Consequences of genetic manipulations of gonadotrophins and gonadotrophin receptors in mice

Conséquences des manipulations génétiques des gonadotrophines et des récepteurs des gonadotrophines chez la souris

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Résumé
Nous avons produit au cours de ces dernières années différents modèles de souris génétiquement modifiées (transgénique [TG], knockout [KO] et knockin [KI]) afin d’étudier les fonctions normales ou aberrantes des gonadotrophines et de leurs récepteurs. Dans cette revue, nous résumons nos constatations les plus récentes dans ces modèles animaux. Le premier point correspond à la cascade des phénotypes extragonadiques engendrée par l’hyperstimulation ovarienne chez les souris TG surexprimant la sous-unité \( \beta \) de l’hCG humain et présentant des taux sériques élevés d’hormone lutéinisante (LH)/hCG bioactive. Des concentrations sériques très élevées de progestérone plutôt que d’estrogènes sont responsables de l’induction de prolactinomes hypophysaires et d’une ascension consécutive des taux de prolactine (PRL). À côté de l’estradiolémie normale avec progestéronémie élevée, les concentrations élevées de prolactine induisent un développement lobulo-alvéolaire de la glande mammaire avec finalement la formation des tumeurs malignes négatives pour les récepteurs aux estrogènes et à la progestérone. Un autre modèle de souris TG exprimant un mutant constitutionnellement actif de récepteur de la FSH (FSHR) se présente avec un phénotype nettement ovarien caractérisé par un développement folliculaire avancé, une dépétition, des follicules hémorragiques, des tératomes et une infertilité. Un troisième modèle de souris TG coexprimant des mutants déficitaires aussi bien dans le domaine de liaison que de signalisation du \( \text{LHCGR} \) sur un fond génétique KO pour le gène du même récepteur, fournit des arguments convaincants en faveur du fait que la complémentarité fonctionnelle à travers une homo-di/oligomérisation est un mécanisme physiologique d’activation des récepteurs couplés aux protéines G (GPCR) de classe A. Considérés dans leur ensemble, les modèles de souris génétiquement modifiées sont de puissants outils pour élucider les fonctions normales et pathologiques des gonadotrophines et de leurs récepteurs.

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Mots clés : Gonadotrophines ; Récepteurs des gonadotrophines ; Hormone lutéinisante (LH) ; Gonadotrophine chorionique humaine (hCG) ; Hormone stimulant la folliculogénèse (FSH) ; Souris transgénique (TG) ; Souris knock-out (KO) ; Ovaires ; Testicules ; Hypophyse ; Lutéome ; Prolactinome ; Récepteurs couplés aux protéines G (GPCR) ; Dimérisation

Abstract
We have produced over the years several genetically modified mouse models (transgenic [TG], knockout [KO] and knockin [KI]) for the study of normal and aberrant functions of gonadotrophins and their receptors. We summarise in the present review some of our recent findings on these animal models. One is the cascade of extragonadal phenotypes triggered by ovarian hyperstimulation in TG mice overexpressing the human choriongonadotrophin (hCG) \( \beta \)-subunit and presenting with elevated levels of serum luteinisng hormone (LH)/hCG bioactivity. Massively elevated levels of serum progesterone, rather than oestrogens, are responsible for the induction of pituitary prolactinomas and the subsequently
1. Introduction

The hypothalamic-pituitary-gonadal (HPG) axis forms the backbone of the endocrine regulation of reproductive functions. The key hormones and receptors (R) functional along this axis are the hypothalamic decapetide gonadotrophin-releasing hormone (GnRH), its pituitary R GnRHR, the two pituitary gonadotropins, follicle-stimulating hormone (FSH), luteinising hormone (LH) and their cognate gonadal R, follicle-stimulating hormone receptor (FSHR) and LHCGR respectively. Although numerous physiological and pathophysiological studies have delineated the functions of GnRH, FSH, LH and their R in detail, we have recently learned many interesting and important new details about their functions from human mutations [1,2] and genetically modified mouse models [3,4]. Human mutations of GnRH, FSH, LH and their cognate gonadal R, follicle-stimulating hormone receptor (FSHR) and LHCGR respectively.

Our laboratory has contributed to some of the genetically modified animal models of aberrant gonadotrophin function, including the LHCGR KO [3], a constitutively activating mutation of FSHR [4], constitutive overexpression of α- and β-subunits of hCG [5,6], and a mouse model demonstrating homo-di/oligomeric LHCGR activation through functional complementation [7].

2. Tumorigenesis in human choriongonadotrophin overexpressing transgenic mice

To find out phenotypic effects of enhanced LH/hCG action, we produced two TG mouse models, one expressing under the human ubiquitin C promoter the human glycoprotein hormone common α-subunit (Cα) minigene [5], the other expressing under the same promoter cDNA of the hCG β-subunit [6]. The female hCGβ-TG mice had about 40-fold elevation of bioactive LH/CG levels in serum, due to dimersisation of the TG hCGβ produced in the pituitary gland with endogenously produced Cα subunit. The double-TG mice (Cα/hCGβ) presented ubiquitous TG expression of both subunits and consequently brought about a 1000-fold elevation in circulating bioactive LH/CG levels. The phenotypes of the two mouse models were rather similar, probably because maximal LH/hCG effect was achieved already in the single hCGβ-TG mice with the moderate LH/hCG elevation.

It was interesting to assess to what extent we could replicate in the mice the phenotypes of human activating mutations in LHCGR, i.e. early-onset precocious puberty in males and no apparent phenotype in females [2]. Conspicuously, the findings on the TG mice were quite different, with no advancement of puberty in males and very strong ovarian and extravascular phenotypes in females. The phenotypes of these mice have been reported in details elsewhere [5,6,8–10].

The female hCGβ-TG mice developed precocious puberty, as monitored by advancement of the age of vaginal opening by about 7 days. Adult mice developed massive obesity weighing about twice as much as their non-TG littermates at 4 months of age [6]. The mice were infertile with signs of dioestrus and pseudopregnancy in the vaginal epithelium. Ovarian histology in adult age revealed massive luteinisation with multiple luteomas and haemorrhagic cysts, and oocytes were often trapped inside the masses of luteoma tissue, reminding the human syndrome of luteinised unruptured follicles (LUF). Uterine weight of adult hCGβ-TG mice did not differ from WT controls but showed signs of strong progesterone action. The ovaries produce peripubertally elevated levels of oestradiol, but later on they were similar to WT controls, apparently because of the strong luteinisation and paucity of nonluteinised granulose cells. In adult age, the main ovarian steroid product was progesterone, reaching levels of >1 μmol/l, and also serum testosterone was about 5-fold elevated (about 5 nmol/l).

The mice developed gradually after puberty massive PRL-secreting pituitary adenomas (Fig. 1). The finding was surprising in the absence of increased oestradiol levels, known to promote the formation of prolactinomas. We therefore examined in greater detail whether progesterone, produced in high concentrations by ovaries of these mice, could be involved in the prolactinoma formation (Ahtiainen et al., unpublished). Indeed, we found several lines of evidence for direct involvement of ovarian steroidogenesis, most likely the combination of normal oestradiol production and massively elevated progesterone, in the induction of the tumours. This was demonstrated for instance by the total lack of pituitary tumorigenesis in gonadectomised mice despite the continuously elevated LH/hCG levels. Cell culture studies showed that a combination of oestradiol plus progesterone was more effective in stimulating pitu-
itary cell proliferation that oestradiol alone (Ahtiainen et al., unpublished).

The normal level of circulating oestradiol along with elevated progesterone and PRL of the hCGβ-TG females was found to boost the lobulo-alveolar development in the mammary gland after puberty, and this enhanced stimulation ultimately resulted in the formation of oestrogen and progesterone receptor-negative, malignant mammary gland tumours (Fig. 1) [9]. These tumours had similar histopathological appearance with those observed in TG mice with activated wnt/β-catenin pathway,
showing increased expression of β-catenin [11], in compliance with human breast tumours. Transdifferentiation was also observed in the TG mammary tumours, accompanied by abnormal expression of the Wnt genes in the tumorous and nontumorous mammary gland tissue. Specifically, increased expression of Wnt5b and up-regulation of Wnt7b and −5b were found in the hCGβ-TG mammary glands at the age of 3 months. These responses of the Wnt cascade appeared to occur independently of the changes in ovarian steroidogenesis. Thus, our TG model revealed a novel link between enhanced hCG action and increased follicle-stimulating hormone action, by expressing exogenous FSH or constitutively active FSHR in mice (Table 1). Typically, increased FSH level and basal R activity lead to biphasic effects on ovarian function. Modest increase in FSH concentration [12] and expression of a constitutively active mFSHR<sub>D580H</sub> [4] bring about improved fecundity in the form of increased litter size, while more progressive and stronger influences eventually lead to infertility [4,12,13]. The ovaries of the former mice, characteristically, contain more corpora lutea (CL) than their WT littermates, demonstrating increased recruitment of growing follicles and ovulation. The mFSHR<sub>D580H</sub> expressing ovaries present with significantly higher proportion of Ki67-positive small follicles than those in WT animals, indicating that the transgene expression increases proliferation of granulosa cells and, thus, growth of primary follicles [4]. In addition to increasing the proliferation of granulosa cells, FSH can decrease apoptosis of granulosa cells by interacting with other signaling pathways such as Activin/Smad3 [14] and PI3K/Akt [15], and the excessive hormone/R activation may therefore also enhance the number of surviving large follicles.

3. Ovarian dysfunction associated with enhanced follicle-stimulating hormone action

We and others have generated several genetically modified mouse models to phenocopy consequences of enhanced FSH action, showing increased expression of β-catenin [11], in compliance with human breast tumours. Transdifferentiation was also observed in the TG mammary tumours, accompanied by abnormal expression of the Wnt genes in the tumorous and nontumorous mammary gland tissue. Specifically, increased expression of Wnt5b and up-regulation of Wnt7b and −5b were found in the hCGβ-TG mammary glands at the age of 3 months. These responses of the Wnt cascade appeared to occur independently of the changes in ovarian steroidogenesis. Thus, our TG model revealed a novel link between enhanced hCG action and increased follicle-stimulating hormone action, by expressing exogenous FSH or constitutively active FSHR in mice (Table 1). Typically, increased FSH level and basal R activity lead to biphasic effects on ovarian function. Modest increase in FSH concentration [12] and expression of a constitutively active mFSHR<sub>D580H</sub> [4] bring about improved fecundity in the form of increased litter size, while more progressive and stronger influences eventually lead to infertility [4,12,13]. The ovaries of the former mice, characteristically, contain more corpora lutea (CL) than their WT littermates, demonstrating increased recruitment of growing follicles and ovulation. The mFSHR<sub>D580H</sub> expressing ovaries present with significantly higher proportion of Ki67-positive small follicles than those in WT animals, indicating that the transgene expression increases proliferation of granulosa cells and, thus, growth of primary follicles [4]. In addition to increasing the proliferation of granulosa cells, FSH can decrease apoptosis of granulosa cells by interacting with other signaling pathways such as Activin/Smad3 [14] and PI3K/Akt [15], and the excessive hormone/R activation may therefore also enhance the number of surviving large follicles.

Table 1

Gain-of-function mouse models for follicle-stimulating hormone pathway.

<table>
<thead>
<tr>
<th>Model</th>
<th>Abbreviation&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Description</th>
<th>Female fertility</th>
<th>Major ovarian abnormalities</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TG (FSHβ)</td>
<td>hFSHβ</td>
<td>TG line with FSHβ minigene</td>
<td>Fertile</td>
<td>No obvious abnormalities</td>
<td>[26]</td>
</tr>
<tr>
<td>TG (Mt1-CGA-Mt1-FSHβ)</td>
<td>MT-hFSH</td>
<td>Cross-breeds of Mt1 promoter-CGA and Mt1 promoter – FSHβ TG lines</td>
<td>Fertile</td>
<td>No obvious abnormalities</td>
<td>[13]</td>
</tr>
<tr>
<td>Low serum concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (Mt1-Cga-Mt1-FSHβ)</td>
<td>MT-hFSH</td>
<td>Cross-breeds of Mt1 promoter-CGA and Mt1 promoter – FSHβ TG lines</td>
<td>Infertile</td>
<td>Haemorrhagic cysts, elevated E2 and P production, disrupted oestrous cycle, no CL</td>
<td>[13]</td>
</tr>
<tr>
<td>High serum concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (rIns2-CGA-rIns2-FSHβ)</td>
<td>TG-FSH</td>
<td>TG line with Ins2-promoter-CGA-Ins2-promoter – FSHβ</td>
<td>Fertile, increased litter size</td>
<td>Increased in size and total CL count</td>
<td>[12]</td>
</tr>
<tr>
<td>&lt;22 w</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (rIns2-CGA; rIns2-FSHβ)</td>
<td>TG-FSH</td>
<td>TG line with Ins2-promoter-CGA-Ins2-promoter – FSHβ</td>
<td>Premature infertility</td>
<td>Increased in size and total CL count</td>
<td>[12]</td>
</tr>
<tr>
<td>&gt;23 w</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG (rAbp-FSHR), Hpg-TG (rAbp-FSHRD567G)</td>
<td>TG-FSH&lt;sub&gt;rwt&lt;/sub&gt;, TG-FSHR&lt;sub&gt;D567G&lt;/sub&gt;</td>
<td>TG lines with rAbp-FSHR and rAbp-FSHRD567G in wt and hpg background</td>
<td>Fertile</td>
<td>No obvious abnormalities</td>
<td>[27,28]</td>
</tr>
<tr>
<td>FVB/N-TG (AMH-FSHR)</td>
<td>mFshr&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>TG line with AMH-promoter-Fshr</td>
<td>Fertile</td>
<td>No obvious abnormalities</td>
<td>[4]</td>
</tr>
<tr>
<td>FVB/N-TG (AMH-FSHRD580H&lt;sup&gt;Y&lt;/sup&gt;, low expression, strong CAM)</td>
<td>mFshr&lt;sub&gt;D580H&lt;sup&gt;Y&lt;/sup&gt;&lt;/sub&gt;</td>
<td>TG line with AMH-promoter-Fshr&lt;sub&gt;D580H&lt;/sub&gt;</td>
<td>Fertile, increased litter size</td>
<td>No obvious abnormalities</td>
<td>[4]</td>
</tr>
<tr>
<td>FVB/N-TG (AMH-FSHRD580H&lt;sup&gt;Y&lt;/sup&gt;, high expression, strong CAM)</td>
<td>mFshr&lt;sub&gt;D580H&lt;sup&gt;Y&lt;/sup&gt;&lt;/sub&gt;</td>
<td>TG line with AMH-promoter-Fshr&lt;sub&gt;D580H&lt;/sub&gt;</td>
<td>Infertile</td>
<td>Haemorrhagic, disrupted oestrous cycle, mildly elevated E2 production, accelerated loss of follicles, occasional teratomas and LUFs</td>
<td>[4]</td>
</tr>
<tr>
<td>FVB/N-Tg (AMH-FSHRD580H&lt;sup&gt;Y&lt;/sup&gt;, Moderate CAM)</td>
<td>mFshr&lt;sub&gt;D580H&lt;sup&gt;Y&lt;/sup&gt;&lt;/sub&gt;</td>
<td>TG line with AMH-promoter-Fshr&lt;sub&gt;D580H&lt;/sub&gt;</td>
<td>Fertile</td>
<td>Haemorrhagic</td>
<td>[4]</td>
</tr>
<tr>
<td>B6; 129-Fshr&lt;sub&gt;D580H&lt;sup&gt;Y&lt;/sup&gt;&lt;/sub&gt;, KI</td>
<td>mFshr&lt;sub&gt;D580H&lt;sup&gt;Y&lt;/sup&gt;-KI&lt;/sub&gt;</td>
<td>KI line with Fshr&lt;sub&gt;D580H&lt;/sub&gt;</td>
<td>Fertile</td>
<td>Haemorrhagic</td>
<td>[4]</td>
</tr>
</tbody>
</table>

CAM: constitutively active mutant; E2: oestradiol; P: progesterone; CL: corpora lutea; LUF: luteinised unovulated follicle; w: week; KI: knockin; TG: transgenic.

<sup>a</sup> Abbreviation of the mouse line as used in the original article.
Robust activation of the FSH signalling, on the other hand, has grave consequences on function of the ovary, leading eventually to infertility. The ultimate example is the metalthiothreonine promoter-drive MT-hFSHβ mouse line producing supraphysiological levels of serum FSH [13]. The females are infertile with disrupted estrous cycle, presenting with severely hemorrhagic and cystic ovaries, and missing late-stage follicles and CL. Less vigorous FSH action, though, can also harm ovarian function; even heterozygous KI mice for a milder form of constitutively activated FSHR, mFshr<sup>D580Y</sup>, develop multiple hemorrhagic ovarian follicles (Table 1) [4]. Furthermore, the progressively rising serum FSH concentration in the MT-hFSHβ mice leads to premature infertility that may be due to accelerated loss of small follicles and recruitment of poor-quality follicles, leading to embryo-fetal resorption [12]. Finally, the mice leads to premature infertility that may be due to progressively rising serum FSH concentration in the MT-hFSHβ mice.

The hemorrhagic follicles with red blood cells leaking from the theca cell layer and infiltrating the granulosa cell layer are also common in all our models with constitutively active FSHR (Table 1) [4]. Hence, the recruitment of poor-quality follicles and/or angiogenetic effects of the constitutively active R resemble observations made in patients with ovarian hyperstimulation syndrome (OHSS).

In addition to the pseudopregnancy-type acyclicity and occasional unsuccessful ovulations of the TG-mFshr<sup>D580H</sup> females, their fertility is compromised by the accelerated loss of small follicles, primordial and primary in particular. The follicles show increased granulosa cell proliferation, and thus, enhanced recruitment of them to the grow pathway [4]. Moreover, a sub-group of the mFshr<sup>D580H</sup> mice develop teratomas associated with almost complete lack of follicles in the affected ovary. Most of the tumours observed are mature benign cysts, but in few cases, cases of immature tissue suggestive of teratocarcinoma or choriocarcinoma have been detected. In addition to the mFshr<sup>D580H</sup> females, TG-hCGαβ+ mice develop ovarian teratomas (Rulli et al., unpublished). Thus, high gonadotrophin stimulation of granulosa cells may interfere with oocyte maturation and subsequently induce parthenogenetic oocyte activation.

4. Functional complementation in vivo of binding and signalling deficient LHCGR mutants

Hormone (FSH, LH or hCG) binding to gonadotrophin receptors results in the activation of intracellular signalling cascades via G-protein coupling to specific intracellular regions of the R. The cyclic AMP/protein kinase A cascade is the most important second messenger in the activation of LHCGR and FSHR. Gonadotrophin receptors belong to the large family of G protein-coupled receptors (GPCR), which are responsible for transmitting signals to all senses (vision, odour, touch), neurotransmitters and a number of hormones. All GPCRs constitute structurally from a seven transmembrane core, which is responsible for signal transmission [16]. A large extracellular ligand-binding domain (about half of size of the R molecule) is a typical feature of the glycoprotein hormone (LH/CG, FSH and TSH) R [2].

Ligand-induced activation is the norm for almost all GPCRs with a few exceptions (see below) and a few orphan R for which no ligand has yet been identified. Whether the GPCRs function as a single-hormone/single R monomer or as R complexes (dimer/oligomer), especially in the case of the large class A of GPCRs, has been a highly debated topic. Although the phenomenon of GPCR di/oligomerisation in cell line experiments in vitro has been known for over 30 years, its importance has only been demonstrated recently on obligatory heterodimeric GPCRs such as GABA<sub>B</sub>, taste (T1R1-3) metabotropic glutamate (mGluR) and calcium-sensing R, where only heterodimers are involved in signal transduction [17,18]. The main questions, however, have remained unanswered, i.e. whether the R are activated in physiological conditions in vivo as monomers or di/oligomers, and whether the two possible modes activate similar or dissimilar signal transduction cascades?

In order to assess the significance of LHCGR function as homodimers in vivo, we took advantage of the complementary molecular models of intermolecular cooperation of gonadotrophin receptors, shown to be functional in vitro by the group of Ji [19–21]. Using LHCGR complementary mutants (one deficient of ligand binding and the other one of signalling), it was possible to partially rescue the cAMP response to LH/hCG stimulation in cell cultures, when both mutants were coexpressed in the same cell, while the mutants were completely inactive when expressed alone.

Using the same principle, similar mutations were introduced to bacterial artificial chromosome (BAC) clones that contain the entire mouse genomic LHCGR gene, including the regulatory regions (promoter, introns, and UTRs) to assure correct spatiotemporal expression in a physiologically meaningful fashion. Two TG mouse lines were created using the modified BACs [7]. As expected, the TG mice expressed LHCGR mutants mainly in gonads, as their WT counterparts, and each line harbouring either a binding-deficient LHCGR or a signalling-deficient LHCGR were crossed with the previously produced LHCGR KO (LuRKO) mice [3], to obtain mice without endogenous LHCGR but expressing either one of the inactive LHCGR mutants. The single LHCGR mutant/LuRKO mice, as LuRKO mice with the LHCGR deletion alone, were hypogonadal and infertile, with defective postnatal development of gonads and genital structures, due to totally missing LH-stimulated gonadal sex steroid production [3]. However, when both mutant R were coexpressed in LuRKO mice, gonadal development and spermatogenesis were completely rescued [7], as demonstrated by their normal
size of testes and extragonadal sex organs (Fig. 2) and normal fertility. The extent of the rescuing could not only be observed at macroscopic level but also at molecular level, where LH-dependent gene expression was nearly fully recovered in double TG but not in single TG mice [7]. The only explanation of this finding is that the two differently inactivated R molecules, when coexpressed in the same cells, can form functional dimers where one defective molecule capable of hormone binding can activate another R mutant that is only able to transmit the signal. To our knowledge, this is the first demonstration of functional relevance of GPCR homodimerisation in physiological conditions.

5. Conclusions

The ability to modify large DNA molecules [22,23], to manipulate the genome [24] and to create genetically modified animal models, provides extremely valuable tools for the characterisation of complex biological phenomena at the physiological level. Indeed, almost 130 years ago Charles R. Darwin envisaged that the understanding of these processes requires experimental *in vivo* models: “...physiology cannot possibly progress except by means of experiments on living animals...” [25].

Increased LH/hCG activity in female mice has serious functional consequences, including ovarian hyperstimulation with luteinisation and massive overproduction of progesterone, in the face of normal oestrogen production. The ovarian stimulation has multiple indirect extragonadal consequences, including the development of pituitary prolactinomas and malignant mammary gland tumours. If extrapolated to humans, and taken the importance of endogenous progesterone and synthetic progestins in female reproductive functions and their pharmacotherapy, it is relevant to revisit the potential role of these hormones in the origin and growth of human prolactinomas and mammary tumours.

Overproduction of FSH and gain-of-function mutations of the *FSHR* have several functional consequences in female mice. While modest increase in FSH action temporarily boosts the fecundity of the mice, more vigorous actions lead to an array of abnormalities in ovarian function. A similar array of phenotypes, such as OHSS, premature ovarian failure, formation of hemorrhagic cysts, abnormal hormone balance of the pituitary-gonadal axis and teratomas, may also be possible in humans expressing constitutively activating mutations of *FSHR*, not yet described.

The molecules of gonadotrophin receptors have been demonstrated to be much more flexible than previously thought, being able to generate multiple interactions with G proteins, resulting in multiple signalling cascades, and possibly even with neighbouring R through homo- and hetero-di/oligomerization. The reasons for such flexibility might involve the previously mentioned functions (transport, coupling, cooperativity, etc), but also signal amplification at the membrane level maintaining R specificity and, in the case of hetero-di/oligomerisation, pharmacological diversity. Yet, the most important finding of our
study was that intermolecular cooperation was able to rescue LHCGR function in the absence of WT R, suggesting that these interactions may also occur in physiological conditions in living organisms. We expect that also other GPCRs will show similar molecular cooperation, either to generate biased signals or the full response.

Conflicts of interest

None.

References