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Transgenic Models for Exploring Gonadotropin Biology in the Male

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Unraveling the selective actions of the two pituitary-derived gonadotropins FSH and LH in vivo is complicated by their very close structural and functional similarities, and their coordinate regulation. Both gonadotrophins are composed of a common α-subunit and distinct β-subunits, which are simultaneously secreted as heterodimers in response to a single trophic hormone GnRH. Functionally, gonadotrophins interact with related receptors and share the same ultimate cellular site in the testis, the Sertoli cell with receptors for both FSH and the androgens synthesized in response to LH. This article will review the strategies to overcome these intimate connections and discuss key findings from transgenic models developed to study gonadotropin actions in the male.

Key Words: Transgenic; FSH; LH; mouse; testis; male.

Introduction

The application of transgenic technology has created significant advances in understanding gonadotropin regulation and function in the male. The ability to transfer individual genes through microinjection into the nucleus of fertilized mouse eggs has been developed to target expression of genes to specific cells in the male reproductive system and the hypothalamopituitary testicular axis. This is achieved by employing promoters, which direct gene expression to the target cells, together with judicious selection of appropriate background strains. Transgenic technology has allowed creation of customized mouse models to study the loss or gain of selected gonadotrophin activities in vivo. These transgenic models provided a precision and selectivity that was previously difficult to achieve in the classical hormone ablation–replacement strategies such as hypophysectomy or blockade of GnRH, LH, and/or FSH using immunological or pharmacological tools. Transgenic models circumvent the non-selectivity of hypophysectomy, which removes all pituitary hormones rather than just gonadotropins, as well as providing more stable, reliably complete, and sustainable effects than antibody or drug-mediated antagonists. Transgenic strategies create genetic models with specific, durable, and complete gonadotrophin deficiency in mice, a species with the advantage of having the most extensive genomic information available of any non-human species. Additionally, although recombinant gonadotropins are now available for experimental use and an advance over hormones purified from biological sources, transgenic hormone expression in vivo offers more durable, consistent, and non-immunogenic hormone delivery. However, the traditional problem of physiological hormone delivery rather than pharmacological dosing remains a challenge for transgene expression.

A key issue in the use of transgenic technology is the selection of the most appropriate gene promoters to direct expression of genes to specific cells. For gonadotrophin research, a variety of promoters have been used to target pituitary gonadotropes, to direct pituitary-independent expression of gonadotrophins, or to express gonadotrophin receptor in the somatic Sertoli cells in the testis. The cell chosen for hormone expression may be a convenient non-reproductive cell type and ectopic transgene expression may tolerable if other expression sites are structurally and functionally remote from the targeted cell. On the other hand, the specificity of cellular expression of hormone receptors must be strict and directed to the physiologically correct reproductive system target cell. In addition to the paramount consideration of specificity of cellular expression, the strength, time of onset, and maintenance of transgene expression during development and maturity are key issues. More recent developments of transgenic technology include the use of constructs featuring switchable promoters that can be activated in vivo by a chemical signal or to express Cre recombinase in specific cells destined to undergo excision of targeted gene segments at sites marked by bracketing LoxP sites.

The choice of mouse genetic background for transgenic gene expression can also be important. In keeping with traditional ablation–replacement strategy, transgene overexpression has greatest utility in a mouse line where it restores the function of a deficient gene. In other circumstances, transgene overexpression on a normal mouse background may only demonstrate pharmacological effects or no phenotype, regardless of the correct cellular expression.

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Isolated Disruption of Gonadotrophin Activity

The *hpg* mouse is a valuable gonadotrophin-deficient model first identified as a naturally occurring mutant mouse with persistence of immature reproductive function *(1)* later shown to be due to a major deletion in the GnRH gene *(2)*. This archetypal model of complete gonadotropin deficiency has mice that are functionally deficient in FSH and LH (and gonadal steroids), while retaining all other pituitary hormones leading to persistent reproductive immaturity *(1)* from birth onwards *(3)*. Confirmation that the *hpg* phenotype was solely due to the GnRH gene defect was provided by the rescue of normal testicular development and fertility by transgenic GnRH *(4)* as well as hypothalamic transplantation of GnRH-containing neurons *(5)* or GnRH-secreting tumor cells *(6)*, administration of GnRH *(7)*, hCG *(8)*, or androgens *(9)*. Functionally, *hpg* testis remains infantile but hormone-responsive with immature Sertoli cells and spermatogenesis arrested at the pachytene stage of meiosis *(1,9)* equivalent to the androgen-insensitive *tfm* testis with a complete congenital lack of androgen action *(10,11)*. Consequently, the dormant *hpg* testis provides a valuable experimental platform, featuring a null reproductive hormone background but with preserved hormone responsiveness, upon which transgenic reconstitution experiments can be superimposed.

Variations on this theme of gonadotrophin-deficient mouse models have been produced by the targeted ablation of pituitary gonadotrophs. A mouse line generated by the complete germline deletion of the glycoprotein hormone α-subunit by homologous recombination produced mice deficient in not only LH and FSH, but also TSH, as all three heterodimeric glycoproteins share the same α subunit. Hence, although the male mice generated were hypogonadal, with comparable phenotype to *hpg* mice due to the absence of FSH and LH, they were also hypothyroid due to the lack of TSH *(12)*. The additional features due to concurrent loss of TSH complicate any interpretation of disrupted gonadotrophins actions on the testis.

A more selective approach has been to employ a toxic transgene to create a selective hormone deficiency state while circumventing the more time-consuming and technically demanding gene knockout approaches using homologous recombination. In one toxic transgene model, 313 bp of the glycoprotein α subunit gene promoter directed expression of diphtheria toxin A chain to gonadotrophs but not thyrotrophs, despite the fact that the α subunit is expressed in both cell lines. Fortuitously, this eliminated gonadotrophins while preserving normal TSH and thyroid function *(13)*. Transgenic males were infertile and exhibited immature testes similar to the gonadotropin-deficient *hpg* mouse. More recent studies showed that toxin A–driven ablation of gonadotrophs during embryonic development reduced prolactin but not growth hormone gene and protein expression, indicating gonadotrophs have a selective paracrine effect upon lactotroph development and prolactin *(14,15)*.

Another toxic transgene approach used an inducible ablation strategy based on a transgenic FSH β-subunit gene promoter to target thymidine kinase (TK) expression to pituitary gonadotrophs *(16)*. Subsequent administration of ganciclovir then led to cytotoxic ablation of transgenic TK+ gonadotropes, assuming no bystander damage to adjacent cells due to local diffusion of the toxic metabolite within the pituitary. This experiment also generated hypogonadal mice with a phenotype resembling the *hpg* mouse. Fetal gonadal development was normal in this model, suggesting FSH and LH (and TSH) are not required for sexual differentiation and genital development *(3)*. However, plasma FSH levels were not completely suppressed, possibly due to mosaic TK expression in gonadotrophs. Furthermore, transgenic TK was detected in the postnatal testis and direct testicular toxicity may confound interpretation using this model.

Transgenic FSH Models

Investigating the selective gain of transgenic FSH activity in mice has provided a valuable approach to examine the role of gonadotropins in testicular function. These transgenic studies include the restoration of FSH to gonadotrophin-deficient mouse models, thereby creating a model to study isolated FSH expression alone or together with androgen replacement, the latter creating a model of isolated LH deficiency.

Transgenic FSH in hpg *Mice*

Studies using *hpg* mice treated with exogenous recombinant human (rh) FSH showed greater effects in neonatal compared with more mature mice indicating early life exposure was important for full FSH effects. These studies were, however, restricted by the inability to deliver exogenous FSH to the fetal testis across the placenta as well as the generation of circulating anti-FSH antibodies *(17,18)*, whereby immunoneutralization may diminish long-term FSH effects *(19)*. To overcome these limitations, transgenic FSH expression was achieved by the simultaneous expression of human FSH beta and alpha subunits in a tandem construct driven by the rat insulin II gene promoter *(20)*. Transgenic FSH expressed in *hpg* mice is independent of GnRH allowing the FSH effects to be studied in isolation from LH effects. This facilitates the study of perinatal and longer-term FSH effects as well as dose-dependent FSH effects in isolation or combined with steroids *(20–22)*. These features provide complementary information to the FSHβ or FSH receptor knockouts in which the secondary effects of elevated blood LH levels and/or effects of exogenous androgens complicate interpretation of pure FSH effects *(23)*.

Transgenic human FSH increased testis weight in *hpg* mice in a dose-dependent fashion with a threshold effect as serum hFSH levels of >1 IU/L were required to increase testis weight *(20)*. Stereological analysis of spermatogenesis from *hpg* mice expressing transgenic hFSH (hFSH/*hpg*)

confirmed the mitogenic effects of FSH on Sertoli cells and spermatogonia, effects that were not significantly affected by maximal testosterone treatment after weaning age (3 wk old) *(22)*. Interestingly, the later finding differed from inhibitory effects of neonatal testosterone treatment on Sertoli cell proliferation in a setting where exogenous FSH stimulated Sertoli cell numbers *(18)*. The contrasting inhibitory effects of neonatal compared with no effects of testosterone treatment at later age supports the view that FSH regulation of Sertoli cell replication is completed during early postnatal life. Furthermore, transgenic FSH restored Sertoli cell numbers in adult *hpg* testes to phenotypically normal mature levels in a dose-dependent fashion, despite the absence of LH and persistently low intratesticular testosterone concentration *(21)*. Yet, in the same mice spermatogonial expansion and meiotic spermatocyte development reached approx 50% of normal, with only minimal post-meiotic spermatid formation *(21)*. Therefore, although FSH alone can stimulate development of a full complement of Sertoli cells, it cannot complete spermatogenesis in gonadotropin-deficient males. This highlights the requirement for additional factors, notably testosterone, for full Sertoli cell function and spermatogenic completion.

The rescue of male fertility using the androgen-treated *hpg* mice provided the first evidence that FSH was not required for murine fertility *(9,24)*, a finding later confirmed by gene knockout models lacking functional FSH β-subunit *(25)* or receptor *(26,27)*. Testosterone or dihydrotestosterone alone in *hpg* mice restored qualitatively normal spermatogenesis *(9)*. However, testis size of these gonadotropin-deficient *hpg* mice remained only approx 30–40% of normal, an observation that could not be attributed to LH deficiency *(8)* making it most likely due to the FSH deficiency. Recent work demonstrated a striking combined effect of FSH and testosterone on germ cell development. Transgenic FSH had additive effects with testosterone on meiotic spermatocyte numbers, and a strong synergistic effect with testosterone on post-meiotic spermatid maturation in *hpg* testes, which increased testis size beyond the maximal threshold of isolated androgen actions *(22)*. Because FSH and androgen receptors are located in Sertoli cells and not the germ cells *(28–31)*, these findings indicate simultaneous FSH and androgen activity promotes the required Sertoli cell function for normal spermatogenesis. One future challenge in this research area will be to elucidate the biological pathways activated by FSH alone or combined with steroids.

Transgenic Activating Mutant Human FSH Receptor in **hpg** *Mice*

A transgenic model has also exploited the gonadotrophin-deficient *hpg* mouse background to verify the physiological effects of the first activating mutation of human FSH receptor (FSHR+) discovered *(32)*. This mutation was discovered in an hypophysectomized man, who demonstrated unexpected persistent spermatogenesis and fertility despite complete FSH and LH deficiency while on androgen replacement therapy alone. The mutated FSH receptor had a single amino acid substitution in the third intracytoplasmic loop that corresponded to the location of ligand-independent activating mutations of the homologous LH *(33)* and TSH *(34)* receptors. It was proposed that this mutation sustained FSH receptor signalling in the absence of its cognate ligand. To evaluate its in vivo activity, transgenic human FSHR+ expression was targeted to Sertoli cells of FSH-deficient *hpg* males using a 1.4 kilobase rat androgen binding protein P1 gene promoter, which contains the necessary elements for Sertoli cell–specific expression in the testis *(35)*. Transgenic FSHR+ doubled *hpg* testis size, and stereological analysis showed that FSHR+ enhanced Sertoli and spermatogonia proliferation in *hpg* testes *(21)*, producing FSH-like effects closely resembling the testicular phenotype in transgenic FSH-*hpg* mice. This model further suggested that the activated FSHR slightly enhanced testosterone production in *hpg* testes independently of LH requirement. Thus, the in vivo actions of transgenic FSHR+ in FSH-deficient *hpg* mice verified the proposal that this mutation provided a constitutive "gain-of-function" FSH receptor. As the man with this activating mutation underwent development of complete spermatogenesis prior to onset of gonadotropin deficiency, more accurate replication of this clinical paradigm therefore required further studies on FSHR+ actions during testicular regression *(36)*.

A parallel transgenic approach expressing the same activating mutant human FSH receptor failed to demonstrate any effects upon gonadal phenotype in normal *(37)* or *hpg* mice (Allan and Handelsman, unpublished finding). Using a different promoter, a 1.5 kilobase fragment of the human FSHR gene, transgene mRNA expression was detected in the testis but it was not possible to detect additional FSH binding attributable to the transgenic FSH receptor, nor was any change in testis size or histology observed due to the transgene. In addition, the FSH receptor promoter region used in the transgene construct produced aberrant transgene expression in the male germ cells, suggesting this DNA fragment contained insufficient regulatory sequences to confer selective Sertoli cell expression.

Transgenic FSH in Normal Mice

In contrast to transgenic FSH having a stimulatory effect in *hpg* testes, the pituitary-independent expression of transgenic FSH (at blood levels of 1–7 IU/L of hFSH) had no effect on testis weight, sperm production, or fertility of normal male mice (Allan et al., unpublished data). In a separate transgenic mouse model, ectopic expression of human FSH via the metallothionein gene promoter also had no phenotypic effect in males at moderate serum levels (48 IU/L), whereas massively supraphysiological FSH levels (151,000

IU/L) increased serum testosterone (19-fold) and epididymal sperm counts (75%) but had no effect on testis weight or histology *(38)*. These extremely high serum FSH levels were associated with male infertility, enlarged seminal vesicles, and altered reproductive behavior, probably due to pharmacological cross-reactivity of transgenic FSH on the LH receptor in which supraphysiological FSH levels sacrifice the specificity of the model. In contrast, pituitary expression of a human FSH-beta subunit transgene generating a functional interspecies heterodimer (human-beta/mouse-alpha) increased both serum testosterone (78%) and testicular weight (22%) relative to normal. In this model, presumably total circulating bioactive FSH levels were elevated and Sertoli and germ cell numbers increased, but these had no reported effect on male fertility *(39)*. A comprehensive explanation for the disparities in testis growth among these distinct transgenic FSH models is still lacking. Whether this difference is a consequence of pituitary-derived FSH versus transgenic pituitary-independent transgenic FSH expression remains unknown. The inability of excessive ectopic transgenic FSH to overstimulate testis size and Sertoli cell proliferation in mice also differs from the actions of exogenous FSH administered to neonatal rats, which showed supraphysiological FSH levels increased final testis weight and Sertoli cell numbers above normal *(19,40)*. A unifying mechanisms explaining these diverse effects on testicular actions of FSH remains to be determined.

In summary, customized transgenic mouse models have provided definitive in vivo evidence regarding the specific role of FSH in testis growth, development, and mature function. It is now clear that FSH is both necessary and sufficient for development of the complete Sertoli cell population of the mature testis, a task it accomplishes by providing the primary mitogenic stimulus for their postnatal replication. However, mature Sertoli cell function to support complete spermatogenesis also requires androgen effects, the latter being crucial for Sertoli cells to support the full complement of post-meiotic germ cell development. Transgenic mouse models have also provided strong in vivo evidence for the ligand-independent activity of the first activating mutation of the human receptor. Currently there are no transgenic models established to verify the effects of the newer activating mutations of the FSH receptor in the testis identified as causing familial recurrent ovarian hyperstimulation syndrome *(41–43)*. Neither have the mechanisms involved in the inactivating mutant FSH receptors been studied, for which the most appropriate mouse background would be the FSHR-null mouse model to allow construction of mouse models allowing the selective examination of the in vivo consequences of different FSHR mutations.

Transgenic LH Models

To date mouse models using transgenic LH have been restricted to investigating LH actions on the hormonal background of normal mice. Transgenic LH mice were first prepared by expressing the bovine LHβ subunit fused with the C-terminal peptide (CTP) of human hCG under the control of the bovine α subunit gene promoter *(44)*. This model created a hybrid dimeric LH comprising the mouse α combined with bovine LH β subunits and demonstrated sexually dimorphic transgenic activity, with LH-CTP males exhibiting normal serum LH and testosterone levels, whereas transgenic LH-CTP females had elevated LH and exhibited infertility, polycystic ovaries, and ovarian tumors *(44)*. The transgenic LH-CTP males also had delayed fertility and smaller testes than non-transgenic controls, but no detailed analysis of the testis phenotype was reported.

A different transgenic mouse model featuring massively increased LH bioactivity producing infertility in males was achieved by expressing dimeric human hCG at high levels *(45)*. This was achieved by creating two transgenic mouse lines, each expressing the α or the β subunits of hCG driven by the universal ubiquitin C gene promoter *(45)*. The hCGβ mice expressed the transgenic hCGβ in many tissues which resulted in blood hCGβ levels of approx 6 mg/L and serum FSH levels reduced by 60%, but had no effect upon testicular testosterone levels or male fertility *(45)*. In contrast, double transgenic hCG-β/α mice exhibited massively increased blood hCG levels (approx 10,000 IU/L), markedly suppressed blood FSH levels (<5%) as well as male and female infertility. In testes of double transgenic mice, Leydig cell hypertrophy and a 14-fold increase in testosterone confirmed elevated LH/hCG activity, but seminiferous tubule morphology remained normal until mice were approx 6 mo old. The infertility of these transgenic hCG-β/α males was reportedly due to copulatory failure. A limitation of this transgenic model is the high circulating levels of transgenic hCG may cross-react with the FSHR and confound interpretation of selective LH actions.

A similar transgenic strategy used mice overexpressing human hCG-β under the control of the mouse metallothionein 1 promoter *(46)*. Three transgenic male founders were infertile despite exhibiting testes with normal morphology and histology. The male infertility in this transgenic model compared to the fertility of ubiquitin C promoter–driven transgenic hCG-β mouse remains unexplained but copulatory behavior was not reported. The generation of transgenic males expressing both hCG subunits using the metallothionein 1 promoter construct produced a phenotype resembling the double transgenic hCG-beta/alpha model above *(45)*, which presented with infertility, high testosterone, and Leydig cell hyperplasia *(46)*. To date there are no transgenic models established to selectively examine the human LHR and its known activating and inactivating mutations. However, potential opportunities include examining activating LHR mutations, ideally on a LH deficient background, or inactivating LHR mutations could also be examined using transgenic expression on the normal mouse background.

In summary, distinct transgenic strategies have allowed the in vivo dissection and characterisation of the separate or combined gonadotrophin responses in the male. It is anticipated new models will be developed to expand the investigation of gonadotrophin biology to in vivo evaluation of gonadotrophin receptor mutations in the testis.

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