2002). When transiently expressed in COS-7 or HEK 293 cells, YHR led to elevated levels of basal cAMP, suggesting that the receptor in this fusion protein was activated by the attached ligand. In this respect, YHR is similar to mutations in LHR that result in constitutively activated receptors. The latter have been implicated in familial male-limited precocious puberty (FMPP), a disorder characterized by increased prepubertal testosterone levels and Leydig cell hyperplasia (Themmen & Huhtaniemi 2000; Shenker 2002). More recently, we have demonstrated that YHR is functional *in vivo* and causes prepubertal increases in testosterone and estrogen in male and female transgenic mice, respectively, in addition to affecting testicular and ovarian development (Meehan *et al.* 2002).

Unlike LHR, quantitative measurements of YHR expression and receptor dynamics cannot be performed by standard ligand binding assays, since the tight association of the covalently attached ligand with the receptor inhibits binding of exogenous hCG (Wu *et al.* 1996). In order to avoid the inherent variability in expression observed with transient transfections, we established a stable cell line of YHR in HEK 293 cells that could be used as a standard cell line for further structure-function and signaling studies. However, it was found that YHR protein expression was readily lost

with continued passage of the cells. Although the cause of this loss is not clear, it was reasoned that chronic activation of the receptor may be responsible.

As a possible solution to this problem, we decided to develop a regulated cell culture system wherein expression of YHR could be controlled and maintained. Additionally, such a system would facilitate further studies on gonadal development in YHR transgenic mice. For this purpose, we chose to develop the tetracycline-regulated system of Gossen and Bujard (Gossen & Bujard 1992). The Tet-on system allows for tight control of gene expression in the presence of the tetracycline analog, doxycycline (Dox) (Gossen *et al.* 1995). Dox binds to the reverse tetracycline repressor protein (rTetR), which is fused to the VP16 activation domain of the herpes simplex virus. This activated fusion protein binds to the tetracycline response element (TRE) that, in turn, drives expression of the protein of interest.

Herein, we describe the establishment and characterization of cells expressing YHR (YHR-tet) in a controlled manner by utilization of the Tet-on system. In YHR-tet cell lines, expression was tightly controlled both by the dose and time of exposure to Dox. The level of YHR expression correlated well with the level of intracellular cAMP indicating that this will be a useful system for further studies on YHR-mediated signaling.

Materials and Methods

Establishment of stable lines in HEK 293 cells

Stable HEK 293 cell lines expressing YHR were established by transfection of YHR cDNA with Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's

instructions, followed by selection with 700 μg/ml Geneticin (Invitrogen) for 2 weeks. The surviving cells were maintained in DMEM (4.5 g/l glucose) containing 10% horse serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 400 μg/ml Geneticin. Expression of YHR was determined by Western blot analysis of total cell membranes and basal cAMP production as described below.

Cloning of pTk-hyg/pTRE-YHR

The pTk-hyg and pTRE plasmids (BD Biosciences Clontech, Palo Alto, CA) were combined by ligating the 1063 bp Ear I fragment from pTRE containing the TRE and multiple cloning site (MCS) into the unique Ear I site in pTk-hyg, thus generating a single plasmid which contains both the hygromycin (hyg) resistance gene and the inducible TRE promoter (Fig. 4.1). YHR cDNA was cloned into the BamHI site of the MCS. YHR consists of the β-subunit of human CG (hCG) linked to the common human α-subunit via its C-terminal peptide (CTP). This complex, in turn, is linked to the rat LHR via an additional CTP and a Factor Xa cleavage site (Fig. 4.1).

Establishment of double stable 293 Tet-on cell lines

HEK 293 Tet-on cells were obtained from BD Biosciences Clontech and grown in α-MEM containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B, and 100 μg/ml Geneticin. Cells at 70% confluency were transfected with 10 μg of either pTk-hyg/pTRE or pTk-hyg/pTRE-YHR utilizing Lipofectamine. Following transfection, cells were selected for antibiotic resistance by incubation with 200 μg/ml hygromycin. Twenty colonies from each transfection were

isolated using cloning cylinders, and clonal cell lines were established that were tested for the expression of YHR in the presence and absence of 2 μg/ml Dox (BD Biosciences Clontech) by Western blot analysis and determination of basal cAMP production. Cell lines established with colonies from the pTk-hyg/pTRE transfection (MT) were utilized as controls.

Total cell membrane preparation and Western blot analysis

Total cell membranes were prepared by homogenization in 50 mM Tris-HCl, pH 7.5, containing 250 mM sucrose and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM *N*-ethylmaleimide, and 2 mM EDTA). A low speed (1000x*g*) centrifugation removed cellular debris and nuclei, and the supernatant was subjected to a high speed spin (48000x*g*) for 45 min to pellet total cellular membranes. The membrane fraction was resuspended in 50 mM Tris-HCl, pH 7.5, containing protease inhibitors, and protein content was determined by BCA assay (Pierce, Rockford, IL). Total cell membrane proteins (10 μg) were resolved on a 10% SDS PAGE gel under reducing conditions and transferred to Immobilon-P (Millipore, Bedford, MA). Following blocking for 2 h (10 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.2% Tween-20, and 3% bovine serum albumin), the blot was incubated for 1 h with a 1/1000 dilution of a polyclonal antibody to the CTP of hCGβ (kindly provided by Dr. Vernon Stevens, Ohio State University). After incubation with an anti-rabbit HRP (Amersham Biosciences, Piscataway, NJ), the blot was developed with SuperSignal ECL (Pierce).

cAMP assay

Cells were plated in a 12-well plate and, after the appropriate treatment with Dox, washed twice in Waymouth/0.1% BSA, followed by incubation for 45 min at 37 ^{o}C in Waymouth/BSA/0.8 mM isobutylmethylxanthine to mimic the stimulation time normally utilized for LHR signaling assays. Intracellular cAMP was collected by treating the cells with 100% ethanol overnight at -20 ^{o}C and dried in a speedvac. cAMP levels were determined by RIA (Perkin Elmer, Boston, MA).

Northern blot analysis

mRNA was prepared from cells using the Fast Track 2.0 kit (Invitrogen), followed by resolution of 4 μg on a 1.2% agarose gel containing 6% formaldehyde. mRNA was transferred to Zeta probe nylon membrane (Bio-Rad, Hercules, CA) by capillary action. Following prehybridization (50% formamide, 5x Denhardts, 5x SSPE, 1% SDS, and 100 μg/ml salmon sperm DNA) at 42 ^{o}C for 4 h, the membrane was probed overnight at 42 ^{o}C in the above buffer with 5 x 10^{6} CPM of a ^{32}P probe made to either the cDNA of YHR or human actin β-subunit by random primed synthesis (Roche, Indianapolis, IN). Following washing (30 min at room temperature in 2x SSC, 0.5 % SDS and 30 min at 50 ^{o}C in 0.1x SSC, 0.1% SDS), the membrane was exposed to film for autoradiography.

Densitometry to determine relative expression levels

In order to determine the relative protein or mRNA expression levels, densitometry was performed on the X-ray film from Western blots and Northern blots utilizing a Fluor-S™ MultiImager (BioRad). Scans of X-ray film were analyzed with Quantity One® software

(Biorad), and the intensity of individual bands was determined and expressed as total optical density (OD).

Curve fitting

Graphs were produced by Prism 3.0 (GraphPad Software, Inc., San Diego, CA). Linear regressions were performed utilizing the least squares fit, and curve fitting for decay experiments was determined using the equation for exponential decay.

Results

Loss of YHR in stable lines

Determination of relative expression levels in YHR-expressing cells during transient transfection is difficult since YHR does not bind exogenously added hormone (Wu *et al.* 1996). In order to normalize the expression levels so that the signaling properties could be better studied, stable lines expressing YHR were established in HEK 293 cells. During the time course of experiments measuring the signaling of YHR, it was determined by Western blot analysis that YHR expression in the stable lines decreased starting in early passages and were undetectable by passage 11 (Fig. 4.2A). The basal levels of cAMP production over mock-transfected levels also decreased with passage number (Fig. 4.2B). Even though the receptor is not detected by Western blot at passage 13, the small increase in cAMP over MT levels suggests that a small amount of YHR is still present.

Generation of tetracycline-responsive clonal lines

In order to establish a cell line with a more constant level of YHR expression, tet-responsive cell lines were generated. After initial attempts to generate stably transfected lines failed due to the necessity of co-transfection of the Hyg resistant plasmid and the TRE containing plasmid into HEK 293 Tet-on cells, the pTRE and pTK-Hyg plasmids were combined (Fig. 4.1), and YHR was cloned into the multiple cloning site. Upon transfection with the pTK-Hyg/pTRE-YHR plasmid, several clonal lines were established. Screening of these lines by Western blots in the presence and absence of Dox demonstrated three different types of clones (Fig. 4.3A). YHRtet-14 showed no expression of YHR; YHRtet-11 and -20 had high basal levels of expression in the absence of Dox; and YHRtet-3, -10, and -16 exhibited no basal expression of YHR but high levels could be induced in the presence of Dox. cAMP production by the clones (Fig. 4.3B) correlated with the expression levels of YHR on the Western blots. Due to its better viability over other lines, YHRtet-16 was chosen for further characterization.

YHR expression responds to both the dose and time of Dox stimulation

In order to characterize the responsiveness of YHR expression to Dox, YHRtet-16 from passage 9 was incubated with varying concentrations of Dox from 0-4 μg/ml for 48 h. Western blot analysis of total membrane preparations revealed that the expression of YHR is detectable with 0.1 μg/ml and maximal with 2 μg/ml Dox (Fig. 4.4A). The production of basal cAMP shows a similar pattern of increase (Fig. 4.4B). The intensity of the YHR bands on the Western blot was quantified by densitometry, and when

graphed against cAMP production, a linear correlation (r^2=0.89) was obtained (Fig. 4.4C).

A time course of Dox stimulation was performed in order to determine the time necessary for maximum YHR expression. YHRtet-16 cells from passage 10 were incubated with 2 μg/ml Dox for 0-48 h. Expression was measured as early as 4 h after Dox addition and was maximal by 24 h (Fig. 4.5A). cAMP production showed a slight increase for the 4 and 8 h time points and was much higher and nearly constant for 18-24 h (Fig. 4.5B). The level of YHR expression estimated from Western blot analysis and cAMP production show a linear correlation (r^2=0.89, Fig. 4.5C). Changing the media after 18 h to replenish the Dox did not have an effect of YHR expression compared to cells where the media was not changed (data not shown), demonstrating that the plateau in YHR expression does not result from a decrease in the concentration of Dox.

Decrease in YHR expression following Dox removal

In order to follow the deinduction of YHR expression, YHRtet-16 cells were incubated for 24 h with 2 μg/ml Dox, then washed to remove any excess Dox, and incubated for various times followed by mRNA isolation. Northern blot analysis was performed to determine YHR mRNA levels (Fig. 4.6A). YHR mRNA levels, normalized to the level of the control β-actin signal, decreased slowly during the first 4 h without Dox, then declined to 40% at 24 h. Similarly, Western blot analysis (Fig. 4.6B) of similarly treated cells showed that YHR protein expression remained constant for the first 4 h, then decreased to 22% at 24 h. These results demonstrate that YHR expression levels in HEK 293 cells are highly dependent on the presence of mRNA (Fig. 4.6C), indicating the rapid

degradation of the constitutively active receptor. These results, however, do not allow for an accurate determination of the turnover rate of YHR because of the possibility of *de novo* protein production from the mRNA still present after 24 h.

Discussion

The unexplained loss of YHR in stably transfected HEK 293 cells prompted us to establish the regulated expression of YHR under the control of a tetracyline-responsive promoter. Characterization of one of the YHR-tet clonal lines revealed the rapid, controlled, and sustained induction of YHR expression by the Tet-on system following the addition of Dox. The decrease in YHR expression following Dox removal was slow with 40% of the mRNA still remaining after 24 h.

The dose response and time course experiments established not only the controlled expression of YHR, but also demonstrated that cAMP production by YHR is directly proportional to the level of receptor expression. Previously, this correlation could not be determined (Wu *et al.* 1996) because of the variability of expression levels in transient transfections. Additionally, these experiments utilized YHRtet-16 cells from later passages, indicating that expression of YHR is consistent over time unlike the HEK 293 stable line for YHR which lost receptor expression when continually passaged.

The protein levels as determined by Western blot analysis closely mimic the levels of mRNA. These results suggest that the turnover rate of the constitutively active YHR is high, which is consistent with previous studies of mutation-induced constitutively active LHR (Min *et al.* 1998; Bradbury & Menon 1999; Min & Ascoli 2000). In the studies with constitutively active LHR, the rates of LHR internalization and down

regulation were determined by following the fate of ^{125}I-hCG bound to the receptor on the surface of the cell. Again, this type of study is not possible in the case of YHR since it does not bind exogenous hCG (Wu *et al.* 1996).

An accurate assessment of the degradation of YHR, however, could not be determined utilizing the Tet-on system because of the slow decline in the mRNA levels following removal of Dox. These results are consistent with a study on the effectiveness of different tetracycline analogs which showed that Dox had the longest delay of all of the analogs tested in returning the system to pre-stimulation levels (A-Mohammadi *et al.* 1997). However, since the Tet-on system only responds to Dox (Gossen *et al.* 1995), no other analogs could be utilized to control YHR expression. Nonetheless, this system is good for turning on and controlling expression but not necessarily the best for following protein decay.

In summary, tetracycline-regulated expression of YHR has been established in HEK 293 cells, and we showed that the levels of expression are tightly controlled by the dose and time of Dox exposure. These results establish the ability to express YHR in a regulated manner which can be further utilized to study the signaling pathways activated by varying levels of constitutive receptor activation in cell culture. The system will also be useful in transgenic mice to determine the reproductive effects of YHR activation at various times in development.

Acknowledgements

This research was supported by the University of Georgia Research Foundation, Inc. and by NIH Research Grant DK33973.

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Figure legends

Fig. 4.1. Schematic diagram of pTk-hyg/pTRE-YHR. A single plasmid was generated containing both the hygromycin resistance gene and the tet-responsive element. YHR, cloned into the multiple cloning site, consists of the hCG β-subunit covalently linked via its CTP to the human α-subunit which is, in turn, linked to rLHR via an additional CTP and a factor Xa cleavage sequence.

Fig. 4.2. Loss of YHR expression from stable lines in HEK 293 cells. The expression of YHR was followed in an HEK 293 stable line between passages 3-13 by : (A) Western

blot analysis on 10 μg of total membrane protein resolved on a 10% SDS PAGE gel under reducing conditions (sizes of molecular mass standards are shown), and (B) basal cAMP production on similar passages of cells. Data shown are mean ± ranges of a representative experiment. MT, mock-transfected.

Fig. 4.3. Establishment of tet-responsive lines expressing YHR. Following transfection of pTK-hyg/pTRE-YHR into HEK 293 Tet-on cells and selection with 200 μg/ml hygromycin, YHR-expressing clones were characterized by Western blot analysis (A) and basal cAMP production (B). Cells from each clone were treated with or without 2 μg/ml Dox for 48 h, and the clones presented here are representative of all those tested. Sizes of molecular mass standards are indicated; basal cAMP production is expressed as fold-increase over mock-transfected basal; and data are mean ± ranges of two independent experiments.

Fig. 4.4. Dose response of Dox on YHR expressing cell lines. YHRtet-16 cells were incubated with increasing concentrations of Dox from 0-4 μg/ml for 48 h followed by (A) Western blot analysis and (B) measurement of basal cAMP production (cAMP values were expressed as fold over "no Dox" basal). Data are mean ± ranges of two independent experiments. (C) Densitometry was performed on the X-ray film from the Western blot, and YHR expression was graphed verses cAMP production using linear least squares analysis.

Fig. 4.5. Time course of Dox stimulation of YHR expression. YHRtet-16 cells were incubated with 2 μg/ml Dox for 0-48 h. Western blot (A) and cAMP production (B) assays were performed to assess the level of YHR expression. Data are mean ± ranges of two independent experiments. (C) Densitometry was performed on the X-ray film from the Western blot, and YHR expression was graphed verses cAMP production and fitted by a linear least squares analysis.

Fig. 4.6. Decrease in YHR expression following removal of Dox. YHRtet-16 cells were incubated with 2 μg/ml for 24 h. Following removal of Dox and washing, cells were incubated for 0-24 h in the absence of Dox. Northern blot (A) and Western blot (B) analyses were performed to assess YHR expression. A β-actin probe was utilized as a control for mRNA levels on the Northern blot. RNA size and molecular mass standards are shown on Northern and Western blots, respectively. (C) Degradation of YHR mRNA and protein. The percentage of YHR mRNA and protein compared to 0 h was calculated from the densitometric quantification of the Northern and Western blots, with the signal from actin being used to normalize the values for the Northern blot. Curves fitted to exponential decay equation: r^2=0.87, RNA; r^2=0.91, protein.

Fig. 4.1

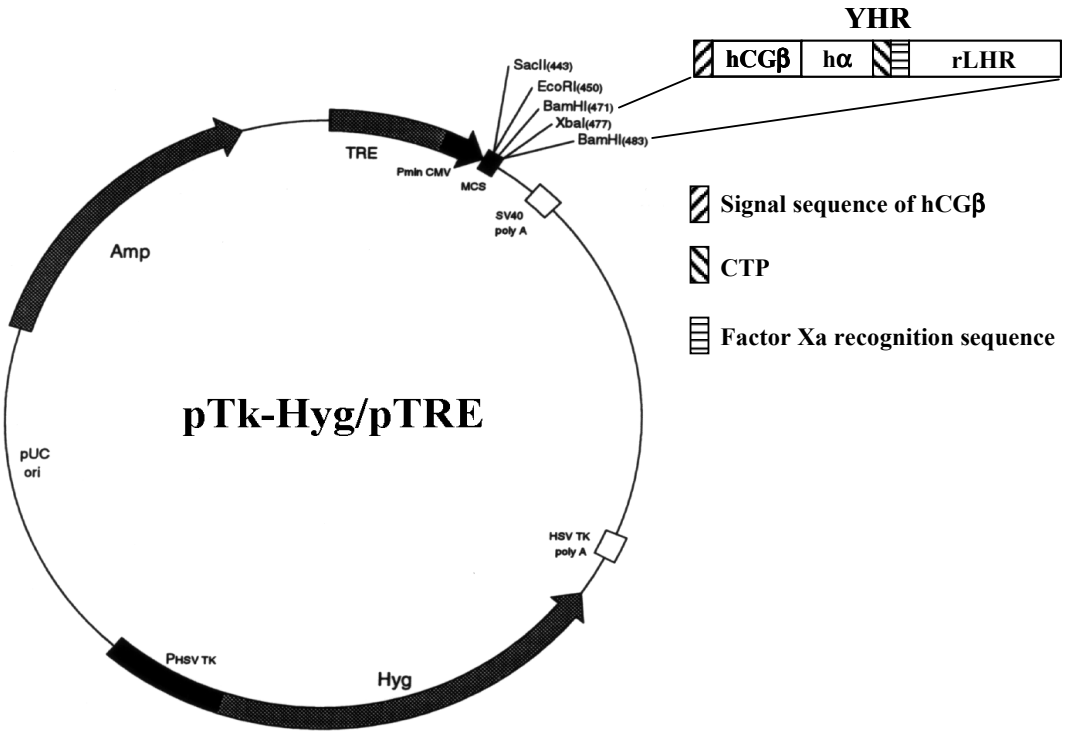
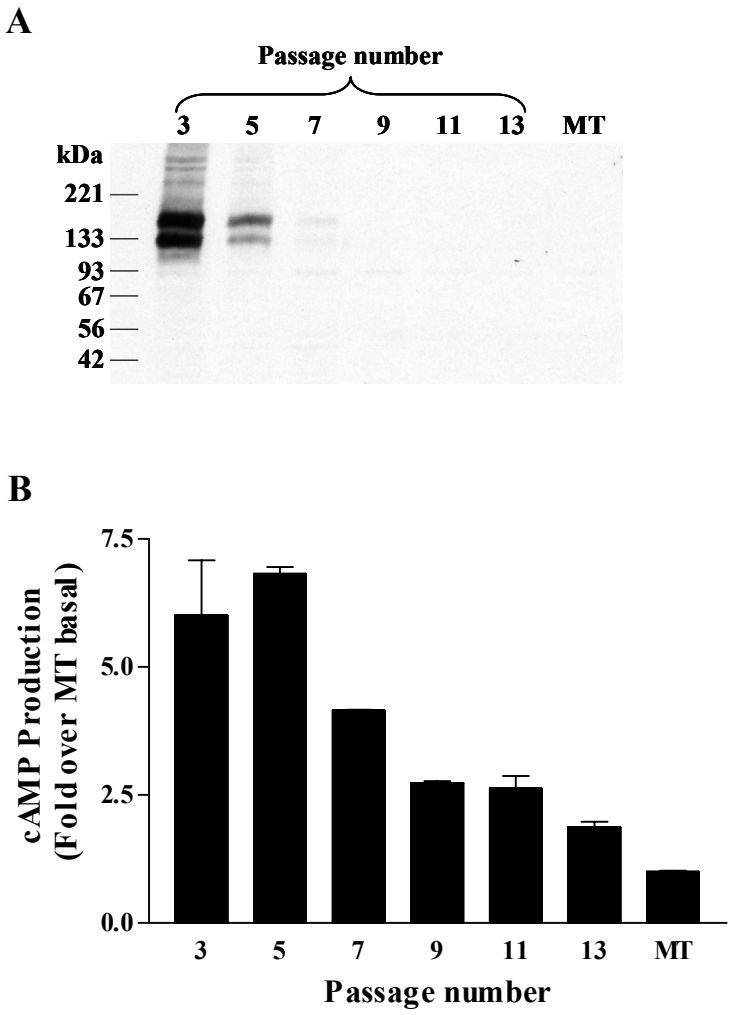

YHR
hCGβ
hα
rLHR
SacII(443)
EcoRI(450)
BamHI(471)
XbaI(477)
BamHI(483)
TRE
Pmin CMV
MCS
SV40
poly A
Amp
pTk-Hyg/pTRE
pUC
ori
HSV TK
poly A
PHSV TK
Hyg
Signal sequence of hCGβ
CTP
Factor Xa recognition sequence

Fig. 4.2


A
Passage number
3
5
7
9
11
13
MT
kDa
221
133
93
67
56
42
B
cAMP Production
(Fold over MT basal)
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5.0
2.5
0.0
3
5
7
9
11
13
MT
Passage number

Fig. 4.3

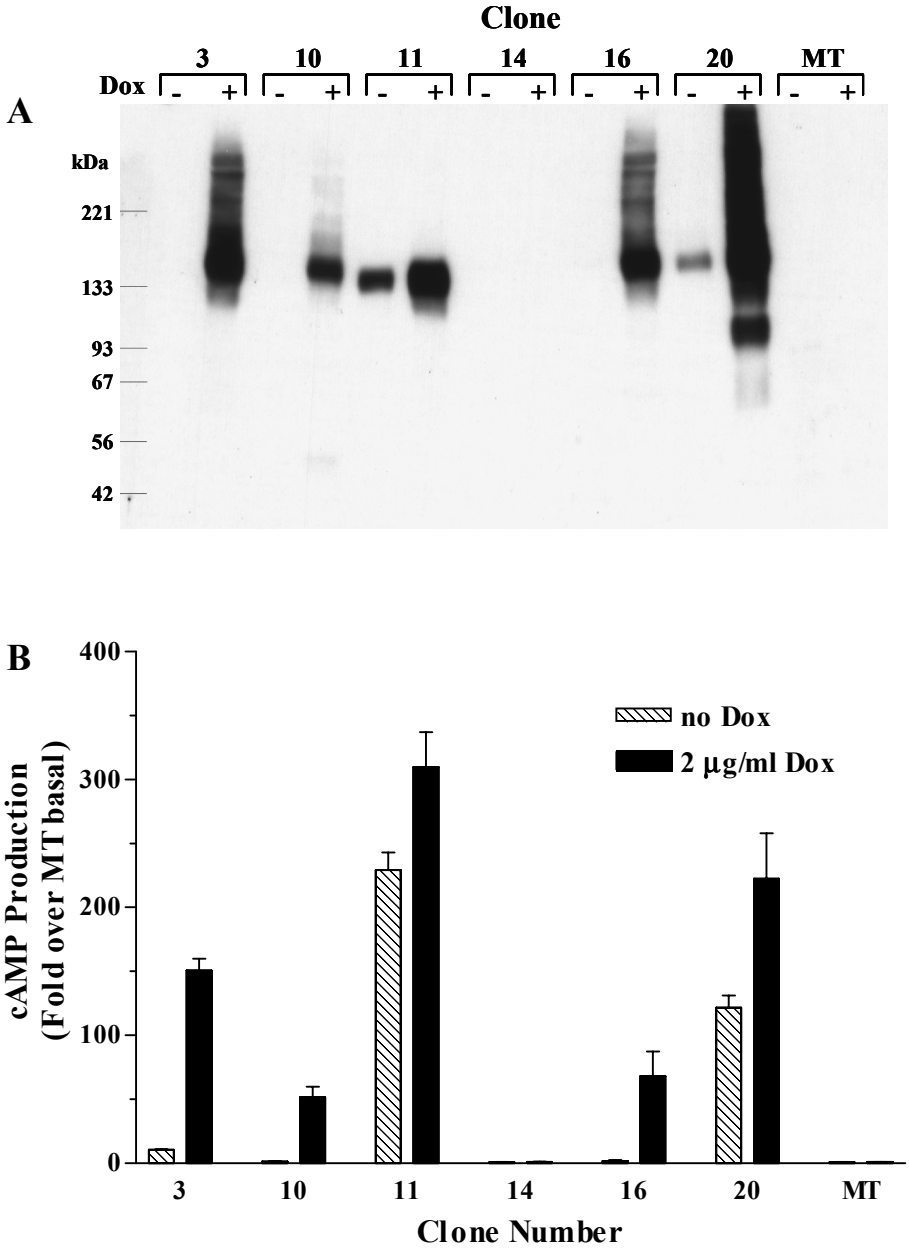

A
Clone
3
10
11
14
16
20
MT
Dox
- + - + - + - + - + - + - +
kDa
221
133
93
67
56
42
B
400
300
200
100
0
cAMP Production
(Fold over MT basal)
no Dox
2 µg/ml Dox
3
10
11
14
16
20
MT
Clone Number

Fig. 4.4

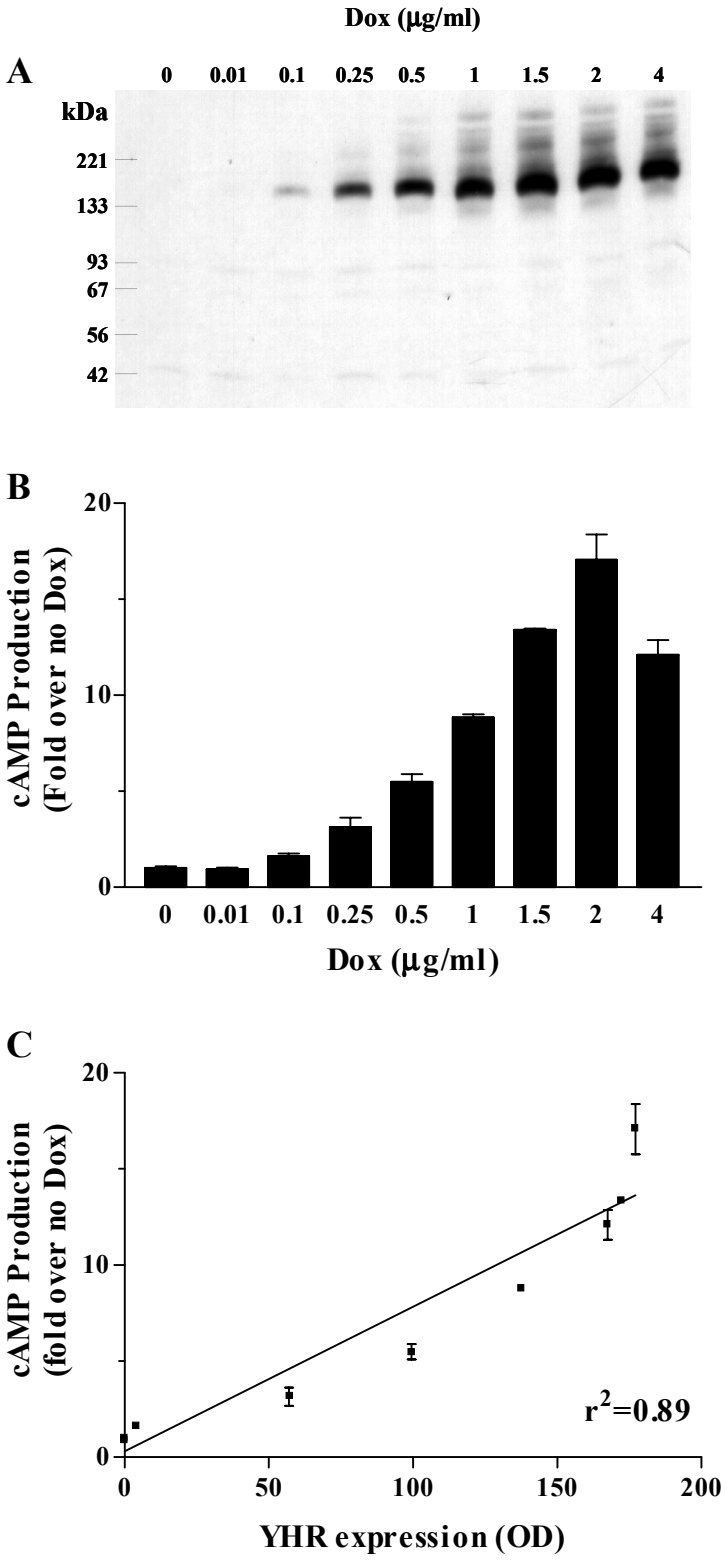

A
Dox (µg/ml)
0 0.01 0.1 0.25 0.5 1 1.5 2 4
kDa
221
133
93
67
56
42
B
cAMP Production
(Fold over no Dox)
20
10
0
0 0.01 0.1 0.25 0.5 1 1.5 2 4
Dox (µg/ml)
C
cAMP Production
(fold over no Dox)
20
10
0
0 50 100 150 200
YHR expression (OD)
r2=0.89

Fig. 4.5

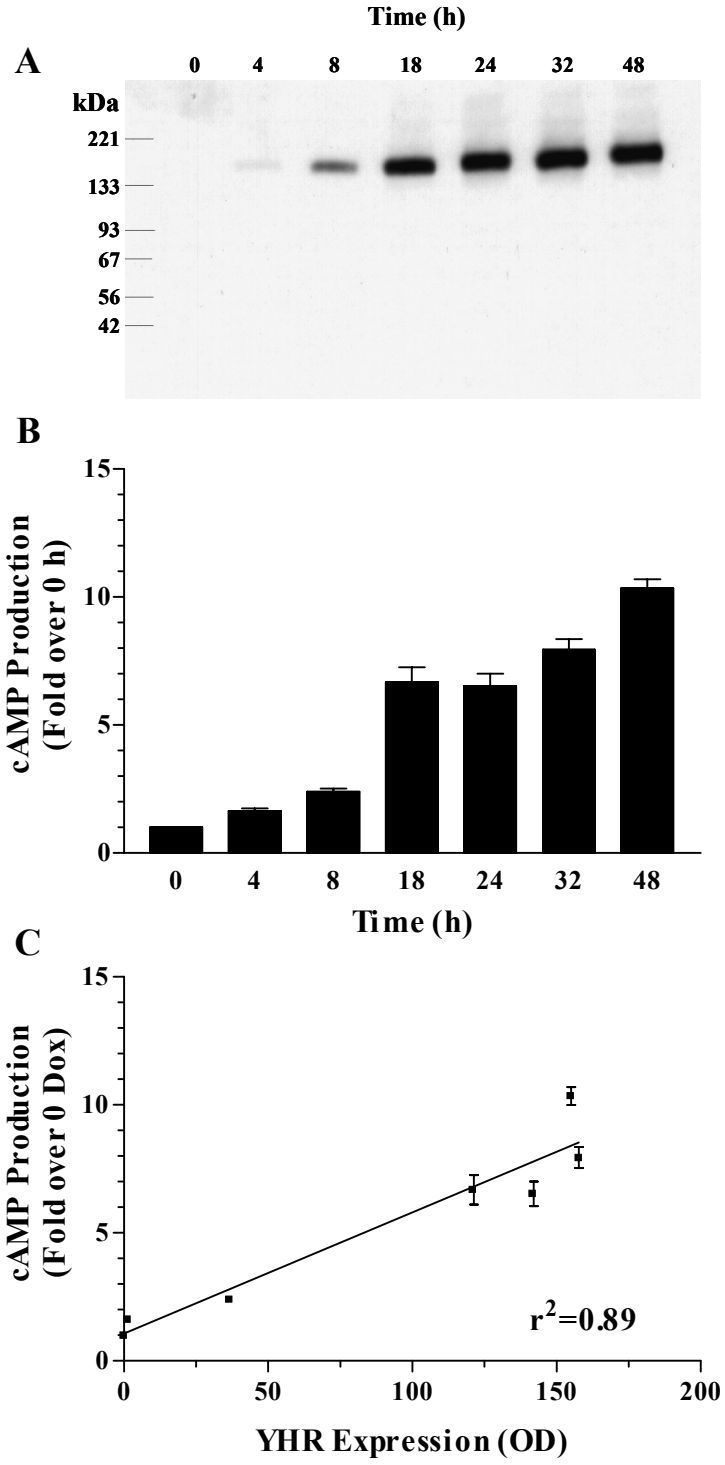
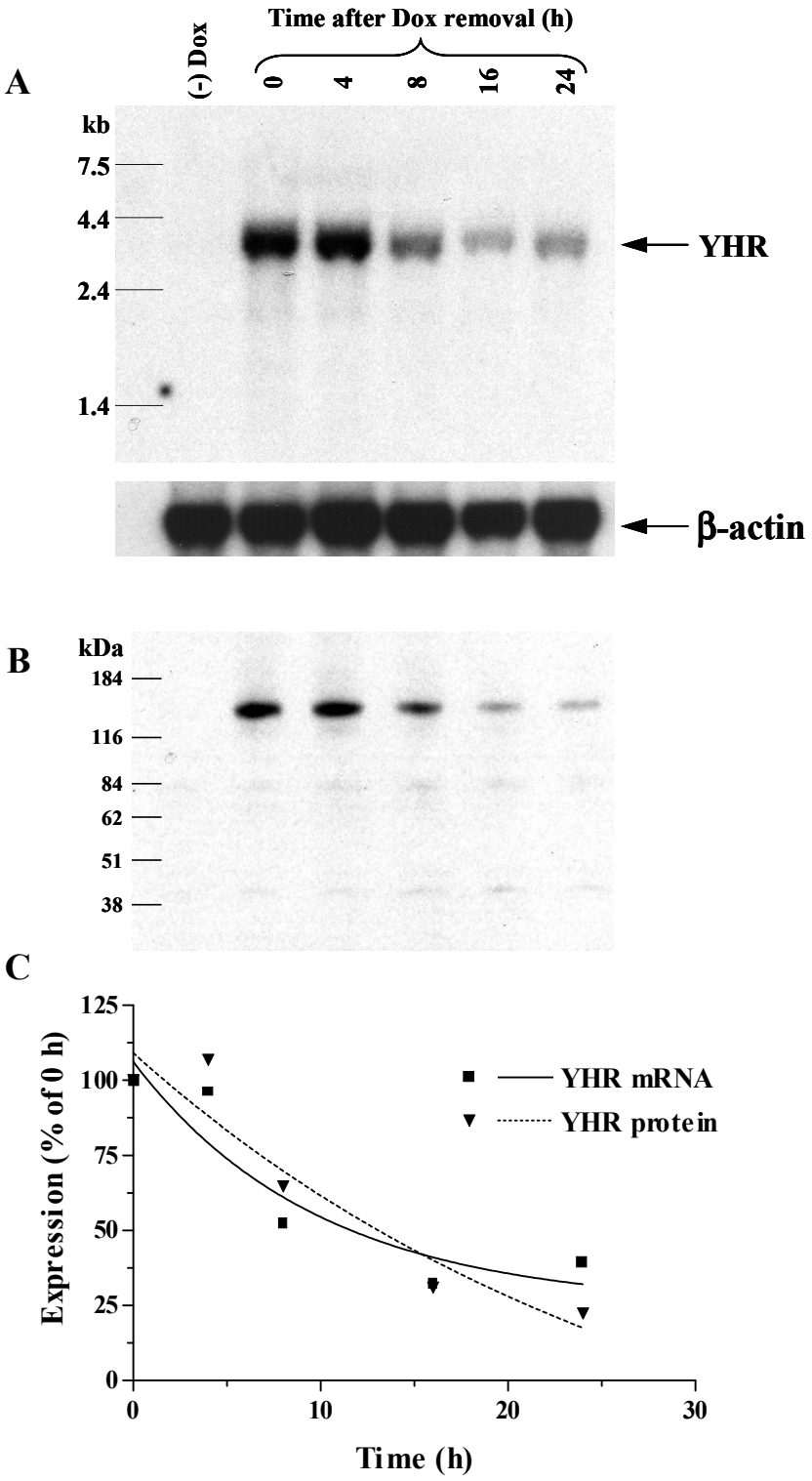

A
Time (h)
0
4
8
18
24
32
48
kDa
221
133
93
67
56
42
B
cAMP Production
(Fold over 0 h)
15
10
5
0
0
4
8
18
24
32
48
Time (h)
C
cAMP Production
(Fold over 0 Dox)
15
10
5
0
r2=0.89
0
50
100
150
200
YHR Expression (OD)

Fig. 4.6


A
Time after Dox removal (h)
(-) Dox
0
4
8
16
24
kb
7.5
4.4
2.4
1.4
YHR
β-actin
B
kDa
184
116
84
62
51
38
C
Expression (% of 0 h)
125
100
75
50
25
0
0
10
20
30
Time (h)
YHR mRNA
YHR protein

CHAPTER 5

CONCLUSIONS

Mouse models have been used extensively to elucidate the roles of gonadotropins and their receptors in mammalian reproduction and physiology. Additionally, naturally occurring mutations in humans have been informative. The human phenotype from these mutations and the corresponding mouse model, however, do not always correlate, demonstrating some of the differences in reproduction in humans and mice.

Towards the objective of creating an *in vivo* model for the actions of constitutively active luteinizing hormone receptors, two activated receptors, D556H LHR and YHR, were cloned under the control of the mouse inhibin α-subunit promoter to target expression to the gonads. As demonstrated in Chapter 2, D556H LHR founder mice were infertile while several transgenic lines were established from YHR founder mice. Interestingly, both D556H LHR and YHR are constitutively active for the cAMP and IP pathways, thereby not elucidating a clear reason for the difference in phenotype. It is important to note, however, that YHR founder mice were subfertile with the line showing the most severe phenotype being derived from a male that only produced a few litters. YHR expressing lines were assayed for the expression of the receptor and two lines were chosen for analysis of the reproductive phenotype. Both lines exhibited a similar phenotype and the data from one line was presented.

Male YHR$^+$ mice (Chapter 2) have prepubertal increases in serum and testicular testosterone levels and seminal vesicle development accompanied by decreases in testicular development and serum gonadotropin hormone levels. The mouse model with YHR has similarities and differences with humans possessing constitutively active LHR. The increase in testosterone is consistent with that found in FMPP patients, but humans have increased testicular volume and Leydig cell hyperplasia as opposed to the decreased testicular development in YHR$^+$ mice. The most likely explanation for these differences occurs in the timing of the ontogeny of the HPG axis. In humans, the HPG axis is quiescent from soon after birth until the beginning of puberty, whereas, in male mice, the axis is active continuously from just prior to birth. This difference is clearly demonstrated in the reduction in gonadotropin levels in YHR$^+$ mice which in humans with FMPP remain prepubertal and undetectable. The reduction in FSH levels in YHR$^+$ mice is the most likely reason for the reduction in testicular development analogous to knockout mouse models of FSH and FSH receptor which contain similar defects. In humans, the effects of constitutively active LHR on the HPG axis and testicular development after puberty have not been analyzed, and it would be interesting to determine if the effects would parallel the mouse model.

Female carriers of constitutively active LHR in families with FMPP patients surprisingly do not develop a distinct phenotype even though LHR plays a critical role in follicle development and ovulation. In contrast, female YHR$^+$ mice undergo precocious puberty with increased steroidogenesis resulting in accelerated ovarian and uterine development leading to the development of follicular cysts and premature ovarian aging (Chapter 3). The most commonly cited explanation for the lack of a phenotype in

females carrying a constitutively active LHR is the understanding that LHR expression does not occur in the ovary until puberty. While this is a plausible explanation, it is also important to note that the mutations that have been studied in females possess only a low level of constitutive activity, whereas YHR and some of the other naturally occurring mutations are more highly active when expressed in cell culture. Also, the mutations which lead to a more severe case of FMPP usually occur sporadically and have not been detected in females, thus indicating that these mutations may not be permissive to germline transmission or that females with these mutations are infertile and have not been properly diagnosed.

Establishment of a tetracycline-regulated system in transgenic mice will allow for the investigation of the role that expression levels have in the severity of the phenotype, as well as determining the role of YHR activation at various times in development. Prior to utilizing a tetracycline-regulated system in mice, controlled expression of constitutively active LHR was demonstrated in HEK 293 cells (Chapter 4). The expression of YHR was controlled by the dose and time of doxycycline induction in clonal lines. The cell lines also demonstrate the correlation between expression of YHR and the levels of basal cAMP production which previously could not be determined. In addition to utilization in mice, the controlled expression of YHR will allow for a more detailed analysis of the signaling properties that are activated by this unique constitutively active receptor.

In summary, this study is the first reported expression of constitutively active LHR in transgenic mice. This mouse model has shed light on pathologies from early and inappropriate activation of LHR and will be useful in future studies on downstream signal

transduction pathways that cause these pathologies. This model, as well as other mouse models of gonadotropin function, will continue to be utilized to elucidate the functions of this important class of molecules in normal reproduction and in disorders found in humans.