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REGULATING MECHANISMS OF GONADAL AND PITUITARY TUMORIGENESIS IN MICE PRODUCING HUMAN CHORIONIC GONADOTROPIN

by

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To my family

ABSTRACT

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Regulating mechanisms of gonadal and pituitary tumorigenesis in mice producing human chorionic gonadotropin

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Human chorionic gonadotropin (hCG) and luteinizing hormone (LH) are structurally and functionally similar glycoprotein hormones acting through the same luteinizing hormone chorionic gonadotropin receptor (LHCGR). The functions of LH in reproduction and hCG in pregnancy are well known. Recently, the expression of LHCGR has been found in many nongonadal tissues and cancers, and this has raised the question of whether LH/hCG could affect the function or tumorigenesis of these nongonadal tissues. We have also previously generated an hCG expressing mouse model presenting nongonadal phenotypes. Using this model it is possible to improve our understanding of nongonadal action of highly elevated LH/hCG.

In the current study, we analyzed the effect of moderately and highly elevated hCG levels on male reproductive development and function. The main finding was the appearance of fetal Leydig cell (FLC) adenomas in prepubertal males. However, the development and differentiation of FLCs were not significantly affected. We also show that the function of hCG is different in FLCs and in adult Leydig cells (ALC), because in the latter cells hCG was not able to induce tumorigenesis. In FLCs, LHCGR is not desensitized or downregulated upon ligand binding. In this study, we found that the testicular expression of two G protein-coupled receptor kinases responsible for receptor desensitization or downregulation is increased in adult testis. Results suggest that the lack of LHCGR desensitization or downregulation in FLCs protect testosterone (Te) synthesis, but also predispose FLCs for LH/hCG induced adenomas. However, all the hCG induced nongonadal changes observed in male mice were possible to explain by the elevated Te level found in these males. Our findings indicate that the direct nongonadal effects of elevated LH/hCG in males are not pathophysiologically significant.

In female mice, we showed that an elevated hCG level was able to induce gonadal tumorigenesis. hCG also induced the formation of pituitary adenomas (PA), but the mechanism was indirect. Furthermore, we found two new potential risk factors and a novel hormonally induced mechanism for PAs. Increased progesterone (P) levels in the presence of physiological estradiol (E2) levels induced the formation of PAs in female mice. E2 and P induced the expression and nuclear localization of a known cell-cycle regulator, cyclin D1. A calorie restricted diet was also able to prevent the formation of PAs, suggesting that obesity is able to promote the formation of PAs. Hormone replacement therapy after gonadectomy and hormone antagonist therapy showed that the nongonadal phenotypes observed in hCG expressing female mice were due to ovarian hyperstimulation. A slight adrenal phenotype was evident even after gonadectomy in hCG expressing females, but E2 and P replacement was able to induce a similar phenotype in WT females without elevated LH/hCG action.

In conclusion, we showed that the direct effects of elevated hCG/LH action are limited only to the gonads of both sexes. The nongonadal phenotypes observed in hCG expressing mice were due to the indirect, gonadal hormone mediated effects of elevated hCG. Therefore, the gonads are the only physiologically significant direct targets of LHCGR signalling.

Key words: chorionic gonadotropin, luteinizing hormone, luteinizing hormone receptor, testis, Leydig cell, adenoma, pituitary adenoma, progesterone, estrogen, cell-cycle, cyclin D1

TIIVISTELMÄ

Petteri Ahtiainen

Sukurauhas- ja aivolisäkekasvainten säätelymekanismit ihmisen istukkahormonia ilmentävässä siirtogeenisessä hiiressä

Fysiologian osasto, Biolääketieteen laitos, Lääketieteellinen tiedekunta, Turun yliopisto
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Ihmisen istukkahormoni (hCG) ja luteinisoiva hormoni (LH) ovat rakenteeltaan ja vaikutuksiltaan samankaltaisia. Ne välittävät biologiset vaikutuksensa saman reseptorin, luteinisoivan hormonin reseptorin kautta (LHCGR). Sekä LH:n vaikutukset lisääntymisbiologian kannalta että hCG:n vaikutukset raskauden aikana tunnetaan hyvin. Viimeaikaiset tutkimukset ovat osoittaneet, että LHCGR ilmentyy myös sukurauhasten ulkopuolella. Täten on arveltu, että LH ja hCG voisivat vaikuttaa myös sukurauhasten ulkopuolella eri kudosten toimintaan ja mahdollisesti lisätä kasvainten ilmaantumista näissä LHCGR:a ilmentävissä kudoksissa. Vastataksemme kysymykseen, onko suurentuneella hCG-pitoisuudella fysiologista merkitystä, teimme ihmisen istukkahormonia ilmentävän siirtogeenisen hiirimallin.

Tässä tutkimuksessa selvitimme kohonneen istukkahormonipitoisuuden vaikutuksia uroshiiren lisääntymistoimintoihin ja niiden kehitykseen. Tutkimuksemme merkittävin löydös oli se, että hCG aiheuttaa sikiötyypin Leydigin soluista (FLC) lähtöisin olevien hyvälaatuisten kasvainten kehittymisen esimurrosikäisille hiiriuroksille. hCG ei kuitenkaan vaikuttanut FLC:en normaaliin erilaistumiseen tai kehitykseen. Tutkimuksessamme osoitimme myös, että hCG:n vaikutus on erilainen FLC:issa ja aikuistyypin Leydigin soluissa (ALC). FLC:issa LHCGR:t eivät epäherkisty tai niiden määrä ei vähene hCG:n sitoutumisen jälkeen, kun taas ALC:issa edellä mainitut tapahtuvat ja LHCGR:ien vaste hCG:lle heikkenee. Tässä tutkimuksessa osoitimme, että kaksi G-proteiinikytkentäisten reseptorien kinaasia ilmentyy selvästi enemmän aikuisessa kiveksessä kuin esimurrosikäisessä kiveksessä selittäen ALC:issa tapahtuvan LHCGR:ien epäherkistymisen ja määrän vähenemisen. Tämä epäherkistymisen puuttuminen FLC:ista mahdollisesti suojelee sikiöaikaista testosteronin tuotantoa ja uroshiiren ilmiasun kehittymistä, mutta myös altistaa FLC:t liialliselle hCG-vaikutukselle, kun taas ALC:t ovat tältä suojassa. hCG:a ilmentävillä uroksilla oli ilmiasu poikkeava myös sukurauhasten ulkopuolella, mutta tämä ilmiasu johtui hCG:n aikaansaamasta testosteronin liikatuotannosta, ei hCG:n suorasta vaikutuksesta näihin kudoksiin.

Tässä tutkimuksessa osoitimme myös, että istukkahormoni aiheuttaa naarashiirille sukurauhaskasvainten kehittymisen LHCGR-välitteisesti. Lisäksi hCG:a ilmentäville naarashiirille ilmaantui hyvälaatuiset aivolisäkekasvaimet. Tässä tutkimuksessa löysimme kaksi uutta aivolisäkekasvainten riskitekijää ja yhden merkittävän molekylaarisen mekanismin aivolisäkekasvainten taustalla. Seerumin suurentunut progesteronipitoisuus (P) yhdessä normaalin estradiolipitoisuuden (E2) kera johti aivolisäkekasvainten kehittymiseen. E2 ja P yhdessä lisäsivät tunnetun solusyklin säätelijän, sykliini D1:n ilmentymistä ja paikantumista tumaan. Lisäksi osoitimme, että vähäkalorinen ruokavalio vähentää aivolisäkekasvainten ilmaantumista viitaten siihen, että lihavuus on yksi aivolisäkekasvainten riskitekijä. Hormonikorvaushoidot ja hormoni-vastavaikuttajien käyttö osoittivat, että sukurauhasten ulkopuolinen ilmiasu hCG:a ilmentävillä naarashiirillä on selitettävissä hCG:n munasarjaan kohdistamilla, munasarjahormonien eritystä lisäävillä vaikutuksilla.

Yhteenvetona voidaan todeta, että istukkahormonin suorat vaikutukset rajoittuvat sukurauhasiin. Lisäksi voidaan todeta, että sukurauhasten ulkopuolinen ilmiasu hCG:a ilmentävillä hiirillä aiheutuu hCG:n epäsuorilla, sukurauhasvälitteisillä, vaikutuksilla. Täten, LHCGR-välitteinen vaikutusmekanismi on fysiologisesti ja patofysiologisesti merkittävää ainoastaan sukurauhasissa.

Avainsanat: istukkahormoni, luteinisoiva hormoni, luteinisoivan hormonin reseptori, kives, munasarja, Leydigin solu, kasvain, aivolisäkekasvain, progesteroni, estradioli, solusykli, sykliini D1

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ABBREVIATIONS

aa	amino acid
ACTH	adrenocorticotrophic hormone
ADP	adenosine diphosphate
AIN60	mouse chow
al	*ad libitum*
ALC	adult Leydig cell
α-MSH	alpha melanocyte-stimulating hormone
ANOVA	analysis of variances
B	bromocriptine
bLH-CTP	fusion protein of bovine LH and hCG C-terminal peptide
bp	base pair
BPS	balano-preputial separation
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CG	chorionic gonadotropin
CPHD	combined pituitary hormone deficiency
CR	calori restricted
CRD	Calori restricted diet
CT	computed tomography
DA	dopamine
DAG	diacylglyserol
DHT	dihydrotestosterone
DPC	density of proliferating cells
E	embryonic day
E2	estradiol
ERE	estrogen responsive element
ERK1	extracellular signal-regulated kinase 1
ERK2	extracellular signal-regulated kinase 2
FIA	fluoroimmunoassay
FLC	fetal Leydig cell
FSH	follicle stimulating hormone
G	gonadectomy
G1	gap1
GCT	Germ cell tumors
GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
h	human
HA	hormone antagonist
hCG	human chorionic gonadotropin
hCGα	TG mouse expressing human CGA subunit
hCGβ	TG mouse expressing human CGB subunit
hCGαβ	TG mouse expressing human CGA and CGB subunit
hpg	hypogonadal
HR	hormone replacement
HRT	hormone replacement therapy
IFMA	immunofluorometric assay
IGHD	isolated growth hormone deficiency
IHC	immunohistochemistry
ILC	immature Leydig cell
IP3	inositol triphosphate
kb	kilo-base
kDa	kilo-Dalton
KI67	antigen identified by monoclonal antibody Ki-67
KO	knockout
LC	Leydig cell
LCH	Leydig cell hypolasia
LCT	Leydig cell tumor
Le	leptin
LH	luteinizing hormone
M	mifepristone (RU486)
MAPK	mitogen-activated protein kinase
MAS	McCune-Albright syndrome
MGI	mouse genome informatics
MRI	magnetic resonance imaging
OC	oral contraception
P	progesterone
PA	pituitary adenoma
PBS	phosphate buffered saline
pc.	*post coitum*
PCR	polymerase chain reaction
PI	proliferation index
PIP2	phosphatidylinositol diphosphate
PI3K	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLA2	phospholipase A2
PLC	phospholipase C
PLCs	progenitor Leydig cells
PMSG	pregnant mares' serum gonadotropin
RAS	Harvey rat sarcoma virus oncogene
RIA	radioimmunoassay
RM1	mouse chow
RP	Rathke's pouch
RT-PCR	reverse transcriptase polymerase chain reaction
S	synthesis
SEM	standard error of mean
SLC	stem Leydig cell
SPECT	single positrone emission computed tomography
SV40	Simian virus 40
T	tamoxifen
TDS	testicular dysgenesis syndrome
Te	testosterone
TG	transgenic
TIDA	tuberoinfundibular dopaminergic cell
TRE	thyroid hormone response element
TSH	thyroid stimulating hormone
WT	wild-type
5-HT	5-hydroxytryptamine (serotonin)

ABBREVIATIONS

Genes

Actb	actin, beta, cytoplasmic
Adrbk2	adrenergic receptor kinase, beta 2
Ahr	aryl hydrocarbon receptor
Aip	aryl hydrocarbon receptor interacting protein
Akr1c18	aldo-keto reductase family 1, member C18
Ar	androgen receptor
Bmp^n	bone morphogenetic protein^n
Ccnd1	cyclin D1
Ccne	cyclin e
Cdk^n	cyclin dependent kinase^n
Cdkn1^n	cyclin-dependent kinase inhibitor 1^n
Cdkn2^n	cyclin-dependent kinase inhibitor 2^n
Cga	glycoprotein hormones, alpha subunit
Cgb	chorionic gonadotropin, beta polypeptide
Crh	corticotropin releasing hormone
Cyp11a1	cytochrome P450, family 11, subfamily a, polypeptide 1
Cyp17a1	cytochrome P450, family 17, subfamily a, polypeptide 1
Dhh	desert hedgehog
Drd2	dopamine receptor 2
E2f^n	E2F transcription factor^n
Egf	epidermal growth factor
Egfr	epidermal growth factor receptor
Esr^n	estrogen receptor^n
Fgf^n	fibroblast growth factor^n
Fgfr^n	fibroblast growth factor receptor^n
Fshb	follicle stimulating hormone beta
Fshr	follicle stimulating hormone receptor
Gal	galanin
Gata4	GATA binding protein 4
Gh	growth hormone
Ghrh	growth hormone releasing hormone
Ghrhr	growth hormone releasing hormone receptor
Gli^n	GLI-Kruppel family member GLI^n
Gnas	GNAS complex locus
Gnrh	gonadotropin releasing hormone 1
Gnrhr	gonadotropin releasing hormone receptor
Grk4	G protein-coupled receptor kinase 4
Hdac1	histone deacetylase 1
Hes1	hairy and enhancer of split
Hesx1	homeo box gene expressed in ES cells
Hmga^n	high mobility group AT-hook^n
Hras1	Harvey rat sarcoma virus oncogene 1
Hsd3b^n	hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase^n
Igf1	insulin-like growth factor 1
Inha	inhibin alpha
Insl3	insulin-like growth factor 3
Isl1	ISL1 transcription factor, LIM/homeodomain
Jak2	Janus kinase 2
Jun	Jun oncogene
Jund	Jun proto-oncogene related gene d
Kiss1r	KISS1 receptor
Lepr	leptin receptor
Lhb	luteinizing hormone beta
Lhcgr	luteinizing hormone chorionic gonadotropin receptor
Lhx^n	LIM homeobox protein^n
Men1	multiple endocrine neoplasia I
Myc	myelocytomatosis oncogene
Nkx2-1	NK2 homeobox 1
Notch^n	notch gene homolog^n
Nr5a1	nuclear receptor subfamily 5, group A, member 1
Nupr1	nuclear protein 1
Pax6	paired box gene 6
Pdgfa	platelet derived growth factor, alpha
Pdgfra	platelet derived growth factor receptor, alpha polypeptide
Pit1	*=Pou1f1*
Pitx^n	paired-like homeodomain transcription factor^n
Pomc	pro-opio-melanocortin
Pou1f1	POU domain, class 1, transcription factor 1 (Pit1)
Prkar1a	protein kinase A type 1A regulatory subunit
Prl	prolactin
Prlr	prolactin receptor
Prop1	paired like homeodomain factor 1
Ptgds	prostaglandin D synthase
Pttg1	pituitary tumor-transforming 1
p15	*=Cdkn2b*
p16	*=Cdkn2a*
p18	*=Cdkn2c*
p21	*=Cdkn1a*
p27	*=Cdkn1b*
Rb1	retinoblastoma 1
Shh	sonic hedgehog
Six^n	sine oculis-related homeobox ^{n} homolog
Smad^n	MAD homolog^n
Sox2	SRY-box containing gene 2
Src	Rous sarcoma oncogene
Star	steroidogenic acute regulatory protein
Stat^n	signal transducer and activator of transcription^n
Tgfa	transforming growth factor alfa
Tgfb^n	transforming growth factor beta^n
Thbs2	thrombospondin 2
Trh	thyrotropin releasing hormone
Tshb	thyroid stimulating hormone, beta subunit
Tshr	thyroid stimulating hormone receptor
Vegf^n	vascular endothelial growth factor^n
Wnt^n	wingless-related MMTV integration site^n
Xrcc6	X-ray repair complementing defective repair in Chinese hamster cells 6

The ^{n} after the gene abbreviation and name stands for a number or alphabet in cases where more than one gene with a similar name is mentioned in the text. In the text, *Gene* refers to a mouse gene, and *GENE* to a human gene. The protein of both mouse and human appear like GENE in the text. Genetic nomenclature is applied from Mouse Genome Informatics (MGI).

LIST OF ORIGINAL PUBLICATIONS

This study is based on the following publications, which are referred to in the text by the Roman numerals (I-III):

I Ahtiainen P, Rulli S, Shariatmadari R, Pelliniemi L, Toppari J, Poutanen M, and Huhtaniemi I (2005) Fetal but not adult Leydig cells are susceptible to adenoma formation in response to persistently high hCG level: a study on hCG overexpressing transgenic mice. *Oncogene 24: 7301-7309.*

II Rulli S, Ahtiainen P, Mäkelä S, Toppari J, Poutanen M, and Huhtaniemi I (2003) Elevated steroidogenesis, defective reproductive organs, and infertility in transgenic male mice overexpressing human chorionic gonadotropin. *Endocrinology 144: 4980-4990.*

III Ahtiainen P, Rulli S, Rivero-Müller A, Mamaeva V, Röyttä M, and Huhtaniemi I. Progesterone induces pituitary adenomas in the presence of physiological estradiol levels. *Submitted.*

This thesis also contains unpublished results.

1. INTRODUCTION

Human chorionic gonadotropin (hCG) and luteinizing hormone (LH) are heterodimeric proteins and members of the family of glycoprotein hormones that share structural and functional similarities. They both share a common α-subunit, and their hormone specific β-subunit also shows remarkable homology (Gharib *et al.*, 1990). Because of the similarities they bind to same luteinizing hormone chorionic gonadotropin receptor (LHCGR). Upon ligand binding, they activate intracellular signalling cascades (Ascoli *et al.*, 2002). LHCGR is mainly expressed in the gonads, and the most important function of LH/hCG is to promote gonadal sex steroid production and the maturation of germ cells. Recently, several normal nongonadal tissues have been shown to express LHCGR, and this has led to speculation regarding the physiological, nongonadal functions of LH/hCG (Filicori *et al.*, 2005; Rao and Lei, 2007; Ziecik *et al.*, 2007). We have recently analyzed he phenotype of luteinizing hormone receptor knockout (*Lhcgr* KO) females, whose ovaries were orthotopically transplanted with wild-type (WT) females. *Lhcgr* KO females with orthotopically transplanted WT ovaries gained normal reproductive capability, and the phenotype of these females was indistinguishable with WT females (Pakarainen *et al.*, 2005b). Testosterone (Te) replacement was also able to rescue the fertility of *Lhcgr* KO males, although they presented with decreased fertility due to the inflammatory reaction seen in epididymides and prostate (Pakarainen *et al.*, 2005a; Savolainen *et al.*, 2007). These results suggest that functional LHCGR has an important physiological role only in the gonads.

Increased levels of hCG/LH have been associated with the development of ovarian cancers both in man and mice (Risma *et al.*, 1995; Wise *et al.*, 1996). Exogenous administration of hCG or substances increasing LH induce Leydig cells tumors in rodents (Cook *et al.*, 1999). In humans, a certain constitutively activating LHCGR mutation leads to Leydig cell adenomas (Liu *et al.*, 1999; Richter-Unruh *et al.*, 2002) highlighting the role of LH/hCG in the development of gonadal neoplasms. It is however unclear, whether the LH/hCG is able to induce gonadal tumors alone or is some contributing factors required. Because the expression of LHCGR was found in many different nongonadal tissues, several studies have tried to prove the role of elevated LH/hCG in nongonadal tumorigenesis (Rao and Lei, 2007; Ziecik *et al.*, 2007). In particular, the role of LHCGR in prostate, breast and uterine cancers has been studied, because of their high incidence after menopause or andropause, situations presenting elevated LH levels (Lei *et al.*, 1993b; Meduri *et al.*, 1997; Tao *et al.*, 1997a). Unfortunately in many cases, the functional evaluation of LHCGR has not been convincing, or then the expression of LHCGR has been negligible compared with the levels in gonads (Stewart, 2001). Most of the studies showing the tumorigenic effect of LH/hCG/LHCGR rely on *in vitro* data performed with already transformed cells or cancer cells. The role of LH/hCG/LHCGR as inducer of nonganadal tumors *in vivo* is completely unclear.

One nongonadal tissue where functional LHCGR has been found is the adrenal gland. However, the normal expression level of LHCGR is very low or totally absent in the adrenal gland (Bielinska *et al.*, 2003). Elevated LH secretion has been shown to

induce LHCGR in adrenal gland causing increased adrenal production of cyclic adenosinemonophosphate (cAMP), progesterone (P) and glucocorticoids (Kero *et al.*, 2000). Elevated LH level is also able to promote tumorigenesis in adrenal gland as shown previously in mice (Bielinska *et al.*, 2003; Mikola *et al.*, 2003), and the expression of *LHCGR* has also been found in human adrenal tumors (Saner-Amigh *et al.*, 2006). Based on those results, adrenal gland might be the only nongonadal tissue, where LH/hCG/LHCGR pathway is able to directly induce tumors.

Transgenic (TG) mice expressing fusion protein of bovine LH and c-terminal peptide of hCG under a pituitary specific promoter has been previously developed (Risma *et al.*, 1995). These mice presented with LH levels of a high physiological/low pathophysiological range (Risma *et al.*, 1995). High LH levels led to development of ovarian, pituitary and mammary gland tumors, and the nongonadal tumors have been shown to be ovarian dependent (Risma *et al.*, 1995; Keri *et al.*, 2000; Milliken *et al.*, 2002; Mohammad *et al.*, 2003). We clarified the role of elevated LH/hCG in the development of gonadal and nongonadal neoplasms by developing two transgenic mouse models presenting pathophysiological and high pharmacological LH-action. The females expressing pathophysiological hCG level presented with increased E2 production early in life, inducing precocious puberty followed by the formation of ovarian dependent pituitary and mammary gland tumors (Rulli *et al.*, 2002).

In the current study, we analysed the effects of moderate and very high hCG levels on gonadal and nongonadal tumorigenesis. We also addressed the molecular mechanism of pituitary tumorigenesis in TG female mice, and paid close attention to the reproductive development and function of TG male mice. We provide new information on the role of hCG in gonadal, nongonadal, and pituitary tumorigenesis and in male reproductive development and function.

2. REVIEW OF THE LITERATURE

2.1. LH, hCG and LHCGR

Gonadotropins secreted in humans are luteinizing hormone (LH), follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG). Together with thyroid-stimulating hormone (TSH) they form a family of glycoprotein hormones sharing structural similarities and a common alpha subunit (CGA). LH and FSH are secreted in a pulsatile manner from the anterior pituitary gland, and these hormones are mainly responsible for normal reproductive development and functions. In humans, primates and equine, but not in mice, the third gonadotropin, chorionic gonadotropin (CG) is secreted by the placenta of the pregnant female, and CG shares high homology with LH. These glycoproteins transmit their intracellular effects through their cognate receptors; LH and hCG through luteinizing hormone chorionic gonadotropin receptor (LHCGR); FSH through follicle stimulating hormone receptor (FSHR); and TSH through thyroid stimulating hormone receptor (TSHR). These receptors also share high structural homology.

2.1.1. Structure of LH, hCG and LHCGR genes and proteins

LH and **hCG** are heterodimeric proteins. As well as FSH and TSH, they share common α-subunit, and a hormone specific β-subunit. The common α-subunit (CGA) in humans is composed of 92 amino acid residues coded by a single gene comprising of 4 exons (Fiddes and Goodman, 1981). The gene for *CGA* is localized on choromosome 6q12.21. In mice, CGA is formed from 96 amino acid (aa) residues with signal peptide of 24 aa, and the gene is located on choromosome 4 and composed of 4 exons (MGI: 88390). The CGAs of human and mouse share 73 % homology in amino acid sequence [Unigene: P01215 (human) vs. P01216 (mouse)].

The biological activity of glycoprotein hormones is determined by the hormone specific β-subunit. In humans, *LHB* and *CGB* genes form a cluster of one *LHB* and six *CGB* genes located on chromosome 19q13.32 (Themmen and Huhtaniemi, 2000). The mouse *Lhb* gene is located on chromosome 7 (MGI: 96782). In mouse, no gene for chorionic gonadotropin exists. In humans, mature LHB protein is composed of 121 amino acid residues, and CGB protein is slightly larger containing 145 amino acid residues. LHB protein shows 83 % homology with CGB protein (if 24 amino acid c-terminal non-homologous extension is excluded). This c-terminal extension of CGB has evolved from LHB protein most probably through a frameshift of the last exon of LHB leading to extension in the reading frame (Fiddes and Goodman, 1980). This c-terminal extension of CGB protein increases the half-life and biological activity of hCG as compared with LH. In mouse, LHB protein is composed of 141 amino acid residues including 20 aa signal peptide sharing 70 % and 65 % homology with human LHB and CGB protein (without C-terminal peptide) [Unigene: P 01229 (human LHB) and NP_149439.1 (CGB) vs. O 09108 (mouse LHB)].

The crystal structure of hCG has revealed that both CGA and CGB contain a cystine knot structure, where each subunit has two long β-hairpin loops on one side, a

central cystine knot and a long loop on the other side. The subunits bind non-covalently, and the interaction is stabilized by a disulfide bridge (Lapthorn *et al.*, 1994). It is most likely, although the crystal structure has not analysed, that LH behaves similarly to hCG. There are no biochemical data on the dimerization of mouse CGA or LHB with human CGA, LHB or CGB, but at least in vivo studies have shown that mouse CGA is able to bind human CGB or fused bovine LHB and C-terminal peptide of hCG, forming a biologically active protein dimer (Risma *et al.*, 1995; Rulli *et al.*, 2002; Matzuk *et al.*, 2003).

LHCGR belongs to the glycoprotein hormone receptor subfamily of G protein-coupled / seven-transmembrane domain receptors (GPCR). *LHCGR* is located on chromosome 2 in humans (Rousseau-Merck *et al.*, 1990), and chromosome 17 in mouse (MGI: 96783). *LHCGR* consists of 11 exons and 10 introns in human, 10 of which code the signal peptide and extracellular part responsible for ligand binding (Dufau, 1998). The last, long exon codes both the seven-transmembrane and G protein-coupled domains. The full-length LHCGR protein consists of 701 amino acid residues corresponding to a molecular weight of approximately 75 kDa, but the mature receptor is 10-20 kDa larger due to extensive glycosylation of the extracellular domain (Ascoli *et al.*, 2002). In mouse, LHCGR consists of 700 amino acid residues and the molecular weight is also ~ 75 kDa (MGI: 96783).

2.1.2. Signal transduction, desensitization and transcriptional regulation of Lhcgr

The function of G protein-coupled receptors (GPCR) has been extensively studied during recent years. The classical view of enhanced signalling cascades followed by ligand binding is still valid, but several new aspects on the function of G protein-coupled receptors have been achieved by crystallographic analysis and studies on gene-modified mice.

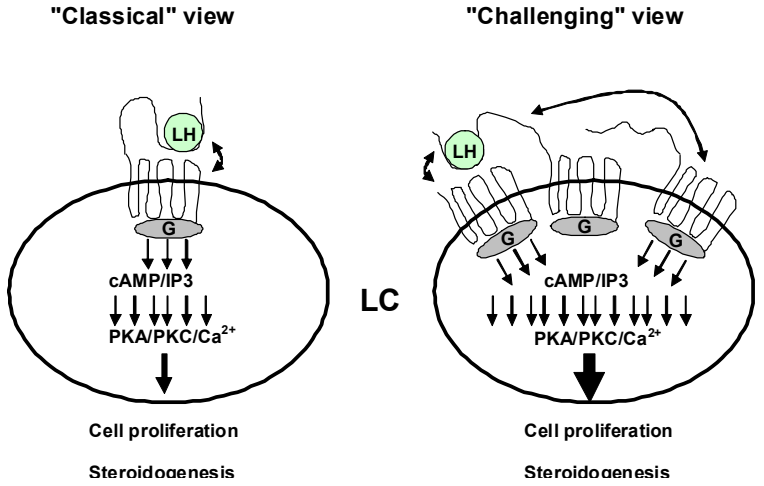


Figure 1. *Schematic representation of the classical and challenging views of GPCR/LHCGR signal transduction. In the classical model, agonist binding induces conformational changes in the receptor leading to activation of signal transduction pathways. Amplification of signal transduction occurs in the signalling pathways. In the challenging model, ligand binding into one GPCR induces the activation of the receptors located in close proximity. Signal transduction pathways of several receptors are activated and the amplification of the signals occurs in each step.*

Classical view of signal transduction: Upon the binding of LH/hCG to the extracellular part of LHCGR, one hormone-receptor complex undergoes extensive conformational changes leading to signal transduction through the cell membrane, and activation of G-proteins. Mutation analysis has revealed that the transmembrane helices TM3, 5 and 6, and intracellular loop IL3 are especially important regions of LHCGR for the activation of G-proteins and signal transduction, and many of the naturally occurring inactivating LHCGR mutations are located in these areas (Ascoli *et al.*, 2002). Amplification of signal transduction is also considered to occur in signal transduction cascades as shown in figure 1.

Challenging view of signal transduction: As shown by crystallographic studies of FSHR (Fan and Hendrickson, 2005), it is possible that some GPCRs are able to dimerize or even oligomerize. This raises the question whether the same occurs in the case of LHCGR. Indeed, co-transfection with binding defective mutant LHCGR and signal-defective mutant LHCGR has been shown to rescue hormone induced cAMP-production, suggesting the physical interaction between these two mutant receptors (Ji *et al.*, 2002; Lee *et al.*, 2002a). The unpublished results from our lab (Rivero-Müller et al. unpublished results) have shown that two receptor mutants together, one without ligand-binding properties and the other without signal transduction properties, in *Lhcgr* KO mice (i.e. without endogenous LHCGR) result in a completely normal reproductive phenotype in contrast to the extensively affected male phenotype seen in normal *Lhcgr* KO males. These findings together suggest the dimerization/oligomerization of LHCGR upon ligand binding. It is also possible that the amplification of signal transduction already occurs at the level of the hormone-receptor complex (Fig. 1). Activated receptor-ligand complex could activate the other receptor(s) in the close proximity leading to activation of the signal transduction system through many LHCGRs.

Signalling cascades: After ligand binding, LHCGR-mediated intracellular events are mostly mediated by the activation of the Gs/ adenylyl cyclase/ cAMP/ PKA pathway especially in Leydig and granulosa cells (Gudermann *et al.*, 1992). In this pathway, ligand binding releases the active G protein α-subunit from the inactive G protein αβγ-trimer. The α-subunit further activates adenylyl cyclase leading to the formation of cyclic adenosine monophosphate (cAMP) and phosphorylation of protein kinase A (PKA). This activated form of PKA further triggers phosphorylations leading to remarkable intracellular events, such as the enhancement of steroidogenesis in granulosa and Leydig cells.

It has, however, become clear that for full activation of intracellular events, crosstalk with GPCRs and epidermal growth factor receptors (EGFR), a process called transactivation, is required especially for coupling the GPCRs and cell-cycle progression (Daub *et al.*, 1996; Prenzel *et al.*, 1999; Rozengurt, 2007). This crosstalk is mediated by activated GPCR induced matrix metalloproteinases driven release of EGFR ligand precursors (extracellular/ cell membrane event), or direct phosphorylation of EGFR by different protein kinases downstream of GPCRs (intacellular event) (Prenzel *et al.*, 1999; Rozengurt, 2007). Activation of EGFR even leads to activation of phosphatidylinositol 3-kinase (PI3K) or RAS/ERK1/2 pathway followed by cell cycle progression (Rozengurt, 2007).

Several recent publications have also shown that transactivation of LH/LHCGR/PKA/cAMP and EGFR pathways are required for normal physiological

function of the gonads of both sexes. It has been shown that LHCGR activation leads to phosphorylation and thus activation of EGFR in LCs followed by activation of ERK1/2 and enhancement of steroidogenesis (Shiraishi and Ascoli, 2006; Evaul and Hammes, 2008; Shiraishi and Ascoli, 2008). The EGFR activation was shown to be predominantly mediated intracellularly, through cAMP/PKA pathway, leading to rapid phosphorylation of EGFR and ERK1/2 followed by enhancement of steroidogenesis (Evaul and Hammes, 2008). The transactivation of EGFR upon LHCGR activation was important in early steroidogenesis, but not for prolonged stimulation, since the early steroidogenesis was completely blocked by using EGFR inhibitors without any effect on prolonged stimulation (Evaul and Hammes, 2008). EGFR inhibitors were also able to attenuate testosterone production *in vivo* suggesting that this transactivation is physiologically significant (Evaul and Hammes, 2008). At the moment, there are no reports of LHCGR and EGFR transactivation on Leydig cell proliferation, but *in vitro* models have shown that the proliferation of rat primary LCs after LHCGR activation is mediated through the ERK1/2 pathway suggesting that EGFR transactivation may also be important in LC proliferation (Shiraishi and Ascoli, 2007). In female mice, it has been shown that transactivation of LHCGR and EGFR is mandatory for normal ovulation (Park *et al.*, 2004), cumulus-cell growth (Park *et al.*, 2004), oocyte maturation (Park *et al.*, 2004; Jamnongjit *et al.*, 2005), and steroidogenesis (Jamnongjit *et al.*, 2005). In the ovaries, the transactivation of LHCGR and EGFR is mediated through extracellular, metalloproteinase-mediated EGFR activation (Jamnongjit *et al.*, 2005). It is thus clear that classical LHCGR/cAMP/PKA pathway alone is not enough for proper gonadal functions, but the transactivation of EGFR is required in both sexes.

Several other signalling cascades have also been shown to be activated upon ligand binding to LHCGR. Recents studies have demonstrated the presence and the importance of MAPK pathway in Leydig cells (Cameron *et al.*, 1996; Hirakawa *et al.*, 2002). It is not thoroughly known, which pathway(s) activate MAPK (ERK1/2) in LCs, but recent studies suggest that PKA and EGFR mediated pathways are important for ERK1/2 activation (Shiraishi and Ascoli, 2007; Evaul and Hammes, 2008; Shiraishi and Ascoli, 2008). It is clear that ERK1/2 is partly activated through LHCGR induced transactivation of EGFR even directly by PKA or indirectly through metalloproteinase released EGFR ligand (Evaul and Hammes, 2008; Shiraishi and Ascoli, 2008). It has been shown that PKA directly activate ERK1/2, since the EGFR inhibitor only partly prevented the hCG induced ERK1/2 activation (Shiraishi and Ascoli, 2007). It is, however, possible that Harvey rat sarcoma virus oncogene 1 (RAS) mediates the PKA induced ERK1/2 activation (Hirakawa and Ascoli, 2003). Activation of ERK1/2 has been convincingly linked to the LC proliferation and steroidogenesis (Shiraishi and Ascoli, 2007; Evaul and Hammes, 2008). Activation of ERK1/2 pathway is able to induce the progression of cell cycle at G1-S transition (Roovers and Assoian, 2000) leading to activation of CCND1/CDK4/6/RB/E2f pathway as more detailed discussed in section 2.3.3.5 and highlighted in Figure 7 (Sears and Nevins, 2002). It is highly possible that this pathway is also activated in proliferating LCs upon LHCGR activation.

LHCGR has also been shown to activate also the phospholipase C (PLC) pathway leading to a ligand binding-induced dissociation of Gβγ (Herrlich *et al.*, 1996). Stimulation of PLC hydrolyses phosphatidylinositol biphosphate (PIP2) to inositol triphosphate (IP3) and diacylglyserol (DAG) (Davis *et al.*, 1986; Dimino *et al.*, 1987).

IP3 mobilizes calcium from intracellular storages by binding to the calcium channels, whereas DAG activates protein kinase C (PKC) (Nishizuka, 1988). In contrast to the cAMP-pathway, the PLC pathway is not recruited in all cells (Ascoli *et al.*, 1989). The activation of the PLC pathway seems to be more prominent in cells having high LHCGR density on the cell-membrane (Zhu *et al.*, 1994). High basal activity of PLC pathway has been found in certain activating LHCGR mutation (D578H) inducing Leydig cell adenoma and testotoxicosis in young boys having this mutation (Liu *et al.*, 1999) suggesting that activation of PLC pathway might be mitogenic in LCs. The importance of PLC pathway *in vivo* is still, however, unclear. In addition to these pathways, LHCGR has been shown to recruit the phospholipase A2 pathway (PLA2) in an older study (Johnson *et al.*, 1991).

An elegant way to study the importance of these specific pathways is to make Leydig cell specific knockout models of each of the G-proteins known to be activated by LHCGR, but at the moment there is no reports on this kind of experiments involving LHCGR signalling.

Desensitization: LHCGR, as many other GPCRs, is desensitized upon binding of an agonist (Ascoli *et al.*, 2002). This means attenuation of receptor responsiveness and function, and in the case of LHCGR, attenuation of steroidogenesis. The mechanisms for desensitization have recently been reviewed and include *i)* uncoupling of the receptors from the G proteins due to receptor phosphorylation, *ii)* internalization of cell surface receptors, *iii)* downregulation of receptor mRNA and protein synthesis, and *iv*) degradation of existing receptors (Fig. 2) (Ferguson, 2001; Moore *et al.*, 2007; Premont and Gainetdinov, 2007). Desensitization usually occurs with high doses and prolonged stimulation of a GPCR agonist.

Several aspects of the desensitization mechanisms of LHCGR have recently been clarified and reviewed (Ascoli *et al.*, 2002). Uncoupling of LHCGR from G-proteins has been shown to occur in mouse, porcine and human LHCGR. Non-visual arrestins, arrestin beta 1 and arrestin beta 2, and ADP ribosylation factor nucleotide exchange factor have shown to be responsible for uncoupling the LHCGR from G proteins (Mukherjee *et al.*, 1999; Mukherjee *et al.*, 2000; Hunzicker-Dunn *et al.*, 2002; Min *et al.*, 2002). All of the studies have suggested that nonvisual arrestins are important for desensitization, but the results of the importance of receptor phosphorylation are contradictory.

Studies carried out with porcine LHCGR suggest a phosphorylation-independent form of desensitization, whereas studies using human and rat LHCGR suggest a phosphorylation-dependent form of desensitization (Ascoli *et al.*, 2002). Nevertheless, the phosphorylation of the GPCRs is mainly mediated by G protein-coupled receptor kinases (GRK) leading to impaired coupling of G proteins with the receptors and thus impaired signal transduction (Ferguson, 2001; Moore *et al.*, 2007; Premont and Gainetdinov, 2007). Another desensitization mechanism, internalization, is also responsible for arrestins and GRKs, and thus phosphorylation of the receptors (Moore *et al.*, 2007). Human, mouse, rat and porcine LHCGR show internalization upon agonist binding via clathrin-coated pits, and this internalization is mediated by arrestins and dynamin (Ascoli *et al.*, 2002).

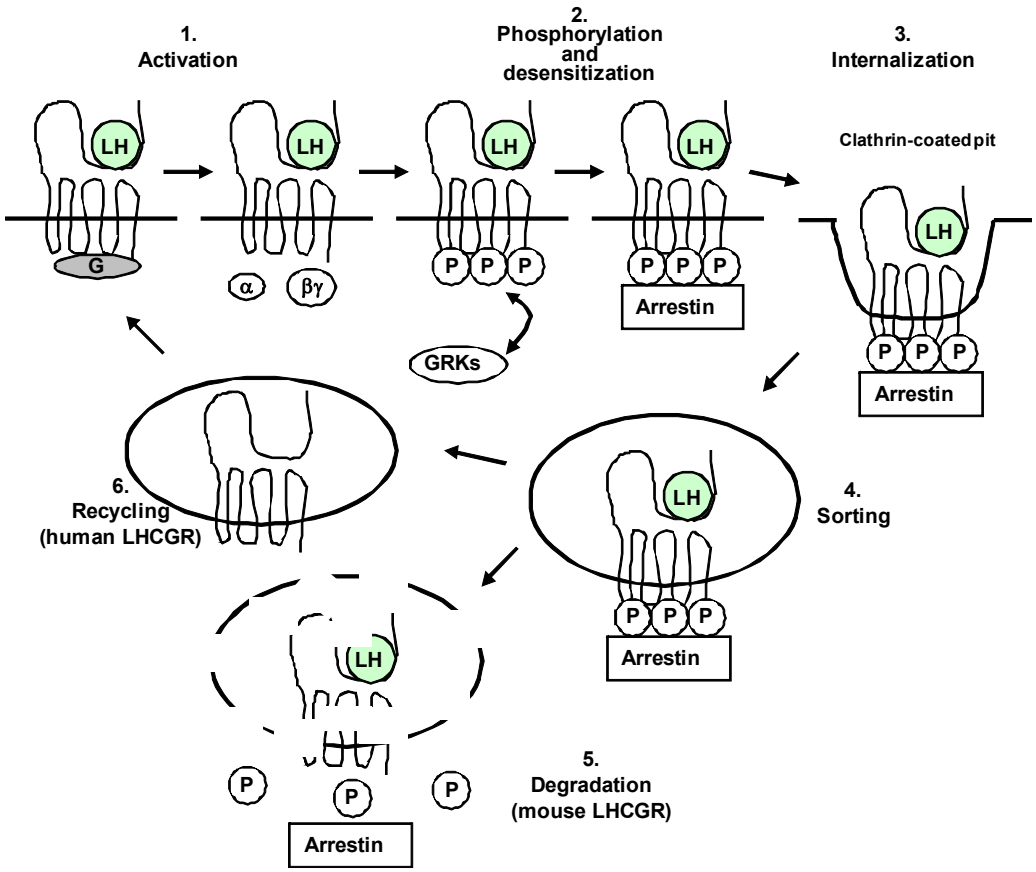


Fig. 2. *Intracellular trafficking of the LHCGR. 1) Activation; LHCGR is activated upon LH/hCG binding. Stimulatory G protein (Gs subunit) dissociates from G-protein complex and activates signalling cascades including adenylyl cyclase. 2) Desensitization; GRKs phosphorylate the intracellular domains of LHCGR, and arrestins bind to the receptor inhibiting the coupling of G-proteins, and signalling is terminated. 3) Internalization; Receptor-arrestin complex is internalized in clathrin-coated pits, and thus the number of cell-surface LHCGR is decreased. 4) Sorting; LHCGR is sorted even for 5) degradation (mouse, rat, and porcine) or 6) recycling back to the cell-membrane (human). (Modified from Moore, C.A.C., Annu. Rev. Physiol. 2007)*

The fate of internalized receptors also plays a role in controlling the cellular response. It is possible, that the agonist-receptor complex is recycled back to the cell membrane and thus becomes active again, or it is routed to lysosomes for degradation (Moore *et al.*, 2007). The complex of mouse, rat or porcine LHCGR and hCG accumulates in the lysosomes for degradation leading to the loss of cell-surface receptors (Ascoli *et al.*, 2002). However, the fate of the human LHCGR-hCG complex seems to be recycling rather than degradation suggesting further the distinctive regulation of desensitization process of LHCGR in different species (Kishi *et al.*, 2001). The classical view of the cell-surface and intracellular events of ligand activated GPCR is presented in Fig. 2.

Transcriptional and translational regulation of LHCGR: Although selection for degradation pathway can be regarded as a downregulation, this section focuses on regulation of LHCGR at the transcriptional and translational level. The best characterised hormone downregulating LHCGR transcription is LH/hCG itself, and at least in Leydig tumor cell line (MA-10) LHCGR downregulation is mediated through the cAMP-pathway (Nelson and Ascoli, 1992; Ascoli *et al.*, 2002). LH/hCG also downregulates LHCGR protein levels (Hsueh *et al.*, 1977). However, in fetal testis LH/hCG has been shown to increase LHCGR content (Huhtaniemi *et al.*, 1981). FSH and E2 have shown to upregulate LHCGR mRNA levels in granulosa cells (Shi and Segaloff, 1995), and PRL increases LHCGR expression in testis and ovaries (Huhtaniemi and Catt, 1981a; Gafvels *et al.*, 1992). P was shown to upregulate ovarian (Jones *et al.*, 1992) and downregulate testicular LHCGR (El-Hefnawy and Huhtaniemi, 1998).

2.1.3. Difference between LH and hCG in receptor binding, signal transduction, desensitization and downregulation

The difference in the amino acid sequence of LH and hCG suggests that there might also be some difference in the function of these two hormones. Indeed, the half-life of hCG is much longer making it biologically more potent than LH. Careful analysis of one hypogonadal male patient showing normal fetal development, impaired induction of puberty, and Te response for hCG stimulation, revealed the deletion of exon 10 in LHCGR (Gromoll *et al.*, 2000). Functional analysis of this mutant receptor revealed normal binding affinity, normal cellular trafficking, and normal desensitization, but impaired cAMP production upon LH stimulation (Muller *et al.*, 2003). hCG stimulation of this mutant receptor produced a normal cAMP-response. This suggests at least some functional difference between LH and hCG in the activation of signal transduction upon binding to LHCGR. Recent publication showing the differential response of cultured luteal granulosa cells for stimulation of LH and hCG supports the functional difference between these two hormones (Becker *et al.*, 2007). It has also been suggested that the binding affinity of hCG to LHCGR is higher than that of LH (McNeilly *et al.*, 1980; Huhtaniemi and Catt, 1981b). Altogether, the potential effects of LH and hCG might be slightly different, although the amplitude of the effects is small.

2.1.4. Regulation of LH and hCG secretion

LH is secreted in a pulsatile manner from the anterior pituitary gland. The main factors responsible for LH secretion are stimulatory gonadotropin releasing hormone (GnRH) secreted from the hypothalamus, and gonadal steroids exerting direct inhibition of LH secretion (Fig. 3). Many other hormones also affect LH secretion, but their actual role *in vivo* is negligible.

GnRH-producing neurons locate in the arcuate nucleus and preoptic area of the hypothalamus. Several neurotransmitters, including kisspeptin, control GnRH secretion to the circulation. Recent studies have revealed that kisspeptin-secreting neurons control the pulsatile secretion of GnRH through KISS1 receptor (KISS1R) (Smith *et al.*, 2006), and the mutations of this receptor cause hypogonadotropic hypogonadism (Seminara *et al.*, 2003). It has also been postulated, that kisspeptin-secreting neurons are the link between gonadal steroids and GnRH secretion (Smith *et al.*, 2006). GnRH

receptors are located in the gonadotrope cells in the anterior pituitary gland. These receptors belong to GPCR/seven-transmembrane domain receptors, and the main signalling pathway responsible for controlling LH synthesis and secretion is mediated through the G_q/PLC-pathway. After GnRH stimulation, gonadotrope cells synthesize and secrete LH, which in turn regulates gonadal functions.

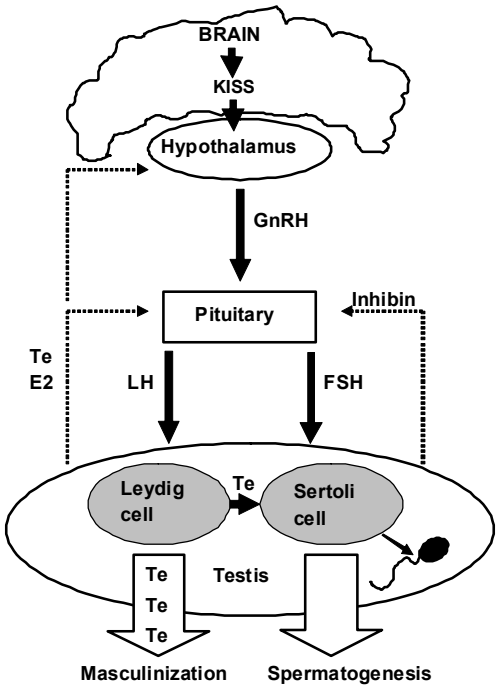


***Figure 3**. Hypothalamic-pituitary-testis axis. GnRH secretion from the hypothalamus is under the control of higher parts of the central nervous system. GnRH secretion is stimulated by kisspeptin though its hypothalamic receptor. GnRH stimulates pituitary secretion of LH and FSH. The main target of LH is Leydig cells, which in turn respond to LH stimulation by producing Te. Te supports Sertoli cells and spermatogenesis. Te also induces male masculinization, and it controls its own secretion by inhibiting the secretion of GnRH, LH and FSH. The main target of FSH is Sertoli cells, the cells needed for proper spermatogenesis. Sertoli cells also produce Inhibin, which negatively regulates FSH secretion. Arrows indicate stimulation, dotted arrows inhibition.*

hCG is secreted by the placenta. CGA is produced by the cytotrophoblastic cells, but the production of both CGA and CGB and thus the formation of dimeric hCG occurs only in the syncytiotrophoblast cells (Muyan and Boime, 1997). hCG can be detected already 8 to 10 days after ovulation, and its concentration peaks at weeks 9 and 10 of pregnancy and decreases thereafter (Muyan and Boime, 1997). Many hormones have been shown to control the secretion of hCG during the pregnancy, but the significance of these factors *in vivo* has remained unknown.

The presence of hCG or CGB in the circulation of a non-pregnant individual is usually a sign of neoplasms. Trophoblastic diseases and germ cell tumors usually produce hCG, and in these conditions hCG is a good diagnostic tool and a biomarker for monitoring the outcome of therapy (Stenman *et al.*, 2004). Furthermore, hCGB is also secreted by some cancers including bladder, renal and prostate cancers, and it usually associates with poor prognosis of the disease (Stenman *et al.*, 2004). However, we need to keep in mind that hCG is also used as an anabolic agent and as a stimulant for evoking testicular steroidogenesis after the misuse of anabolic steroids.

2.1.5. Physiological nongonadal effects of LH/hCG

Thus far, it has been considered that LH/hCG/LHCGR have an important role only in reproductive function. In recent years, as seen in Table 1, several studies have shown

the expression of LHCGR in normal extra-gonadal tissues in many species. This has led to speculation that LH/hCG might also have some physiological effects in non-gonadal tissues (Rao, 2001; Shemesh, 2001; Fields and Shemesh, 2004; Filicori *et al.*, 2005; Berndt *et al.*, 2006). The contentious issue has been whether the receptor is functional or not. Basically, the expression level of nongonadal LHCGR has been much lower than the level seen in the ovaries, and the length of the transcript and the protein has been varied in non-gonadal tissues suggesting that the receptor might not be functional (Stewart, 2001). One interesting finding has been the expression of LHCGR in the placenta, uterus and cervix, where LHCGR might be important during pregnancy, when high amount of natural ligand is present (Reshef *et al.*, 1990; Han *et al.*, 1997; Zhang *et al.*, 2001b; Lin *et al.*, 2003; Filicori *et al.*, 2005). Recently, we have shown that female mice lacking LHCGR are able to become pregnant, maintain pregnancy and deliver and nurse healthy pups, after their own ovaries were replaced by normal WT ovaries (Pakarainen *et al.*, 2005b). This result suggest that there is no physiologically important role for uterine LHCGR, although we have to keep in mind the physiological difference between mice and man, the first not having chorionic gonadotropin and the latter with extremely high hCG levels during pregnancy. Subjects who have inactivating mutation in their LHCGR present only a gonadal phenotype, and the nongonadal phenotypes observed can be explained by the impaired gonadal function (Themmen and Huhtaniemi, 2000).

2.1.6. Effects of LH/hCG on nongonadal neoplasms

The role of elevated LH and LHCGR in the development of ovarian cancers is well established (Simon *et al.*, 1983; Wise *et al.*, 1996; Parrott *et al.*, 2001). High cancer incidence after menopause has raised the question, whether the increased gonadotropin level participates in the tumorigenic processes. The role of LH/hCG/LHCGR in the development of prostate and mammary gland and uterine cancers has been recently implicated (Lei *et al.*, 1993b; Meduri *et al.*, 1997; Tao *et al.*, 1997a) by showing only the presence of LHCGR in the respective tissues. The best evidence of the function of LH/LHCGR in promoting tumorigenesis is obtained from adrenal cancers. It has been shown that normal human adrenal cortex expresses LHCGR, and some human adrenal tumors also express LHCGR and show response for gonadotropin (Pabon *et al.*, 1996b; Couzinet *et al.*, 2001; Lacroix *et al.*, 2001; Christopoulos *et al.*, 2005; Saner-Amigh *et al.*, 2006; Bourdeau *et al.*, 2007). Several genetically modified mouse models show adrenal tumorigenesis as a response to high circulating LH levels. *Inhibin* α (*Inha*) KO mice and TG mice expressing SV40 T-antigen under the *Inha* promoter develop adrenal tumors after gonadectomy (Kumar *et al.*, 1996; Rilianawati *et al.*, 1998). Recently it was shown that gonadectomy-induced LHCGR expression in the adrenal gland is similar in tumorigenic and non-tumorigenic strains of mice (Bernichtein *et al.*, 2008b). However, authors found a complex multigenic trait driven by epistatic loci underlying the tumorigenic phenotype and suggested that this loci affects downstream of LHCGR (Bernichtein *et al.*, 2008b). hCG was also shown to induce adrenal tumorigenesis in nude mice, in which Chinese hamster ovary cells expressing and secreting hCG was xenografted (Bielinska *et al.*, 2005).

As in the case of the physiological significance of nongonadal LH/LHCGR, the role of LH/LHCGR in the development of nongonadal neoplasms (except adrenal neoplasms) is still poorly known. It is possible that some cancers express LHCGR, and thus LH could modulate cancer progression through its receptors. However, according to the current literature, we can conclude that elevated LH is not a major contributor in tumorigenesis after the menopause/andropause.

Table 1. *LHCGR found in nongonadal tissues of humans and rodents.*

Tissue	*Species*	*References*
male reproductive tissues		
prostate	rat	*(Reiter et al., 1995; Tao et al., 1995)*
seminal vesicles	rat	*(Tao et al., 1998b)*
sperm	human	*(Eblen et al., 2001)*
female reproductive tissues		
cervix	human	*(Lin et al., 2003)*
oviduct	human	*(Lei et al., 1993c; Han et al., 1996)*
	mouse	*(Zhang et al., 2001b)*
placenta	human	*(Reshef et al., 1990)*
uterus	human	*(Reshef et al., 1990; Han et al., 1997)*
	mouse	*(Zhang et al., 2001b)*
Other tissues		
adrenal gland	human	*(Pabon et al., 1996b)*
	mouse	*(Bielinska et al., 2003; Bernichtein et al., 2008b)*
	rat	*(Apaja et al., 2005)*
blood vessel[a]	human	*(Reshef et al., 1990)*
	mouse	*(Berndt et al., 2006)*
brain	rat	*(Lei et al., 1993a; Apaja et al., 2004)*
fetal tissues[b]	human	*(Abdallah et al., 2004)*
kidney	rat	*(Apaja et al., 2005)*
lymphocyte	human	*(Lin et al., 1995)*
mammary gland	rat	*(Tao et al., 1997b)*
skin	human	*(Pabon et al., 1996a; Venencie et al., 1999)*
spinal cord	rat	*(Rao et al., 2003)*
umbilical cord	human	*(Rao et al., 1993)*
urinary bladder	human	*(Tao et al., 1998a)*

a; endometrial and myometrial blood vessels, b; fetal kidneys, liver, pancreas, lung, small and large intestines and adrenals.

2.2. LH/hCG in male reproductive functions and testicular neoplasms

Recently, several studies have been carried out to reveal the role of LH/hCG/LHCGR in male reproductive development. The importance of mutations found in LH and LHCGR in relation to human male reproductive development and function in adult age has recently been clarified. Also, several activating LHCGR mutations have been found in humans, and one of them has been linked to LC adenomas (Liu *et al.*, 1999). Although the prevalence of testicular neoplasias has increased, elevated LH-action has not been regarded as the cause of it, but rather as a sign of a plethora of impaired testicular functions termed testicular dysgenesis syndrome (TDS) (Skakkebaek *et al.*,

2007). Together with these clinical findings, several different genetically modified mouse models have been created to improve the understanding of the role of LH/hCG/LHCGR on male reproduction. These mouse models have shown important differences between mice and man in the respect of reproductive function. Therefore, the following review focuses on mice, and highlights the most important differences between man and mouse.

2.2.1. Testicular effects of LH/hCG

The direct testicular effect of LH and hCG is limited in the LCs, the only testicular cells expressing LHCGR. There are also several reports of the expression of LHCGR in the nongonadal tissues of males including the prostate, seminal vesicles and sperm (Reiter *et al.*, 1995; Tao *et al.*, 1995; Eblen *et al.*, 2001). This raises the question of the important role of nongonadal LH/hCG action for male reproductive function.

2.2.2. LH/hCG in the development of male phenotype

In humans, functional hCG and LHCGR are needed for the normal masculinization of the male fetus. Their effect takes place after primary sex determination, i.e. gonadal differentiation, and the main mediator is Te, produced by LCs upon LH/hCG stimulation. Clinical cases have demonstrated that inactivating mutations of LHCGR lead to Leydig cell hypolasia (LCH), the severity of which is associated with the degree of the mutations. Phenotypes vary from complete male pseudohermaphroditism to micropenis with hypospadias, indicating that Te is needed for the formation of male type external genitalia and secondary sex characteristics. (Themmen and Huhtaniemi, 2000) As expected, no inactivating mutations of the CGB subunit have been found, because it is deletorious for pregnancy. Four men with inactivating mutation in LHB-subunit have been described (Weiss *et al.*, 1992; Valdes-Socin *et al.*, 2004; Lofrano-Porto *et al.*, 2007). They were normally masculinized at birth, but spontaneous puberty was missing and men were infertile (Weiss *et al.*, 1992; Valdes-Socin *et al.*, 2004). These findings demonstrate that intact hCG is able to induce fetal Te synthesis for normal fetal masculinization.

In mice, the situation is different as they do not have chorionic gonadotropin. Analysis of mouse models with mutated LHCGR and LHB has revealed that LH and LHCGR are not necessary for normal fetal development indicating that fetal Leydig cells in mouse produce Te in a gonadotropin independent manner (Charlton, 1984; Kendall *et al.*, 1995; El-Gehani *et al.*, 1998; Lei *et al.*, 2001; Zhang *et al.*, 2001a; Ma *et al.*, 2004). This highlights the special function of fetal LCs.

2.2.3. Fetal Leydig cells

Two types of LCs appear in the testis. The first LCs are called fetal LCs (FLCs), and their main function is to produce Te to induce masculinization of the male urogenital system. Several factors responsible for FLC development and differentiation have been found (Habert *et al.*, 2001; Wu *et al.*, 2007). Briefly, after primary sex differentiation, FLCs arise from three possible sources; 1) the coelomiec epithelium, 2) the migrating mesonephric population including neural crest cells or 3) the common early precursor

of the adrenal steroid cells (Habert *et al.*, 2001). At the moment, platelet-derived growth factor receptor alpha (PDGFRA), desert hedgehog (DHH), X-linked aristaless-related homeobox gene and transcription factor GATA-4 have been shown to be necessary for FLC differentiation (Kitamura *et al.*, 2002; Yao *et al.*, 2002; Brennan *et al.*, 2003; Bielinska *et al.*, 2007). One important factor responsible for normal steroidogenic potential of FLC is steroidogenic factor 1 (*Nr5a1*). It is directly regulated by DHH through its receptor Patched-1 and indirectly by PDGF-A/PDGFRA (Yao *et al.*, 2002; Brennan *et al.*, 2003). It was shown that the failure of FLC development in DHH KO mice was due to impaired induction of steroidogenic factor 1 and P450 side chain cleavage (CYP11A1) and thus impaired testosterone production (Yao *et al.*, 2002). When steroidogenic factor 1 is properly induced, FLCs develop normally and start to produce a sufficient amount of Te at day 13 *post coitum* (*pc*.) in mice to cause secondary sex determination and masculinization of male fetus. This secondary sex determination includes the formation of male external genitalia, accessory sex organs and proper testicular descent together with insulin like factor 3 (Insl3) (Nef and Parada, 1999). It is notable that FLCs and their Te production are independent of LH/LHCGR and of the whole pituitary gland, as suggested by the presence of normal male mice at birth, when the pituitary gonadal axis is disturbed (Charlton, 1984; Kendall *et al.*, 1995; Lei *et al.*, 2001; Zhang *et al.*, 2001a; Pakarinen *et al.*, 2002; Ma *et al.*, 2004). It is noticeable that LHCGR appears in FLCs around day 16 *pc.* in mice and rats (Zhang *et al.*, 1994; O'Shaughnessy *et al.*, 1998). Therefore, FLCs are able to respond to LH by increasing Te production. At the end of the fetal period, FLCs begin to regress functionally and the current view is that they also remain silent in adult rat testis (Kerr and Knell, 1988; Ariyaratne and Chamindrani Mendis-Handagama, 2000).

In humans, the FLCs show cytodifferentiation and the expression of LHCGR until the tenth week of fetal life (Huhtaniemi and Pelliniemi, 1992), but testicular Te production beguns already at week seven (Tapanainen *et al.*, 1981). After the appearance of LHCGR into FLCs at week ten in fetal life, FLCs start to produce Te in hCG dependent manner (Habert *et al.*, 2001). After the week 22 in fetal life, Te production is at least partly dependent on the functional pituitary-gonadal axis and thus the production of LH (Habert *et al.*, 2001). Disturbances of proper fetal Te production due to inactivating mutation in LHCGR lead to LCH with complete male pseudohermaphroditism in most severe cases as already mentioned above. However, even in most severe cases, the structures (vasa deferentia, epididymides) derived from the Wolffian ducts are found indicating that human fetal testis is also able to produce Te in LH/hCG independent manner (Habert *et al.*, 2001), since the men having complete androgen insensitivity syndrome show male pseudohermafroditism but no signs of Wolffian ducts derived structures due to complete block of androgen action (Hannema *et al.*, 2004). FLCs appear in human testis also after the birth and they are responsible for testosterone surge at the age of three months regressing thereafter (Habert *et al.*, 2001). Taken together, the clear functional difference between human and mouse FLCs is that the sufficient Te production to induce formation of external genitalia and masculinazation requires intact LHCGR/hCG axis in the human FLCs.

At the moment, the switch determining the regression of FLC is not known. It is possible that the low gonadotropin levels during childhood and before puberty is responsible for the regression of FLCs, but there is no solid data on this. However, the

appearance of fully differentiated ALCs seems to associate with the normal puberty with increased LH levels, since the mice lacking functional LHCGR, ALCs are not present (Zhang *et al.*, 2001a). Also the mice having an inactivating mutation in LHB subunit, adult Leydig cells are not developed, but as in the case of men having inactivating LHB mutation, hCG injections were able to rescue pubertal development suggesting that ALCs appear into the testes upon hCG stimulation (Ma *et al.*, 2004; Valdes-Socin *et al.*, 2004).

The most important difference between adult and fetal Leydig cells is the lack of desensitisation of LHCGR in FLCs (Huhtaniemi *et al.*, 1982b; Huhtaniemi *et al.*, 1985b; Pakarinen *et al.*, 1990). This is a peculiar phenomenon among the G-protein coupled receptors, because they often tend to show desensitization upon agonist binding (Premont and Gainetdinov, 2007). The mechanism for this is not known, but it might have a role in the protection of fetal masculinization. Neither is it known whether other GPCRs show similar properties in FLCs, thus prompting further evaluation of GPCR desensitization in FLCs.

2.2.4. LH/hCG in adult males

All the known functions of LH in adult males are mediated by Te. Appropriate LH-levels are needed for normal Te synthesis, for normal reproductive function, and for normal masculinization and development of secondary sex characteristics. This has been shown by treating *hpg*-mice and *Lhcgr* KO mice with Te. In both models, secondary sex characteristics were recovered by Te treatment (Singh *et al.*, 1995; Pakarainen *et al.*, 2005a). Our results demonstrate that Te replacement therapy for *Lhcgr* KO males was able to partially rescue the fertility of *Lhcgr* KO males (Pakarainen *et al.*, 2005a). Decreased fertility of Te treated *Lhcgr* KO mice is most probably caused by the inflammatory reaction seen in Te treated epididymides and prostate (Pakarainen *et al.*, 2005a). This reaction was recently shown to be mediated through Te aromatization and ESR1 activation (Savolainen *et al.*, 2007). Lei et al. also carried out Te replacement therapy of *Lhcgr* KO males, but they did not detect recovered fertility of Te treated males (Lei *et al.*, 2003). Two explanations for this discrepancy are the later starting age (21 d vs. 30 d) and the shorter duration (60 d vs. 21 d) of the Te replacement compared with our study (Lei *et al.*, 2003; Pakarainen *et al.*, 2005a). Old male mice without functional LHCGR start to produce sperm, which is inhibited by antiandrogens indicating that propgression of spermatogenesis needs only small quantities of Te (Zhang *et al.*, 2003). There have also been several male contraception trials which use androgens in some form to decrease the secretion of LH and testicular Te production. This has been an efficient way to decrease sperm counts, but there has been a difficulty to achieve complete azoospermia. These results demonstrate that LH-action is needed only for production of Te, which is able to drive all necessary male reproductive functions. Clinical data of four men having an inactivating mutation of the LHB subunit and data of *Lhb* KO males also suggests the same. Both men and mice lacking functional LH responded to exogenous hCG-treatment with the beginning of puberty and development of secondary sex characteristics and spermatogenesis (Weiss *et al.*, 1992; Ma *et al.*, 2004; Valdes-Socin *et al.*, 2004; Lofrano-Porto *et al.*, 2007).

2.2.5. Adult Leydig cells

In rodents, adult Leydig cells (ALC) are not derived from FLCs. ALCs are derived from undifferentiated precursor cells. These cells proliferate neonatally and form a population of spindle-shaped undifferentiated "mesenchymal-like" cells (Hardy *et al.*, 1989). Recently, some of these spindle-shaped cells were shown to be different than the progenitor Leydig cells (PLCs) in that they did not express *Hsd3b1* or *Lhcgr,* but were *Pdgfra* positive (Ge *et al.*, 2006). These cells were able to proliferate and expand indefinitely, differentiate into the LCs, and colonize the testicular interstitium and differentiate into LCs (Ge *et al.*, 2006). It has been shown that these cells are stem Leydig cells (SLC) (Ge *et al.*, 2006). Upon appropriate stimulus, these SLCs commit to a Leydig cell lineage, are transformed into a spindle-shaped progenitor Leydig cells, and start to express some markers for Leydig cells including 3β-hydroxysteroid dehydrogenase, cytochromes P450scc and P450c17 (CYP11A1 ja CYP17A1), LHCGR and produce androgens (Hardy *et al.*, 1990; Ariyaratne *et al.*, 2000). Around day 28 in rats, PLCs are transformed into immature Leydig cells (ILC), where the activity of steroidogenic enzymes mentioned above is increased (Dupont *et al.*, 1993; Shan *et al.*, 1993). After dividing once between the ages of 28-56 days, ILCs differentiate to mature ALCs having the capacity to synthesize Te. Several factors have been shown to mediate the development, normal maturation and differentiation of ALC. These include functional LH/LHCGR-pathway, androgens, DHH, and PDGFA (Kendall *et al.*, 1995; Clark *et al.*, 2000; Gnessi *et al.*, 2000; Lei *et al.*, 2001; Zhang *et al.*, 2001a; O'Shaughnessy *et al.*, 2002; Ma *et al.*, 2004).

2.2.6. Testicular neoplasm

In recent decades the incidence of testicular cancer, and especially testicular germ cell tumors (GCT) has increased dramatically, but the reason for this has remained unsolved (Skakkebaek *et al.*, 2007). The age-adjusted incidence of GCT is now 10 per 100000 in high risk countries like Denmark and Norway (Skakkebaek *et al.*, 2007), and the lifetime risk of man to get testicular cancer is 1% among Norwegian and Danish men (Skakkebaek *et al.*, 2007). GCTs are the most common testicular cancers representing 90-95 % of cases. A clear demographic variation in the incidence of testicular cancer exists. In Finland the risk is clearly lower than in Denmark and Norway, but the risk is still substantially higher than some decades ago. Potential risk factors for GCT are low birth weight, premature birth, birth order, high maternal estrogens or bleeding during the pregnancy, high maternal age, high maternal body weight, and jaundice (Rajpert-De Meyts *et al.*, 2006). Recently, it was also shown that high risk of cryptorchidism is associated with high risk of testicular cancer suggesting the similar pathogenic mechanism between these two diseases (Boisen *et al.*, 2004). Furthermore, one reproductive disorder increases the individual's risk to gain another reproductive disease excluding sexually transmitted disease. Chromosomal abnormalities and androgen insensitivity syndrome are also related to GCTs. As expected, unilateral testicular tumor increases the risk of development of new primary tumor in contralateral testis. One important risk factor for GCT is low sperm production, and thus a lower fertility rate (Jacobsen *et al.*, 2000a; Jacobsen *et al.*, 2000b).

To explain the testicular cancer risk and the other male reproductive disorders, the term TDS has been created (Skakkebaek *et al.*, 2001). This term integrates all the facets of male reproductive disorders under one broader term, the purpose of which is to emphasize that all the individual disorders could be explained by a common, yet unknown origin.

2.2.7. Leydig cell tumors

The incidence of Leydig cell tumors (LCT) is very low in humans, the age-adjusted rate is 0.4 per million (Gilliland and Key, 1995) although they are commonly found in laboratory animals, especially in rats. In humans, LCTs are rarely malignant, and often nodular in structure. Because the disease is rare, pathological definitions between hyperplasia and adenoma do not exist, but an arbitrary criterion has been created. When a LC nodule is larger than the diameter of seminiferous tubules, it can be regarded as an adenoma (Clegg *et al.*, 1997). In humans, a certain activating LHCGR mutation leads to LC adenomas and precocious puberty (Liu *et al.*, 1999; Richter-Unruh *et al.*, 2002).

Recently, the presence of LC micronodules in the testis of men with impaired spermatogenesis, and with a decreased Te/LH ratio highlights the importance of the role of elevated LH/hCG in LC tumorigenesis (Holm *et al.*, 2003). Furthermore, an activating mutation in stimulatory G protein has been found in LCT (Fragoso *et al.*, 1998). Recently, two different *fumarate hydratase* mutations were found in two LCT patients (Carvajal-Carmona *et al.*, 2006).

In contrast, several pharmacological compounds have been shown to induce LCT in laboratory animals. These compounds affect Leydig cell tumorigenesis mainly by increasing LH levels. They inhibit testicular Te synthesis (enzyme inhibitors, estrogens), decrease Te action on peripheral tissues [5α-reductase inhibitors, androgen receptor (AR) blockers] or directly affect the pituitary gland by increasing LH secretion (aromatase inhibitors) (Cook *et al.*, 1999) (figure 4). Some genetically modified mouse models have also been shown to develop LCT. These include aromatase overexpression (Fowler *et al.*, 2000; Sirianni *et al.*, 2007), and SV40 T-antigen expression under *Inha* promoter (Kananen *et al.*, 1996; Mikola *et al.*, 2003). All these studies emphasize that LH/LHCGR action is important mediator for LCT development. Because many of the pharmacological compounds inducing LCT also decrease circulating androgens and/or prevent androgen action at a tissue level by blocking its receptors, it is also possible that the lack of androgens is the growth stimulus for LCs. Sertoli cell specific *androgen receptor* (*AR)* knockout mouse have already been created (De Gendt *et al.*, 2004), and LC specific AR knockout mice could elucidate the role androgens in LCTs, if the normal development of LCs is not prevented.

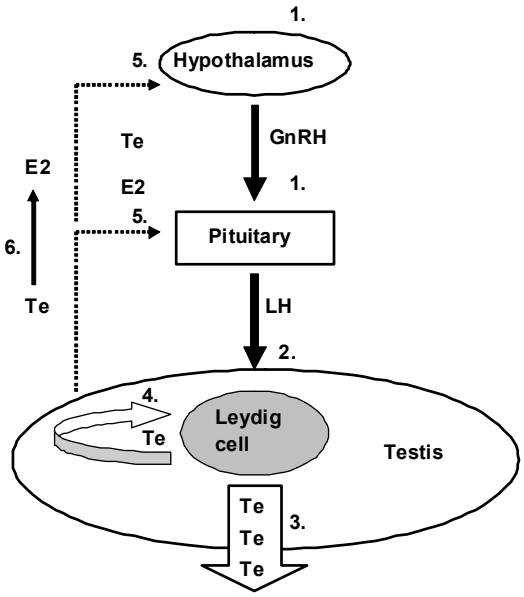


Figure 4. *The suggested sites of disruption of hypothalamic-pituitary-testis axis leading to Leydig cell tumors. 1) Agents or tumors leading to increased secretion of GnRH and LH. 2) Activating mutations of LHCGR or agents increasing the number and activity of LHCGR. 3) Agents decreasing Te production leading to decreased negative feedback regulation and elevated LH levels. 4) Impaired LC autoregulation due to decreased Te production or Te bioavailability (AR blocking, AR mutations). 5) Decreased negative feedback regulation and elevated LH levels due to AR blocking. 6) Decreased negative feedback regulation and elevated LH levels due to blocking the aromatization of Te to E2.*

2.3. Pituitary gland and LH/hCG

The pituitary gland is the operational center of many necessary endocrine functions. It assembles multiple signals coming from the central nervous system through the hypothalamus. These signals are transformed into an understandable format and forwarded to the periphery by secretion of approximately 10 important hormones, which we need in our daily life. The pituitary gland also receives information of the function of peripheral target organs and it is able to modulate its own secretory function by using feedback information (Figure 5).

The pituitary gland is divided into two anatomically and functionally separated areas; the adenohypophysis (the anterior and intermediate lobes) and neurohypophysis (posterior lobe). The adenohypophysis contains six different hormone-producing cell types classified by the hormones they secrete. Somatotropes secrete growth hormone (GH), lactotropes secrete prolactin (PRL), corticotropes produce adrenocorticotrophic hormone (ACTH), thyrotropes synthesize thyroid stimulating hormone (TSH), and gonadotropes secrete follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Melanotropes in the intermediate lobe produce α melanocyte-stimulating hormone (α-MSH). Folliculostellate cells are epithelioid, non-secretory cells in the anterior pituitary, and they have been thought to communicate with, and mediate the secretory signals between, the other cell types (Ooi *et al.*, 2004). Neurohypophysis secretes oxytocin and vasopressin. The peripheral functions of the hormones above are shown in Figure 6. In the following, most attention is paid on the development, function and tumorigenesis on lactotroph cells.

2.3.1. Pituitary development

The development of the pituitary gland in mammals is well characterized. In mammals, the development of the anterior pituitary gland begins when the roof of the oral cavity is invaginated towards the ventral diencephalon forming an anatomical structure called Rathke's pouch (RP) (Zhu *et al.*, 2007). A proper spatiotemporal expression of certain transcription factors and the induction of certain signal transduction pathways are needed for the normal development of the pituitary gland. Interference of this system by the gene knockout approach or by introducing certain signal transduction systems or transcription factors even at the wrong magnitude, time or place have been shown to lead impaired development of pituitary gland.

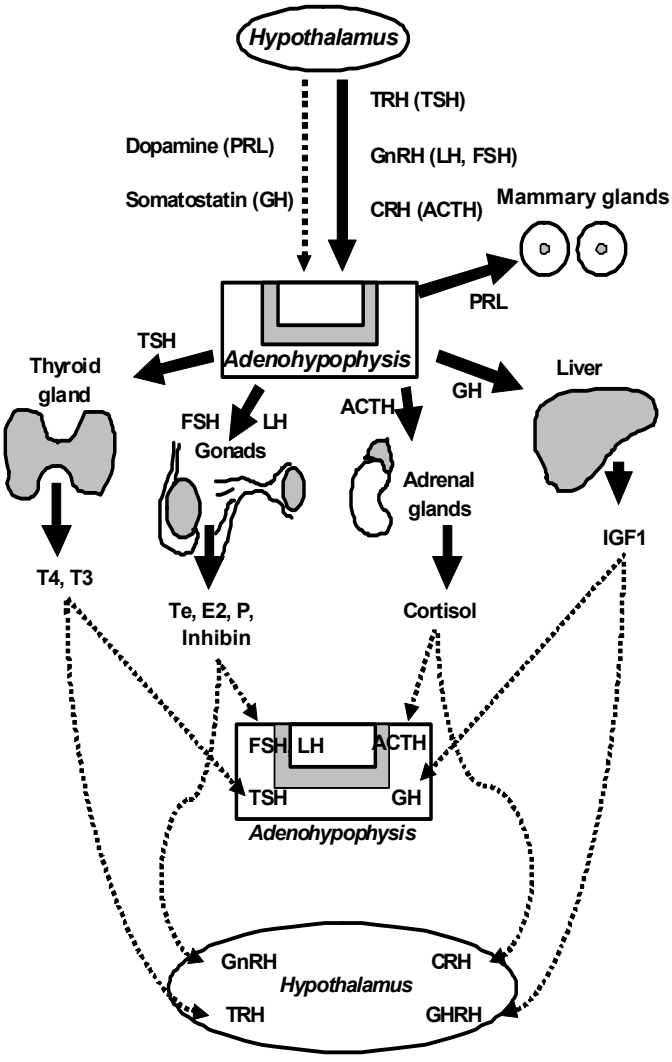


Figure 5. *Hypothalamic-pituitary-target-organ-axes. The hypothalamus controls the pituitary gland by secreting mainly stimulatory hormones. The PRL is regulated mainly by inhibitory hormone dopamine, and somatostatin inhibits GH secretion. Target organs secrete several hormones, which affect in the peripheral tissues. These hormones exert negative feedback effect to the pituitary gland and to the hypothalamus to control their own secretion. Hormone(s) in parenthesis indicate(s) the target hormone. Arrows indicate stimulation and dotted arrows inhibition.*

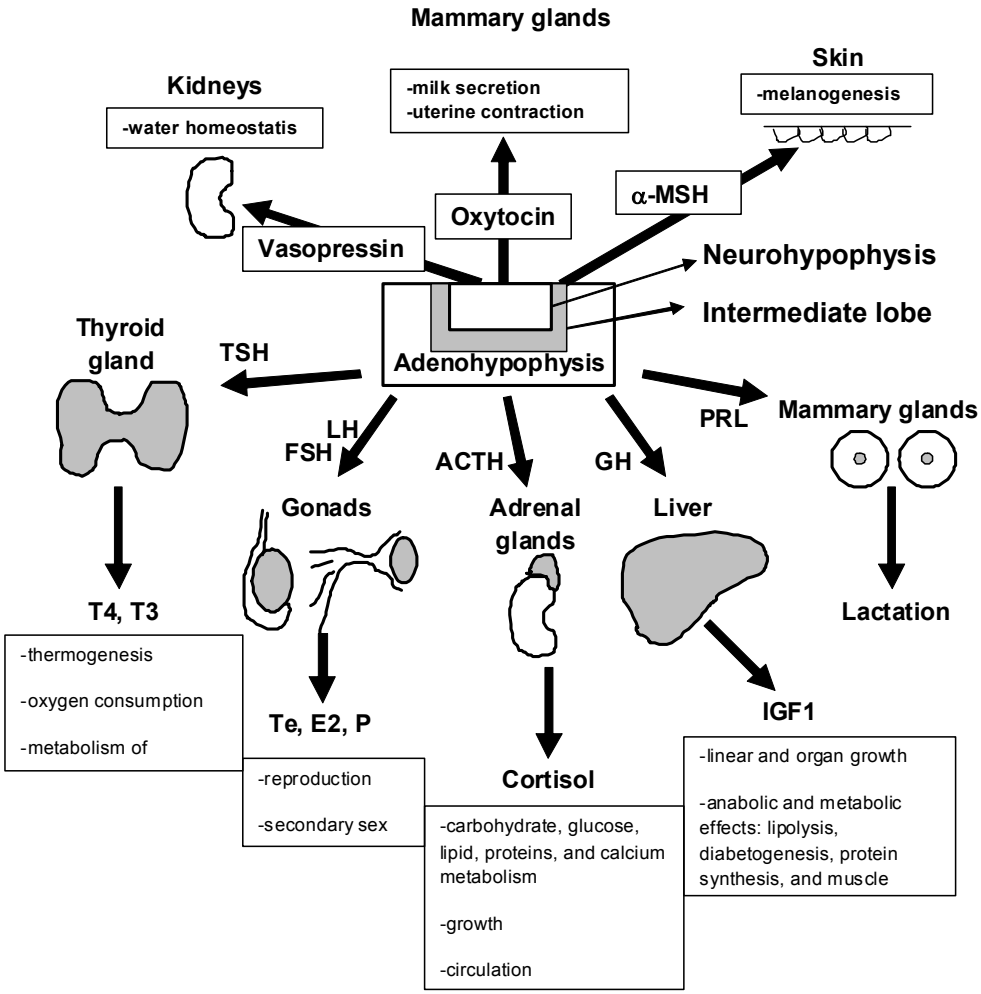


Figure 6. *The pituitary gland – master regulator of endocrine functions. Direct target organs and the most important functions of the pituitary gland are presented in this figure. Adenohypophysis secretes 6 important hormones controlling many important and vital endocrine systems. Intermediate lobe secretes α-MSH especially in animals, but the importance of this hormone in humans is controversial. Neurohypophysis secretes 2 important hormones responsible for water balance and milk secretion.*

2.3.1.1. Transcription factors needed for anterior pituitary gland development

Several transcription factors are needed for normal pituitary development. This section will focus on some of the most important factors responsible for the early development of anterior pituitary gland, and they are listed in order of appearance in the pituitary gland. Transcription factors important for the differentiation of the Pit1 cell lineage are also mentioned. A short review of these transcription factors is shown in Table 2.

Table 2. *Short summary of transcription factors needed for pituitary development and the phenotype of inactivating mutations in humans.*

Transcription factor	***Pituitary phenotype of KO model***	***Pituitary phenotype of inactivating mutations in humans***
Pitx1	Decreased expression of Lhb, Fshb, and Tshb, and increased expression of Pomc	Not found
Pitx2	Full KO; no anterior lobe, some corticotropes exist Hypomorphic: no gonadotropes, decreased somatotropes and thyrotropes	Rieger sdr 1; IGHD
Isl1	Impaired RP proliferation	Not found
Lhx3	RP is formed, only residual corticotropes present, other lineages not exist	CPHD; Intact ACTH levels, decreased GH, PRL, FSH, LH and TSH levels
Lhx4	Pituitary hypoplasia: decreased somatotropes, thyrotropes, gonadotropes, and corticotropes	CPHD; decreased GH, ACTH and TSH levels
Hesx1	Bifurcated pituitary gland, 5% lacks pituitary gland completely, pituitary hypoplasia; IGHD to CPHD	IGHD to CPHD
Prop1	Decreased somatotropes, lactotropes and thyrotropes, delayed gonadotropes	Most common cause of CPHD; decreased GH, PRL and TSH levels
Pit1	Decreased somatotropes, lactotropes and thyrotropes	CPHD; decreased GH, PRl, TSH (often delayed onset)
Gli2	Embryonally lethal, normal RP exist	Pituitary anomalies
Six3	Failure of the development of pituitary gland	Not found
Six6	Pituitary hypoplasia	Not found
Pax6	Expansion of ventral cell types, especially thyrotropes, decreased lactoropes and somatotropes	Not found

IGHD; isolated growth hormone deficiency, CPHD; combined pituitary hormone deficiency.

Paired-like homeodomain transcription factor 1 and ***2*** (***Pitx1*** and ***Pitx2***). They are expressed at embryonic day (E) 8.5 in the oral plate, which gives rise to RP (Gage *et al.*, 1999a). Thereafter, they are expressed in the anterior and intermediate lobes of the pituitary gland during proliferation and differentiation of the anterior pituitary cell lineages (Suh *et al.*, 2002). The expression pattern of these two transcription factors are similar, and they have been shown to redundantly control pituitary cell proliferation, survival and differentiation (Kioussi *et al.*, 2002; Suh *et al.*, 2002; Charles *et al.*, 2005). Knocking out the *Pitx1* gene leads to severe hindlimb defects, cleft palate, and lethality soon after the birth (Lanctot *et al.*, 1999; Szeto *et al.*, 1999). The pituitary glands of

these mice show decreased expression of *Fshb*, *Lhb*, and *Tshb*, and increased expression of *Pomc* (Lanctot *et al.*, 1999; Szeto *et al.*, 1999). No human disease showing inactivation of PITX1 has been identified.

Pitx2 deficient mice display a failure of the body-wall closure, right pulmonary isomerism, and defects in heart, tooth, eye, and pituitary development, and homozygosity of Pitx2 null allele is embryonally lethal (Lin *et al.*, 1999; Lu *et al.*, 1999). In these mice, the pituitaries show the development of RP, but it fails to expand and develop anterior lobe, most probably due to failure of maintaining expression of *Hesx1*, *Lhx3*, and *Prop1*, thus showing only few corticotropes and no cells from Pit1 lineage (Gage *et al.*, 1999b; Lin *et al.*, 1999). Mice with hypomorphic *Pitx2* have been created, and these mice showed lack of gonadotropes and decreased number of somatotropes and thyrotropes, but unaffected corticotropes (Gage *et al.*, 1999b). In humans, inactivated mutation of *PITX2* leads to Rieger syndrome 1, an autosomal dominant condition showing defects in the development of the eye, teeth, umbilicus and pituitary gland together with isolated GH deficiency (IGHD) (Semina *et al.*, 1996).

LIM-type homeodomain transcription factors (***Isl1***, ***Lhx3***, and ***Lhx4***). The *ISL1 transcription factor, LIM/homeodomain (**Isl1)*** gene is detected in the oral ectoderm at E8.5, and thereafter in the RP at E9.5, and especially the ventral part of the RP at E10.5. Its expression is induced by ventral signal BMP2 and repressed by dorsal signal FGF8/FGF2 (Ericson *et al.*, 1998). Inactivation of *Isl1* leads to embryonic lethality at E10 with the presence of primitive RP which consists of a very thin epithelium suggesting the role of Isl1 in proliferation and differentiation of pituitary progenitor cells (Takuma *et al.*, 1998).

LIM homeobox protein 3 (Lhx3) gene is expressed in the RP starting at the age E9.5 and its expression also persists in the adult pituitary gland, especially in the anterior and intermediate lobes (Zhadanov *et al.*, 1995; Zhu *et al.*, 2007). Mice without functional *Lhx3* die during the first 24 hours of their life. They lack the anterior and intermediate lobe of the pituitary gland. Pituitary gland development is arrested after the formation of Rathke's pouch, because of the decreased expression of *Hesx1* and a lack of induction of *Pit1*. Only the residual corticotropes appear in the pituitary gland, the rest of the cell types are missing suggesting the importance of *Lhx3* for lactotrope, somatotrope, thyrotrope and gonadotrope specification. These studies also suggested that *Lhx3* acts downstream of *Pitx1* and *Pitx2* for preventing apoptosis and promoting cell survival. (Sheng *et al.*, 1996; Zhao *et al.*, 2006) Humans with inactivating mutations in *LHX3* show combined pituitary hormone deficiency (CPHD) with impaired production of GH, PRL, FSH, LH and TSH, but with intact ACTH levels, exactly like patients with a *PROP1* mutation (Netchine *et al.*, 2000).

LIM homeobox protein 4 (Lhx4) gene is expressed in the RP at E9.5 and its expression is restricted to the parts which become the anterior lobe of the pituitary gland at E12.5. At E15.5 its expression is downregulated, but it is also found in the anterior and intermediate lobes of the adult pituitary gland, although the expression levels are far below that of *Lhx3*. (Li et al., 1994) Knockout mice for *Lhx4* have been created, and these mice die immediately after birth due to the problems in the lungs (Li *et al.*, 1994). In their pituitary gland, cell death is increased between E12.5-14.5, but at E18.5 cell differentiation has occurred, although the number of somatotropes, thyrotropes, gonadotropes, and corticotropes are clearly reduced leading to pituitary

hypoplasia (Raetzman *et al.*, 2002). It has also been suggested that Lhx4 acts upstream to *Lhx3* and *Isl1*, because in *Lhx4* KO mice, the expression of these genes are impaired (Raetzman *et al.*, 2002). Double knockout mice of *Lhx3* and Lhx*4* present with a more severe phenotype than either knockout mice alone suggesting that these two factors act redundantly to control the formation of RP (Sheng *et al.*, 1997). Patients with inactivating *LHX4* mutation are short in stature and present CPHD with affected GH, ACTH and TSH secretion completely in line with *Lhx4* KO mice (Machinis *et al.*, 2001).

Homeo box gene expressed in ES cells (Hesx1) gene belongs to the paired-like class of homeodomain genes. In the pituitary gland, expression is restricted to the ventral diencephalons and RP at E9.5 (Hermesz *et al.*, 1996). Expression is maintained until E12, after which expression declines (Hermesz *et al.*, 1996). This decreased expression coincides with increased expression of *Prop1* followed by differentiation of the pituitary cell lineage. Transcription factor *Lhx3* maintains the expression of *Hesx1*, while its expression is decreased by transcription factor *Prop1* (Olson *et al.*, 2006). *Hesx1* knockout mice present with defects in the central nervous system, absence of optic vesicles, impaired olfactory development, and hypopituitarism like the humans with septo-optic dysplasia (Dattani *et al.*, 1998). The pituitary phenotype varies greatly, 5 % of mice present complete lack of pituitary gland, but most of the mice present bifurcated pituitary glands with hypopituitarism ranging from CPHD to IGHD (Dattani *et al.*, 1998).

Paired like homeodomain factor 1 (Prop1) (Prophet of Pit-1) gene is a homeodomain-containing transcription factor, the expression of which is completely limited in RP (Sornson *et al.*, 1996). Expression begins at day E10 in mice, reaches a maximum at E12, and diminishes by E14.5 (Sornson *et al.*, 1996). Naturally occurring inactivating *Prop1* mutation in mice ($Prop1^{Ames}$) leads to a severely decreased number of somatotrope, lactotrope, and thyrotrope cells, and delayed appearance of gonadotropes. This is due to the dysmorphogenesis of RP and failure of the expression of the *Pit1* gene, the downstream effector of *Prop1*. (Andersen *et al.*, 1995; Gage *et al.*, 1995) These mice are short in stature due to GH deficiency, but they live twice as long as their WT littermates (Brown-Borg *et al.*, 1996). In humans, mutations in the *PROP1* gene are the most common cause of familial CPHD, and were first found in patients with familial short stature and with impaired production of GH, TSH and gonadotropins (Wu *et al.*, 1998). At the moment, several mutations in the *PROP1* gene leading to CPHD have been identified (Mody *et al.*, 2002).

POU domain, class 1, transcription factor 1 (***Pou1f1***=official symbol, ***Pit1***=used in text) is a transcription factor expressed exclusively in the pituitary gland. It is expressed at E13.5 in the ventral part of the anterior pituitary gland, and expression persists in adult and is colocalized with GH, PRL and TSH. Spontaneous mutation of this transcription factor is found in mice (*Snell* mice), where the single nucleotide mutation interferes with the DNA binding capacity of PIT1 (Li *et al.*, 1990). The phenotype of homozygous mice presents dwarfism and infertility due to loss of somatotrope, lactotrope and thyrotrope cell lineages and their hormonal products, indicating the important role of *Pit1* in terminal differentiation of these cell lineages (Li *et al.*, 1990). Mutations of *PIT1* gene has also been found in humans with CPHD, originally in patient with cretinism due to impaired secretion of GH, PRL and TSH

(Tatsumi *et al.*, 1992). Subsequently, many other mutations of Pit1 gene have been reported, with a wide variety of phenotypic appearance (Parks *et al.*, 1999). Mostly, somatotropes and lactotropes are affected, but TSH deficiency is often delayed.

GLI-Kruppel family member GLI1, 2 and ***3*** (***Gli1***, ***2***, and ***3)*** are zinc-finger transcription factors known to mediate the downstream effects of SHH. They are expressed in the ventral diencephalon and developing RP (Hui *et al.*, 1994). *Gli2* knockout mice present with the variable loss of the pituitary gland with normal development of RP, and they die at birth due to neural and skeletal defects (Ding *et al.*, 1998; Motoyama *et al.*, 1998). Double knockout mice of *Gli1* and *Gli2* have severe defects in the development of the pituitary gland, and around half of double mutants present with a complete lack of RP at E12.5 due to loss of SHH signalling (Park *et al.*, 2000). Double knockout mice of *Gli2* and *Shh* present with a similar phenotype to *Shh* mutant alone indicating that *Gli2* acts downstream to *Shh* (Bai *et al.*, 2002). In humans, mutation in GLI2 is associated with pituitary anomalies and holoprosencephaly (Roessler *et al.*, 2003).

Sine oculis-related homeobox 6 homolog (Drosophila) (***Six1***, ***3***, ***4***, and ***6)*** are members of six homeodomain transcription factors expressed in the pituitary gland (Zhu *et al.*, 2007). Six6 shows a dorsal-ventral gradient during early pituitary development, and deletion of Six6 leads to hypoplastic pituitary gland with a decreased number of differentiated cells (Li *et al.*, 2002). Six3 is a upstream activator of the WNT pathway, and Six3 knockout mice present failure of the development of the ventral diencephalons and pituitary gland (Lagutin *et al.*, 2003).

Paired box gene 6 (Pax6) is a transcription factor expressed in the RP and anterior pituitary gland between E10-E17.5. It shows a dorsal-ventral gradient and has been suggested to oppose SHH-signalling. *Pax6* KO mice have a dorsal expansion of ventral cell types, especially thyrotropes, and decreased number of dorsal cell types, lactotropes and somatotropes (Kioussi *et al.*, 1999).

2.3.1.2. Signalling pathways responsible for anterior pituitary gland development

A summary of signalling pathways responsible for pituitary development is given in Table 3.

SHH (Sonic hedgehog) is an important factor for embryo patterning. In the early embryo, the major source of Shh is the notochord (Echelard *et al.*, 1993). Surgical ablation of notochord or deletion of the *Shh* gene leads to disturbed embryo axis patterning and, for example, the absence of RP (Zhu *et al.*, 2007). Although three transcription factors, Gli1, 2, and 3, acting downstream of SHH, are expressed in the ventral diencephalons and RP, the ablation of SHH signalling seems to affect pituitary development by causing a general defect in midline structures rather than causing Gli-related defects in the pituitary primordium (Hui *et al.*, 1994; Park *et al.*, 2000). Mutations affecting SHH signalling result in the development of lens tissue instead of pituitary tissue or hypoplasia of the pituitary gland (Kondoh *et al.*, 2000; Herzog *et al.*, 2003; Sbrogna *et al.*, 2003).

During pituitary development in mice, SHH is present in the ventral diencephalons and oral ectoderm, but not in the RP. However, its downstream target *Patched1* is expressed during pituitary development. Blocking of the effect of SHH by inducing its

antagonist prevents the growth of RP and the appearance of endocrine cell lineages. The overexpression of SHH induces pituitary hyperplasia with an increased number of ventral gonadotrope and thyrotrope lineages with modified *Lhx3* levels indicating that SHH also has an important role in the proliferation and determination of cell types (Treier *et al.*, 2001).

FGFs (Fibroblast growth factors) are a large family of growth factors playing an important role in organogenesis and cell type differentiation. At least FGF8, -10 and -18 have been found in the ventral diencephalons and posterior lobe of the pituitary gland during development, and FGF8 and FGF10 play an important role in pituitary development (Ericson *et al.*, 1998; Maruoka *et al.*, 1998; Ohuchi *et al.*, 2000). When *FGF10* or its receptor FGFR2 IIIb is knocked out, the RP is originally formed, but then it undergoes severe apoptosis leading to agenesis of the anterior pituitary gland at E14.5 (Ohuchi *et al.*, 2000). This indicates the role of FGF10 signalling in pituitary cell survival. Mice deficient for *Fgf8* die at E8.5, before the pituitary gland is formed. The overexpression of *Fgf8* induces ectopic *Lhx3* followed by pituitary hyperplasia of the cell lineage producing POMC and inhibition of other cell types (Treier *et al.*, 1998; Treier *et al.*, 2001). FGF8 has been shown to affect the patterning of RP by opposing the ventral signal of BMP2 and by inducing proliferation of dorsal cell types (Ericson *et al.*, 1998; Norlin *et al.*, 2000).

BMP4 and **BMP2** (bone morphogenic protein 2 and 4) have been shown to participate in the development of the anterior pituitary gland. Initially, BMP4 is expressed in the ventral diencephalons, a structure overlying the RP at E8.5 serving a ventral gradient for invagination of the oral ectoderm and development of the RP (Takuma *et al.*, 1998). In TG mice overexpressing BMP4 antagonist *noggin*, pituitary development is arrested at E10 just after the RP is formed (Treier *et al.*, 1998). In these mice, all the other cell types are missing except some corticotropes (Treier *et al.*, 1998). Oral ectoderm invagination also occurs in *NK2 homeobox 1 (Nkx2-1*) knockout mice, where BMP4 is still present at E9.5 (Kimura *et al.*, 1996; Takuma *et al.*, 1998). These results suggest that BMP4 is critical for the continuation of pituitary development.

BMP2 is expressed in the most ventral part of anterior pituitary at E9.5 expanding throughout the whole Rathke's pouch at E12.5 (Treier *et al.*, 1998). BMP2 is able to induce the expression of *Isl1* and *Cga,* the ventral markers of pituitary gland suppressing the expression of ACTH (Ericson *et al.*, 1998). Continuous expression of BMP2/4 under the *Cga*-promoter induce the expansion of ventral cell types into the dorsal regions of the pituitary gland (Treier *et al.*, 1998). Thus, BMP2 is needed for the induction of ventral cell types, and its spatio-temporal expression pattern opposes the dorsal signal of FGF8 inducing typical pattern of transcription factors for cell lineage specification events (Ericson *et al.*, 1998; Treier *et al.*, 1998). However, BMP2 has a dual role in pituitary cell type differentiation, as the sustained expression of BMP2 prevents the terminal differentiation from occurring (Treier *et al.*, 1998).

NOTCH (Notch gene homolog) signalling is present in early pituitary development and declines at E13.5, when lineage commitment begins in the anterior pituitary gland (Raetzman *et al.*, 2004; Raetzman *et al.*, 2006; Zhu *et al.*, 2006). Inactivation of NOTCH signalling causes premature differentiation of progenitor cells due to decreased *hairy and enhancer of split 1* (*Hes1*) expression. This is followed by

induction of Pit1 lineage into the corticotrope lineage due to downregulation of *Prop1*, a transcription factor needed for the proper development of Pit1 lineage (Zhu *et al.*, 2006). These results indicate that NOTCH signalling is required for proper lineage commitment. However, attenuation of Notch signalling is necessary for terminal differentiation, as sustained NOTCH1 activity under Pit1 regulatory elements blocks the terminal differentiation of all Pit1 lineages, and sustained NOTCH2 under Cga regulatory elements prevent terminal differentiation of thyrotropes and gonadotropes (Raetzman *et al.*, 2006; Zhu *et al.*, 2006).

WNTs (wingless-related MMTV integration site) have been shown to be active in the developing pituitary gland (Treier *et al.*, 1998). When WNT signalling is disturbed by targeted inactivation of β-catenin, the pituitary glands are smaller and do not express *Pit1* leading to a lack of all Pit1 lineages and a reduced number of gonadotropes (Olson *et al.*, 2006). Also, an aberrant activation of β-catenin leads to *Hesx1* repression and thus pituitary agenesis (Olson *et al.*, 2006). Ten *Wnt* genes have been detected in the developing pituitary gland, and especially *Wnt4* and *Wnt5a* have been shown to be effective (Treier *et al.*, 1998; Olson *et al.*, 2006). *Wnt5*a is expressed in the ventral diencephalons and infundibulum at E9.5. *Wnt5a* KO mice show morphologically distorted pituitary glands with an enlarged intermediate lobes (Cha *et al.*, 2004). *Wnt4* is detected in RP until E14.5, and *Wnt4* KO mice present pituitary hypoplasia with affected gonadotrope, thyrotrope and somatotrope lineages (Treier *et al.*, 1998). These results suggest that the main function of WNT signalling is the determination and expansion of Pit1 lineages.

EGF (epidermal growth factors) signalling has an impact on many developmental processes. In the pituitary gland, the proper expression of EGF during development is needed for normal expansion of somatotropes and lactotropes (Roh *et al.*, 2001). In mice expressing a dominant negative form of EGFR lacking its kinase domain, dwarfism occurs due to the decreased number of somatotropes (Roh *et al.*, 2001).

Table 3. *Summary of signalling pathways responsible for pituitary development.*

Signalling pathway	***Action during the pituitary development***	***Pituitary phenotype of signalling deficient mice***
SHH	Ventral signal, proliferation, and determination	General midline defect leading to disrupted pituitary development, development of lens tissue instead of pituitary gland, pituitary hypoplasia
FGFs	Dorsal signal, cell survival	Apoptosis of RP
BMPs	Ventral signal, development of RP, proliferation of anterior pituitary cells	Arrested pituitary development after formation of RP, impaired induction of ventral cell types
NOTCHs	Lineage commitment, terminal differentiation	Premature differentiation of progenitor cells, induction of Pit1 lineage to corticotrope lineage
WNTs	Determination and expansion of Pit1 lineages	Pituitary hypoplasia, no Pit1 lineages, reduced gonadotropes
EGFs	Proliferation	Impaired expansion of somatotropes and lactotropes

2.3.1.3. Differentiation of lactotropes and somatotropes

As already mentioned, transcription factor *Pit1* is needed for terminal differentiation and expansion of three pituitary cell lineages (lactotropes, somatotropes, and thyrotropes), but also for repressing the gonadotrope lineage (Li *et al.*, 1990; Dasen *et al.*, 1999). *Pit1* is also needed for transcription of *Gh*, *Prl*, *Tshb*, and *Ghrhr*. The main regulators of *Pit1* expression during fetal development are *Prop1* and WNT signalling, and their correct temporal expression pattern is also crucial for normal differentiation and expansion of *Pit1* lineages (DiMattia *et al.*, 1997; Olson *et al.*, 2006). Starting at E16.5, the maintenance of Pit1 expression is regulated through the vitamin D receptor binding site, and retinoic acid response elements, but also by autoregulation through three Pit1 binding sites (Rhodes *et al.*, 1993).

Determination of somatotrope and lactotrope lineages occurs through induction and repression of *Gh* or *Prl* in the somatotropes or lactotropes (Zhu *et al.*, 2007). The minimal promoter inducing *Gh* expression into the somatotropes and restricting it from the lactotropes is the proximal 320 bp promoter containing binding sites for Pit1, Sp1 transcription factor, thyroid hormone response element (TRE), a zinc finger protein Zn-15, and a yet unidentified protein (Zhu *et al.*, 2007). All of the binding sites mentioned are needed for proper induction of *Gh* into the somatotropes (Zhu *et al.*, 2007), but only Pit1, TRE and a yet unidentified factor are needed for repressing *Gh* from the lactotropes (Scully *et al.*, 2000). The expression of GH is induced by thyroid hormones and retinoic acids through TRE (Zhu *et al.*, 2007). GHRH induces and controls the expression of GH through GHRHR by activating the PKA pathway (Lin *et al.*, 1993).

The promoter inducing *Prl* into the lactotropes and restricting it from the somatotropes is 3 kb long region upstream of the gene (Zhu *et al.*, 2007). Furthermore, distal enhancer and proximal promoter region is required for high expression of *Prl* in lactotropes (Crenshaw *et al.*, 1989). The high expression of *Prl* in the lactotroph cells is driven by Pit1 and estrogens through classical EREs and four Pit1 binding sites (Day *et al.*, 1990; Zhu *et al.*, 2007). The effects of estrogens are mediated through estrogen receptor 1 (ESR1), as mice lacking Esr1 shows lower PRL and the lower number of lactotropes than the WT mice (Scully *et al.*, 1997). Estrogen induces PRL also through the MAPK pathway (Watters *et al.*, 2000). In adult mice, dopamine (DA) is the major regulator of PRL (Ben-Jonathan and Hnasko, 2001).

2.3.2. Functions of the pituitary gland

As previously mentioned, the pituitary gland is necessary for many important and vital endocrine functions, and thus it should have the capacity to recover after the minor diseases. The pituitary gland shows plasticity throughout its lifespan. It adapts the desired number of hormone producing cells to fulfil the demands of peripheral target organs (Fauquier *et al.*, 2008). A recent study showed that SOX2-expressing progenitor cells can differentiate to all the hormone producing pituitary cell types and, therefore, these progenitor cells can explain the plasticity of the pituitary gland (Fauquier *et al.*, 2008). Following section focuses on lactotroph cells and the functions of PRL.

2.3.2.1. Prolactin

The ancient Greeks noticed the correlation between milk production and reproductive disturbances, but the actual hormone responsible for this was not identified until the last century. In 1928, a pituitary factor responsible for the induction of milk secretion in rabbits was identified, and in 1933 a pituitary hormone was shown to control milk secretion of other mammals and was named "pro – lactin" (Striker and Grueter, 1929; Riddle *et al.*, 1933). Only after the development of a radioimmunoassay for PRL in the 1970s (Hwang *et al.*, 1971) has the characterization of diverse effects of PRL in many important physiological functions been possible.

2.3.2.2. Biochemical characteristics of prolactin

Prolactin, growth hormone and placental lactogens are members of same protein family sharing a common ancestral gene, from which they have evolved by duplication (Niall *et al.*, 1971). GH and PRL lines have diverged several hundred million years ago (Cooke *et al.*, 1980; Cooke *et al.*, 1981). In humans, *PRL* is coded by a single gene on chromosome 6 (Owerbach *et al.*, 1981). The gene itself consists of 5 exons and 4 introns with the total size of 10 kb (Cooke *et al.*, 1981; Truong *et al.*, 1984). Transcription of the *PRL* gene is driven by different promoter regions, the proximal 5000 bp promoter is responsible for pituitary specific expression and the more upstream promoter drives extrapituitary *PRL* expression (Berwaer *et al.*, 1991; Berwaer *et al.*, 1994). The transcript itself is 914 bp long, with a coding sequence of 681 nucleotides, resulting in a prohormone of 227 aa, with a 28 aa signal peptide (Sinha, 1995). Thus, the PRL secreted by the anterior pituitary gland contains 199 aa.

2.3.2.3. Prolactin receptor and signal transduction

Prolactin receptor (PRLR) belongs to the type 1 cytokine receptor family (Cosman *et al.*, 1990). These receptors present with a large extracellular domain for ligand binding, short transmembrane-spanning sequence and intracellular domain for signal transmission (Bole-Feysot *et al.*, 1998). PRL, bound to one receptor, docks with other receptor to form a dimer with one PRL and two receptors (Bole-Feysot *et al.*, 1998). Ligand binding and dimerization induce conformational changes needed for the full activation of intracellular events. The main event in proper signal transduction is the phosphorylation of tyrosine residues in the intracellular part of the receptor. Janus kinase 2 (JAK2) phosphorylates these residues and also the residues in the kinase itself after ligand binding. This is followed by phosphorylation and activation of STAT5, the main mediator of PRL signalling. (Campbell *et al.*, 1994; Gao *et al.*, 1996; Parganas *et al.*, 1998; Teglund *et al.*, 1998) Phosphotyrosines are also the docking sites for other signal transduction proteins, such as Src-kinases, mitogen-activated protein kinases (MAPKs) and protein kinase C (PKC) (Bole-Feysot *et al.*, 1998).

2.3.2.4. Synthesis and secretion of prolactin

Prolactin is mainly synthesized by lactotroph cells in the anterior pituitary gland, but also other sites of PRL synthesis, such as the brain and deciduas, are described (Freeman *et al.*, 2000). The best characterized mechanism for controlling PRL

synthesis and secretion is the dopaminergic system. Contrary to the stimulation-driven control of other pituitary hormones, PRL synthesis and secretion is controlled by DA mediated inhibition. DA is synthesized in the hypothalamus by tuberoinfundibular dopaminergic cells (TIDA) in the *nucleus arcuatus*, and DA is released into the eminentia media and pituitary stalk (Ben-Jonathan and Hnasko, 2001). In addition to this, the tuberohypophysial dopaminergic system located in the rostral *nucleus caudatus* and in the *nucleus paraventicularis,* synthesizes DA and secretes DA into the posterior pituitary gland (Ben-Jonathan and Hnasko, 2001). DA synthesis and secretion is under the control of the higher parts of the brain. Synthesis is mainly controlled by the activity of tyrosine hydroxylase converting the tyrosine to levodopa followed by conversion into active DA by aromatic amine decarboxylases (Ben-Jonathan and Hnasko, 2001). Several other PRL releasing factors (for example E2, TRH, oxytocin, galanin, EGF and FGF2) have been described, but the actual physiological significance of these factors in the synthesis and secretion of PRL is not always properly documented (Freeman *et al.*, 2000). Causes of hyperprolactinemia are listed in Table 4.

DA exerts its effects through dopamine receptor 2 (DRD2). DRD2 belongs to G protein-coupled receptors (GPCR)/seven-transmembrane domain receptor. There exist two isoforms of the receptor: the long form (the full length receptor) and the short form (the splice variant lacking exon 6 from the third cytoplasmic loop) (Dal Toso *et al.*, 1989; Giros *et al.*, 1989). The long form is predominant in the pituitary gland of rat and also in the normal human pituitary gland (Dal Toso *et al.*, 1989; Renner *et al.*, 1998). The activation of DRD2 leads to reduced intracellular cAMP levels through a pertussis toxin-sensitive mechanism indicating the involvement of $G_{i/o}$ proteins (Enjalbert *et al.*, 1988). This mechanism is the main inhibitor of *PRL* gene expression (Ben-Jonathan and Hnasko, 2001). In addition to the cAMP-pathway, DRD2 has been suggested to affect IP3 production possibly through the PLC pathway (Caccavelli *et al.*, 1992). The rapid, secretory action of DRD2 has been associated with the action of ion channels. DRD2 is able to activate G protein-coupled potassium channels leading to hyperpolarization of the cell membrane and closure of voltage gated calcium channels, and thus decreased intracellular calcium levels followed by decreased exocytosis (Gregerson *et al.*, 2001). Recently, the MAPK-pathway has been shown to affect the control of lactotroph proliferation and PRL transcription, although some of the studies showed induction of ERK1/2, while some studies showed inhibition of ERK1/2 responsible for the effects (Banihashemi and Albert, 2002; Iaccarino *et al.*, 2002; Liu *et al.*, 2002; Kim *et al.*, 2004; Van *et al.*, 2007). The lack of 29 aa in DRD2 short form has been shown to change the signal transduction pathways and specificity for certain G-proteins. Furthermore, the *in vivo* effect of these splice variants is different suggesting that the expression balance of these two receptor isoforms are important for intracellular effects (Usiello *et al.*, 2000; Senogles *et al.*, 2004).

Table 4. *Causes of hyperprolactinemia.*

Cause of hyperprolactinemia	***Suggested mechanism***
Physiological conditions	
Pregnancy and puerperium	E2, P, Neuronal
Physical activity	Neuronal
Stress	Neuronal
Medication	
Dopamine receptor blockers	
Serotonin reuptake inhibitors	5-HT receptor 2 in TIDA neurons
Cimetidine	Histamine 2 receptor blocking
Tricyclic antidepressants	5-HT receptor 2, Drd2 receptor blocking
Verapamil (Ca^{2+} blocker)	N-type Ca2+ channel inhibition, low DA
Pathological disease	
Pituitary disease	
Prolactinoma	
Somatotropinoma	
Hypothalamic disease	
Tumors	Preventing dopamine inhibition
Inflammatory diseases	
Granulomatous diseases	
Stalk section	
Vascular disease	
Other diseases	
Primary hypothyroidism	TRH
Adrenal Insufficiency	
Uremia	PRL clearance, DA
Cirrhosis	E2
Paraneoplastic	Ectopic production
Idiopathic	Other reasons ruled out

The list is incomplete. 5-HT; 5-hydroxytryptamine (serotonin).

2.3.2.5. Functions of prolactin

PRL has shown to be involved in many physiological processes (Freeman *et al.*, 2000). However, we have to keep in mind that the pharmacological doses of PRL used especially in older studies might explain many of these effects.

Women/females: As the name "prolactin" states, the hormone is mandatory for normal development of mammary glands and lactation. PRL is indirectly required for mammary ductal side branching, since in *Prlr* KO female mice presenting with low P, ductal side branching was recovered by P treatment or transplanting the mammary gland into female mice with normal endocrine levels (Brisken *et al.*, 1999; Binart *et al.*, 2000). Similar results were obtained in *Prl* KO mice (Naylor *et al.*, 2003). During pregnancy, alveolar morphogenesis and milk secretion was disrupted in pregnant PRLR KO females, and similar results were obtained from *Jak2* KO and *Stat5* KO mice indicating their role as downstream effectors of PRLR (Liu *et al.*, 1997; Brisken *et al.*, 1999; Wagner *et al.*, 2004). These results are in line with the results obtained from women with isolated PRL deficiency presenting puerperal alactogenesis. This shows the importance of normal PRL secretion in mammary gland development during and after pregnancy (Kauppila *et al.*, 1987).

Most of the data obtained from PRL action on ovaries is derived from mouse models. PRL is a luteotrophic hormone. The main functions of PRL are to prevent apoptosis in the corpus luteum and to protect the appropriate P synthesis to ensure implantation and pregnancy. Both *Prl* and *Prlr* KO females are infertile, which is associated with the disturbed oestrous cycle and impaired function of corpus luteum (Horseman *et al.*, 1997; Binart *et al.*, 2000; Grosdemouge *et al.*, 2003). The luteotrophic actions of PRL in the *corpus luteum* are the prevention of apoptosis, the induction of angiogenesis, the prevention the P metabolism by decreasing aldo-keto reductase family 1, member C18 (20α-hydroxysteroid dehydrogenase) (Akr1c18), and the induction of steroidogenic enzymes involved in P synthesis (Feltus *et al.*, 1999; Grosdemouge *et al.*, 2003; Bachelot and Binart, 2007). In *Prlr* KO females, P replacement therapy rescues implantation, but not full term pregnancy, suggesting that the decidual action of PRL is important for full term pregnancy (Binart *et al.*, 2000). In humans, decidua produces PRL and it has been suggested that this is important for implantation and early pregnancy (Jabbour and Critchley, 2001). The reproductive difficulties in women with isolated PRL deficiency suggest that PRL is also important for proper ovarian function in humans (Kauppila *et al.*, 1987).

Furthermore, PRL has been associated in behaviour, haematopoiesis, ion and calcium balance and metabolism, but only in laboratory animals (Freeman *et al.*, 2000). It is possible that these actions are induced only by pharmacological PRL levels.

Men/males: At the moment, PRL seems not to have any particular function in man, but it has been suggested that PRL might have some role in the development of prostate cancer (Nevalainen *et al.*, 1997; Bachelot and Binart, 2007). The reproductive capability of *Prlr* KO males was normal, and they did not present any obvious phenotype (Binart *et al.*, 2003).

2.3.2.6. Effects of high and low levels of prolactin

Low PRL: In humans, isolated PRL deficiency is a very rare condition, but some women having this disease have been described (Kauppila *et al.*, 1987; Zargar *et al.*, 1997). These women present with reproductive difficulties and failure of lactation, but no other clinically relevant changes. These results raise the question whether normal PRL also has some permissive action in normal reproductive functions in women. It is also possible, that medication increasing dopaminergic tone causes drug-induced hypoprolactinemea. In men, no reports on the effect of low PRL have been described.

High PRL: Contrary to low PRL levels, high PRL levels cause obvious changes in both sexes. The most drastic abnormalities are observed in reproductive functions in both sexes. High PRL has been shown to inhibit GnRH and LH secretion leading to impaired gonadal function (Zarate *et al.*, 1975; Sauder *et al.*, 1984). In women, this usually means a short luteal phase, anovulatory cycles, oligo- and amenorrhea. In man, only severe hyperprolactinemia tends to cause hypogonadism, and this is usually associated with the other signs of macroprolactinomas, i.e. visual defects and headaches (Zarate *et al.*, 1975). Another sign of increased PRL secretion is galactorrhea, which is more common in women than in men. Osteoporosis is also a common finding in person with severe hyperprolactinemia, but this is rather mediated by hypogonadism than high PRL itself (Greenspan *et al.*, 1986; Kayath *et al.*, 1993).

The causes of hyperprolactinemia are listed in Table 4, and pituitary neoplasms are discussed below.

2.3.2.7. Secretion of the anterior pituitary hormones

Briefly, all the other pituitary hormones are secreted from the pituitary gland upon stimulation of "releasing" hormone, but in some case inhibitory substances also modify the outcome. Growth hormone-releasing hormone (GHRH), Corticotropin-releasing hormone (CRH), gonadotropin-releasing hormone (GnRH), and thyrotropin-releasing hormone (TRH) are the hypothalamic "releasing" factors involved in the secretion of GH, ACTH, LH and FSH, and TSH, respectively.

2.3.3. Pituitary neoplasms

Pituitary neoplasms generally arise sporadically from clonal expansion. They account for approximately 15% of all intracranial tumors, and they are usually benign adenomas, but aggressive behaviour is also occasionally seen (Ezzat *et al.*, 2004). The morbidity they cause is due to hormonal secretion or local compressive effects. Recently, the prevalence of clinically relevant pituitary adenomas was documented to be as high as 1/1000 individuals in the Belgium population, and a systematic review combining data obtained from radiological and autopsy studies showed the prevalence as high 16.7% (Ezzat *et al.*, 2004; Daly *et al.*, 2006). These numbers indicate that pituitary adenomas are common, although many of them are asymptomatic.

There are two ways to classify pituitary adenomas. One is based on the size of the adenoma; microadenomas are < 10 mm in diameter, and macroadenomas are > 10mm in diameter. Another possibility is to classify pituitary adenomas according to the hormones they secrete, in which case the most common tumors are PRL secreting tumors, prolactinomas. Prolactinomas account for more that 40% of all pituitary adenomas (McComb *et al.*, 1983; Ezzat *et al.*, 2004). Like all the other pituitary adenomas, they tend to be benign. The morbidity they cause is due to high PRL secretion affecting hypothalamic-pituitary axis and decreasing pituitary secretion of gonadotropins and thus sex steroid production, a condition called hypogonadotropic hypogonadism. In the case of macroadenomas, other clinical manifestations may also occur, including visual defects, headache and lack of some other pituitary hormones due to the compression effects (Poon *et al.*, 1995; Abe *et al.*, 1998; Mah and Webster, 2002).

GH secreting adenomas called somatotropinomas and non-functioning pituitary adenenomas account for 13% and 15% of adenomas, respectively (Daly *et al.*, 2006). Somatotropinomas are also benign, and the morbidity they cause is due to increased GH and insulin like growth factor 1 (IGF1) production leading to acromegaly and increased risk of coronary artery disease and type 2 diabetes. Furthermore, ACTH secreting adenomas occur with a frequency of 6% and are responsible for the Cushing's disease, culminating in the overproduction of cortisol (Daly *et al.*, 2006). These are also benign, and sometimes so small, that not detected by MRI. Therefore, invasive blood sampling from petrosal sinuses is needed. The morbidity caused by Cushing's disease is due to elevated cortisol production leading to central obesity, type 2 diabetes, muscle weakness, and many other symptoms. Adenomas secreting TSH and gonadotropins are rare. Symptoms caused by these tumors are, for example, thyroid

hormone induced cardiac arrhythmias or sex steroid induced precocious puberty, but generally gonadotroph adenomas tend to be nonfunctional. The factors responsible for PAs in mouse or man are summarized in Table 5.

2.3.3.1. Aetiology of pituitary adenomas

The aetiology of pituitary adenomas is not properly known. Female sex is the only well characterized risk factor and this elevated risk is associated with the higher amount of circulating estrogens. Some transsexual men after special surgery and oral estrogen substitution have also developed pituitary adenomas. In the case of microprolactinomas, the female to male ratio is 20:1, but in macroprolactinomas 1:1 (Faglia, 1993; Mindermann and Wilson, 1994). Some valid mechanisms for pituitary tumors have been found, but they in total account for only a minor portion of pituitary tumors (Heaney and Melmed, 2004). Further, some inherited forms of pituitary adenomas occur and these are discussed below.

Table 5. *Summary of genetic disorders, aberrant oncogene or tumor suppressor gene expression, and hormones found in pituitary adenomas of mouse and man. Mouse models, corresponding human diseases and types of PAs are also presented.*

Cause of pituitary adenomas	***Mouse models***	***Human disease or pharmacological intervention***	***Type(s) of pituitary adenomas***
MEN1	*Men1* KO	Inactivating mutation of *MEN1* (Multiple endocrine neoplasia 1)	Mouse; PRLoomas Human; PRL-and GHoomas
CDKN1B	*Cdkn1b* (p27) KO	Inactivating mutation of *CDKN1B* or reduced expression in PAs	Mouse; intermediate lobe tumors, Human; GHoomas
RB1	*Rb1* KO	Decreased expression and increased methylation of *RB1*	Mouse; intermediate lobe tumors, Human; invasive GHoomas
CDKN2A (p16)	*Cdkn2a* (p16) KO	Decreased expression and increased methylation of *CDKN2A*	Mouse; no PAs Human; many types of PAs
CDKN2C (p18)	*Cdkn2c* (p18) KO		Mouse; intermediate lobe tumors
AIP	Not exist	Inactivating mutations of *AIP*	Human; GHoomas
PRKAR1A	*Prkar1*a KO	Inactivating mutations of *PRKAR1A*	Mouse; no PAs Human; GHoomas
GNAS	Not exist	Activating mutation of *GNAS*	Human; MAS, GHoomas rarely
DRD2/DA	*Drd2* KO	Medication decreasing DA	Mouse; PRLoomas Human; PRL↑
HMGA1 and 2	*Hmga1 and 2* overexpression	*HMGA2* amplification	Mouse; PRLoomas and GHoomas, Human; PRLoomas
PTTG1	*Pttg1* overexpression	Overexpression of *PTTG1*	Mouse; PRLoomas Human; many types
CCND1/CDK4	Increased expression of CCND1/CDK4	Increased expression of CCND1/CDK4	Human; many types Mouse: PRL- and TSHoomas
Estrogens	E2 treatment Aromatase overexpression bLH-CTP TG mice	Estrogen replacement, lactotroph hyperplasia during pregnancy	Mouse; PRLoomas Human; PRLoomas
Progestins	P treatment		Mouse; stumlation and inhibition of many types of PAs
TGFA	Pituitary *Tgfa* overexpression		Mouse; PRLoomas
Hypothalamic releasing factors			Corresponding PAs
FGFR4	Overexpression of human PAs derived *FGFR4*	Aberrant *FGFR4* expressed in PAs	Not known
PRL/PRLR	*Prl* and *Prlr* KO		Mouse; PRLoomas

The list is not complete.

2.3.3.2. Genetic disorders in pituitary adenomas

At the moment, five inherited genetic disorders have been shown to predispose the formation of pituitary adenomas.

Multiple endocrine neoplasia 1 (MEN1): One of the first characterised tumor suppressor genes was *MEN1*, and this syndrome is inherited as an autosomal-dominant trait. This syndrome predisposes to the development of pituitary adenomas, parathyroid hyperplasia, and pancreatic endocrine tumors. The familial form of MEN1 is characterized by the presence of two of these tumors and a positive family history in first-degree relatives (Marx *et al.*, 1999). Sporadic *MEN1* mutations without family history also exist. Pituitary adenomas are present in 25-30% on MEN1 patients (Burgess *et al.*, 1998), but the MEN1 mutation is rarely found in sporadic pituitary adenomas (Zhuang *et al.*, 1997). Pituitary tumors are mostly prolactinomas (60%) and somatotropinomas (20%), but other types are also present (Thakker, 1998). *MEN1* encodes a protein called MENIN, which is known to interact with at least transcription factors JUND and JUN to inhibit JUN-mediated transcription (Agarwal *et al.*, 1999; Yumita *et al.*, 2003). Conditional inactivation of Men1 in mouse leads development of insulinomas and prolactinomas in line with human disease (Biondi *et al.*, 2004).

Cyclin-dependent kinase inhibitor 1B (CDKN1B, p27): Recently, a nonsense mutation of *CDKN1B* was found in human with MEN1-like syndrome and pituitary adenoma. Although genetic analysis failed to show loss of the wild-type allele, the CDKN1B protein was not detected in tumor tissue (Pellegata *et al.*, 2006). After this, inactivating *CDKN1B* mutation with premature stop codon was found in one Dutch patient with MEN1-like syndrome (Karhu and Aaltonen, 2007). This patient presented with pituitary adenoma, parathyroid tumor, and neuroendocrine carcinoid tumor. CDKN1B is a nuclear protein negatively regulating the cell-cycle by inhibiting cyclins and cyclin-dependent kinases. *Cdkn1b* KO mice present pituitary intermediate lobe tumors and enhanced growth with multiorgan hyperplasia (Fero *et al.*, 1996). A very similar phenotype was observed in *Cdkn1b* KO knockout rats (Pellegata *et al.*, 2006).

Aryl hydrocarbon receptor interacting protein (AIP): Recently, two families with pituitary adenomas were studied for germline mutation in the *AIP* gene, and two different mutations were found (Vierimaa *et al.*, 2006). Subsequently, several different mutations of the AIP gene have been found in the families with familial predisposition to the pituitary adenomas (Karhu and Aaltonen, 2007). It seems that the mutations in this gene predispose for the development of somatotropinoma, and patients with these mutations present signs of somatotropinomas at a young age (Vierimaa *et al.*, 2006; Georgitsi *et al.*, 2007). However, in sporadic pituitary adenomas, germline mutations in *AIP* do not seem to play an important role (Barlier *et al.*, 2007), but abnormal pituitary expression of AIP was found in sporadic pituitary tumors (Leontiou *et al.*, 2008). AIP interacts with the aryl hydrocarbon receptor (AHR) known to be a high affinity receptor for dioxins. It has been shown that AHR interacts with retinoblastoma protein (RB), heat shock proteins, phosphodiesterase 4A5, peroxisome proliferation-activated receptor-α, survivin, and thyroid receptor β1 (Karhu and Aaltonen, 2007).

Protein kinase A type 1A regulatory subunit (PRKAR1A): PRKAR1A is a tumor suppressor functioning downstream of cAMP to inhibit the PKA mediated phosphorylation of cAMP target genes (Bossis and Stratakis, 2004). Inactivating

mutations in PRKAR1A cause a rare genetic disorder called Carney Complex (Kirschner *et al.*, 2000; Boikos and Stratakis, 2007a). These patients develop slowly progressive acromegaly quite often accompanied with Cushing's syndrome (primary pigmented nodular adrenocortical disease) (Boikos and Stratakis, 2006). Three different mouse models for *Prkar1a* deficiency have been developed, but these mice did not develop pituitary adenomas (Boikos and Stratakis, 2007b), nor have PRKAR1A mutations been detected in sporadic pituitary adenomas.

GNAS complex locus (GNAS): Mosaicism for mutations in the GNAS gene causes a disease called McCune-Albright syndrome (MAS). This disease is characterized by polyostotic fibrous dysplasia, pigmented lesions in the skin, and the increased activity of many endocrine glands (Akintoye *et al.*, 2002). Around 20% of these patients present elevated GH levels, and some of them have hyperplasia of GH- and PRL-producing cells, but occult pituitary adenomas are rare (Boikos and Stratakis, 2007b). *GNAS* is an abundantly expressed gene coding for stimulatory G protein. It is needed for the activation of adenylyl cyclase and thus the generation of cAMP. The mutations of the *GNAS* gene responsible for MAS lead to high adenylyl cyclase activity and increased intracellular cAMP levels mainly due to impaired reassembly and inactivation of stimulatory G protein (Vallar *et al.*, 1987; Weinstein *et al.*, 1991; Lania *et al.*, 2003).

2.3.3.3. Hormones and medications responsible for pituitary adenomas

Estrogens: The mechanism of estrogen induced pituitary tumorigenesis has been studied extensively. There is a large body of evidence suggesting that estrogens play a pivotal role in pituitary tumorigenesis in humans: 1) Pituitary adenomas (prolactinomas) are more common in women than in men (Faglia, 1993; Drange *et al.*, 2000), 2) estrogens induce PRL secretion (Lieberman *et al.*, 1978; Maurer, 1982) 3) the presence of physiological pituitary hyperplasia during pregnancy (Gonzalez *et al.*, 1988), 4) increased pituitary tumor growth or relapse during the pregnancy (Molitch, 1999), 5) development of lactotroph adenomas in transsexual men during pharmacological estrogen treatment (Gooren *et al.*, 1988; Kovacs *et al.*, 1994; Serri *et al.*, 1996), and 6) the presence of ESRs in pituitary adenomas and the correlation between ESRs expression and the size of pituitary adenomas (Nakao *et al.*, 1989; Jaffrain-Rea *et al.*, 1996). Contrary to these findings, it was previously shown that hormone replacement therapy does not increase the risk of pituitary adenomas (Wingrave *et al.*, 1980). A recent large-scale clinical study showed increased risk for pituitary and central nervous system tumors in women used/using oral contraceptive pills (Hannaford *et al.*, 2007). However, major caveat in this study was that central nervous system and pituitary tumors were combined, and the direct evidence of oral contraceptive pills induced pituitary tumors were not achieved (Hannaford *et al.*, 2007). One case report showed that in man treated for macroprolactinoma-induced hypogonadism with Te, PRL levels rose rapidly after the initiation of Te treatment, but recovered after adding aromatase inhibitor into the treatment (Gillam *et al.*, 2002). Nevertheless, it is clear that the estrogens alone are not responsible for pituitary tumors, but additional factors are involved.

In rodents, estrogen administration after birth or exposure to diethylstilbestrol have been shown to induce pituitary adenomas (Walker and Kurth, 1993; Heaney *et al.*, 1999). Also in three different mouse models, gonadectomy has been shown to prevent pituitary tumorigenesis implicating the vital role of estrogens in pituitary tumor development (Hentges and Low, 2002; Rulli *et al.*, 2002; Mohammad *et al.*, 2003). However, in one of these models, E2 replacement after gonadectomy was not able to recover tumorigenesis (Hentges and Low, 2002), although another model showed tumorigenesis after E2 replacement (Mohammad *et al.*, 2003). The third model is analyzed in current study.

The mechanism of estrogen induced pituitary tumorigenesis has been extensively studied in rodents. Estrogens have been shown to induce pituitary expression of pituitary tumor transforming gene (PTTG1), fibroblast growth factor 2 (FGF2), vascular endothelial growth factor (VEGF), transforming growth factors α and β (TGFA, TGFB), epidermal growth factor (EGF) and to modulate the action of bone morphogenic protein 4 (BMP4) through interaction with Smad 1 and 4 (McAndrew *et al.*, 1995; Heaney *et al.*, 1999; Hentges and Sarkar, 2001; Heaney *et al.*, 2002; Paez-Pereda *et al.*, 2003). All these factors have been shown to affects the proliferation of pituitary cells.

Progestins: The role of progestins in the formation of pituitary adenomas is unclear. Most studies were conducted in the 1980s or earlier, and have shown the inhibitory role of progestins in pituitary tumorigenesis (Haug and Gautvik, 1976; Lamberts *et al.*, 1985; Lamberts *et al.*, 1987; Caronti *et al.*, 1993; Piroli *et al.*, 1998; Piroli *et al.*, 2001). However, there are some studies which suggest that progestins could actually promote pituitary tumorigenesis. Prolonged oral administration of progestins led to pituitary tumors in non-gonadectomized mice, and medroxyprogesterone acetate induced tumor growth in non-gonadectomized somatomammotrophic tumor bearing male rats (Poel, 1966; Winneker and Parsons, 1981). More evidence of the role of progestins in the formation of pituitary adenomas has been obtained by mifepristone treatment, which has been shown to inhibit the growth of transplantable ACTH/PRL-producing rat pituitary tumors and the growth of estrogen induced pituitary tumor growth in intact, but not in gonadectomized rats (Lamberts *et al.*, 1985; Sakamoto *et al.*, 1987). Altogether, results are not uniform, and in a same study set-up both progestins and mifepristone can show inhibitory effects. This can, of course, be mediated through the blockade of glucocorticoid receptors, a known effect of mifepristone. One recent study demonstrated that pituitary adenomas appear in dopamine 2 receptor knockout mice, but are prevented by gonadectomy and not recovered with estrogen replacement (Hentges and Low, 2002). This particular study suggested that ovaries secrete some other factor(s) than estrogens responsible for pituitary tumors. One hormone secreted by the ovaries is P, a hormone present also in oral contraceptive pills.

Dopamine and dopamine receptor 2 (DA, DRD2): The role of the dopaminergic system in controlling lactotroph proliferation and development of prolactinomas is well characterized especially in rodents. Two mouse models lacking *Drd2* have been shown to develop lactotroph hyperplasia followed by adenomas (Kelly *et al.*, 1997; Saiardi *et al.*, 1997), but the development of lactotroph adenomas in these mice is a rather late event as compared, for example, to *Prlr* KO mice (Schuff *et al.*, 2002). An opposite effect was observed in mice lacking *dopamine transporter*. These mice exhibited

increased pituitary dopaminergic tone leading to downregulation of DA binding sites and thus pituitary hypoplasia (Bosse *et al.*, 1997). Medication acting as a DA antagonist (neurolepts, antipsychotics, antidepressants) lead to increased secretion of PRL, and the magnitude of PRL secretion might manifest as clinical hyperprolactinemia with reproductive disturbances (Bronstein, 2006). DA agonists commonly used for treatment of prolactinomas also decrease the size of pituitary adenomas, especially those producing PRL (Bronstein, 2006). However, some of the prolactinomas are resistant to dopaminergic medication. Therefore, new therapeutic approaches to treat prolactinomas are needed.

The exact mechanism for DA inhibitory signalling is controversial. One suggested mechanism is that increased dopaminergic tone induces the expression of TGFB1 responsible for the inhibition of proliferation in pituitary cells (Sarkar *et al.*, 2005). The lack of dopaminergic signalling in *Drd2* KO mice leads to increased VEGF expression facilitating the development of pituitary tumors, although increased VEGF alone is not responsible for cell proliferation (Cristina *et al.*, 2005). The effects of different splice variants of DRD2 and their different role in MAPK-signalling have been discussed earlier (2.3.3.4.1. Synthesis and secretion of prolactin*)*. However, at the moment it is clear that the loss of dopaminergic tone is only a weak stimulus for lactotroph proliferation, and additional or contributing factors are needed for pituitary tumorigenesis. This was highlighted in *Drd2* KO females, who did not develop pituitary tumors after gonadectomy (Hentges and Low, 2002).

Transforming growth factor alpha and beta (TGFA, TGFB): The best evidence of TGFA in the induction of pituitary adenomas was derived from TG mouse model expressing Tgfa under the rat *Prl* promoter. Female mice developed PRL producing pituitary adenomas at the age of 12 months (McAndrew *et al.*, 1995). It has been shown that TGFA is a downstream effector of E2, but whether some other factors are able to induce TGFA expression in pituitary is not known (Borgundvaag *et al.*, 1992). The clinical relevance of TGFA is not known.

Several isoforms of TGFB have been found in the pituitary gland. Estrogens have been shown to induce the expression of mitogenic TGFB3 and decrease the expression of inhibitory TGFB1 in the pituitary gland, and these changes partially explain estrogen-driven pituitary tumorigenesis (Pastorcic *et al.*, 1995; Hentges *et al.*, 2000a; Hentges *et al.*, 2000b; Hentges and Sarkar, 2001).

Hypothalamic factors and impaired negative feedback regulation: Extremely rarely, pituitary adenomas arise due to hypersecretion of hypothalamic factors necessary for maintaining normal secretion of pituitary hormones. These factors include TRH, GHRH, CRH and GnRH, and they are responsible only for <1% of pituitary adenomas. The inactivating mutations of thyroid hormone receptors lead to impaired negative feedback regulation and thus overproduction of TSH.

2.3.3.4. Obesity and pituitary adenomas

Obesity increases the risk of many cancers (Renehan *et al.*, 2008). Obesity is also associated with pituitary adenomas, especially prolactinomas, but whether obesity is a cause or a consequence, is not clear (Greenman *et al.*, 1998). High PRL levels can cause hypogonadotrophic hypogonadism, a disease associated with obesity (Bronstein,

2006). Leptin receptors (LEPR) are expressed in pituitary adenomas (Shimon *et al.*, 1998; Jin *et al.*, 1999), and a mutation of the LEPR causes pituitary dysfunction in humans (Clement *et al.*, 1998). Le acts through its receptors and activate JAK2, STAT3, and MAPK pathways (Fruhbeck, 2006). Le has been shown to induce mammary tumor cell proliferation, and the possible pathways involved are STAT3 and ERK1/2 followed by induction of CCND1 and VEGF (Hu *et al.*, 2002; Gonzalez *et al.*, 2006). At the moment, there are no studies on the molecular mechanism of Le action in the pituitary glands and adenomas, but the simultaneous increase in obesity and in pituitary adenomas observed in Western countries suggests that obesity possibly through Le could be one factor involved in the induction of pituitary tumorigenesis (Daly *et al.*, 2006). However, we need to keep in mind that the technical development and availability of proper diagnostic tools for microadenomas definitely explains at least partly the increased incidence of pituitary adenomas (Daly *et al.*, 2006).

2.3.3.5. Molecular mechanisms responsible for sporadic pituitary adenomas

Cell cycle regulators: Many cancers present aberrant activation of the cell cycle at G1-S phase transitions due to enhanced activation or lack of inhibition of this regulatory pathway (Massague, 2004). Briefly, mitogenic stimuli induce the expression of cyclin Ds, which in turn assembles with CDK4 and CDK6 (Murray, 2004). Eventually, cyclin D/CDK4/6 complex phosphorylates and inactivates RB (Sherr and Roberts, 1999; Murray, 2004; Sherr, 2004). Upon RB phosporylation, E2F transcription factors are activated, and they in turn induce the expression of Cyclin E and other genes supporting the DNA replication (Sears and Nevins, 2002; Stevaux and Dyson, 2002). At the cellular level, the G1-S phase is controlled by CDK4 inhibitors, for example CDKN1A (p21), CDKN1B (p27), CDKN2A (p16), CDKN2B (p15), and CDKN2C (p18) (Sherr and Roberts, 1999). CDKN1A and B are able to bind cyclins D, E and A, and also CDKs to prevent cell cycle progression. CDKN2A, B and C bind only CDK4 and CDK6 and inhibit their catalytical units (Sherr and Roberts, 1999). A schematic representation of G1-S phase is shown in Figure 7. The picture is simplified, and the factors involved also have an important role in cellular senescence and apoptosis. They also tend to show redundancy in the regulation of G1-S phase progression (Massague, 2004).

KO mouse models for *Rb1*, *Cdkn1a-b*, and *Cdkn2a* and *c* are available. The lack of these inhibitory molecules leads to impaired control of G1-S phase transition, increased proliferation, organomegaly, enhanced growth, and hyperplasia and cancers of different tissues including the pituitary gland (Jacks *et al.*, 1992; Deng *et al.*, 1995; Fero *et al.*, 1996; Nakayama *et al.*, 1996; Serrano *et al.*, 1996; Franklin *et al.*, 1998). Overexpression of CCND1 and mutated CDK4 knockin mouse models develop cancers indicating that activation of the cell-cycle at the G1-S phase leads to tumorigenesis in mice (Wang *et al.*, 1994; Sotillo *et al.*, 2001).

One of the main regulators of G1-S phase transition is RAS (Sears and Nevins, 2002). It is a downstream target of many receptor tyrosine kinases (Massague, 2004), and activation of RAS leads to induction of the ERK pathway (Sears and Nevins, 2002). The activated ERK pathway stabilizes MYC, a transcription factor inducing CCND1 and inhibiting CDK inhibitors leading to cell proliferation (Sears and Nevins, 2002).

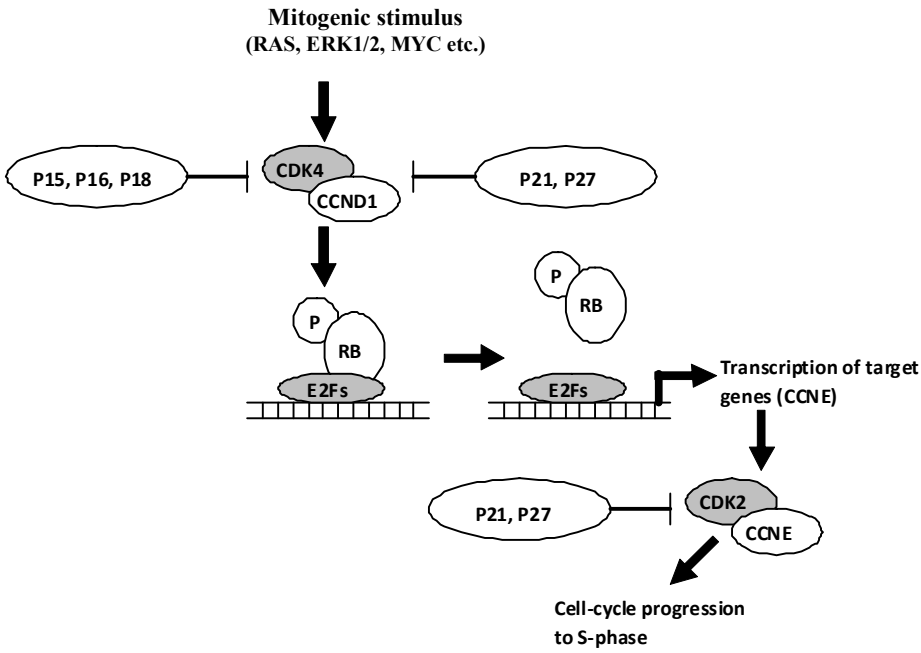


Figure 7. *Simplified view of the cell-cycle regulation at G1-S phase transition. Mitogenic stimulus induce CCND1/CDK4 complex to phosphorylate RB leading to dissociation of E2Fs. Activation of E2Fs is followed by transcription of target genes including CCNE eventually leading to the progression of the S phase. CDKNs inhibit the progression of cell-cycle.*

At the moment, abnormal G1-S phase cell-cycle control is associated with pituitary adenomas. Pituitary tumors appear in mice deficient of *Rb1*, *Cdkn1b*, and *Cdkn2c* (Jacks *et al.*, 1992; Fero *et al.*, 1996; Nakayama *et al.*, 1996; Franklin *et al.*, 1998). In the case of RB deficient mice, the pituitary tumor phenotype is rescued by deficiency of E2F transcription factors 1, 3 and 4 (Yamasaki *et al.*, 1998; Lee *et al.*, 2002b; Ziebold *et al.*, 2003). Interestingly, the pituitary adenomas of these mice arise from the intermediate lobe of the pituitary gland. However, in mice expressing mutated (activated) CDK4, pituitary adenomas arise from the anterior lobe, and 15% of the pituitary adenomas were transformed into carcinomas producing PRL (Sotillo *et al.*, 2001). Mice expressing aberrant thyroid hormone receptor β also develop pituitary adenomas derived from thyrotropes due to induction of the CCND1/RB/E2F1 pathway (Furumoto *et al.*, 2005). One explanation for the different cellular origin of pituitary adenomas in these models can be that the functions of cell-cycle inhibitors and activators are different in specific cell types.

In human pituitary adenomas, an increased expression of CCND1 and its downstream target CCNE has been found (Jordan *et al.*, 2000; Turner *et al.*, 2000; Morris *et al.*, 2005). Reduced expression or increased methylation of RB, CDKN1B, CDKN2A and CDKN2C has also been found in pituitary tumors (Woloschak *et al.*, 1996; Simpson *et al.*, 2000; Korbonits *et al.*, 2002; Simpson *et al.*, 2004; Morris *et al.*, 2005; Scheithauer *et al.*, 2006; Boikos and Stratakis, 2007b). All these results indicate that the loss of G1-S phase control is important for pituitary tumorigenesis, and factors/hormones affecting this pathway need to be recognized.

High mobility group AT-hook 2 (HMGA2): HMGA-group proteins are architectural transcription factors able to bind the minor groove of AT-rich DNA sequences and to mediate the assembly of multiprotein transcription factor complexes (Thanos and Maniatis, 1992; Fusco and Fedele, 2007). These factors are highly expressed during embryogenesis, but show absent or low expression in adult tissues, and HMGA2 expression has not been detected in any adult tissues tested (Zhou *et al.*, 1995; Rogalla *et al.*, 1996). HMGA proteins have been shown to promote tumorigenesis and are often overexpressed in human tumors of mesenchymal origin (Reeves, 2001; Fusco and Fedele, 2007). Transgenic mice overexpressing *Hmga1* and *2* develop tumors especially in the pituitary gland (Fedele *et al.*, 2002; Fedele *et al.*, 2005). Surprisingly, HMGA proteins have also been shown to induce cellular senescence, a completely opposite process than tumorigenesis (Narita *et al.*, 2006). However, HMGA-induced senescence is bypassed by the expression of CDK4 suggesting that HMGA proteins do not induce tumorigenesis *per se*, but additional factors are required (Narita *et al.*, 2006; Fusco and Fedele, 2007).

In human pituitary tumors, overexpression of HMGA2 has been found, and this is usually achieved by abnormal dosage of chromosome 12 (Finelli *et al.*, 2002). Interestingly, human *cyclin-dependent kinase 4* (*CDK4*) gene is located near the *HMGA2* gene and thus co-amplified with *HMGA2*. This co-amplification might be necessary for bypassing HMGA2 induced senescence and tumorigenesis (Finelli *et al.*, 2002; Narita *et al.*, 2006). HMGA2 overexpressing mice develop mixed PRL and GH producing adenomas (Fedele *et al.*, 2002). Recently, a molecular mechanism capitulating HMGA2 induced and E2F1 dependent pituitary tumorigenesis was described (Fedele *et al.*, 2006). HMGA2 binds to the RB/E2F/HDAC complex and displaces histone deacetylase 1 (HDAC1) leading to acetylation of histones and opening of the chromatin structure. After acetylation, HMGA2 and RB dissociate from the complex and E2F1 is stabilized and activated by acetylation leading to enhanced transcription of the target genes (Fedele *et al.*, 2006). However, at the moment it is not known whether some hormonal factors could contribute to the regulation of HMGA2. As shown in human pituitary adenomas, chromosomal aberrations are accompanied with HMGA2 overexpression, suggesting that the mechanism triggering pituitary tumorigenesis and chromosomal aberrations is not HMGA2, but some other factor (Finelli *et al.*, 2002).

Pituitary tumor transforming 1 (PTTG1): This gene was isolated in the rat GH4 pituitary cell line, and human homologue was also cloned (Pei and Melmed, 1997). *PTTG1* overexpression was shown to induce cell transformation, tumor growth and the expression of FGF2 (Zhang *et al.*, 1999). PTTG1 expression was also induced by estrogens, and high expression of PTTG1 coincides with the expression of FGF2 and VEGF in pituitary tumors (Heaney *et al.*, 1999; Heaney *et al.*, 2002). PTTG overexpression has been found in many human pituitary tumors, and the level of expression seems to correlate, for example, with KI67 labelling index (Zhang *et al.*, 1999; McCabe *et al.*, 2003). The overexpression of human *PTTG1* under pituitary specific *Cga* promoter also led to focal pituitary tumorigenesis in rodents (Abbud *et al.*, 2005). After these original studies, the oncogenic role of PTTG1 in many cancers has been clarified (Heaney *et al.*, 2002). PTTG1 induces transformation through the MAPK pathway, and it also induces the expression of MYC (Pei, 2000, 2001). Normal

cellular PTTG1 functions as a securing protein to ensure normal chromosome separation during mitosis (Zou *et al.*, 1999). Recent study also demonstrated the importance of PTTG in the regulation of G1/S phase transition (Tong *et al.*, 2007). It has been speculated that PTTG1 might also be responsible for repairing DNA damage, since it has been shown to associate with XRCC6 (KU70), the regulatory subunit of DNA-dependent protein kinase (Romero *et al.*, 2001). Based on these normal functions, it is understandable that the abnormal expression of PTTG1 can participate tumorigenesis by inducing cell proliferation, transformation, aneuploidy, and tumor microenvironment (induction of FGF2 and VEGF), and by inhibiting apoptosis (Vlotides *et al.*, 2007).

Fibroblast growth factors (FGFs): Recent studies have characterized the important role of fibroblast growth factors/fibroblast growth factor receptors (FGFRs) in pituitary tumorigenesis. Four FGFRs have been identified and they belong to the tyrosine kinase receptor family. Furthermore, more than 20 FGF ligands have been identified (Asa and Ezzat, 2002). These factors are involved in the cell proliferation, angiogenesis and normal development. In particular, the role of FGF2 (or bFGF) has been highlighted in pituitary tumorigenesis. FGF2 is a estrogen-sensitive gene showing high expression in estrogen-induced pituitary tumors (Heaney *et al.*, 1999), and high expression of FGF2 has also been found in human pituitary adenomas (Asa and Ezzat, 2002). FGF4 has been shown to induce lactotroph proliferation and transforming FGF4 sequences have been identified in human pituitary tumors (Gonsky *et al.*, 1991; Shimon *et al.*, 1996). Recently, an isoform of FGFR4 derived from human pituitary tumor was identified. The overexpression of this isoform was able to transform pituitary cell lines and led to pituitary adenomas in mice overexpressing it (Ezzat *et al.*, 2002).

Bone morphogenic protein 4 (BMP4): In addition the important role of BMP4 in pituitary development (see above), BMP4 expression has been found in pituitary adenomas. BMP4 is overexpressed in prolactinomas derived from *Drd2* KO mice, in estrogen induced rat prolactinomas, and in human prolactinomas (Paez-Pereda *et al.*, 2003). In *Drd2* KO mice, BMP4 inhibitor noggin showed clear downregulation as compared with the WT pituitaries (Paez-Pereda *et al.*, 2003). The mechanism for BMP4 action is not completely clear, but it has been shown to induce known oncoprotein MYC through the interaction of ESR1 and SMAD4 (Paez-Pereda *et al.*, 2003; Giacomini *et al.*, 2007).

Galanin (GAL): Galanin is a neuropeptide, which is synthesized in lactotroph cells and it is a sensitive marker of estrogen status of mice (Hyde *et al.*, 1991). Estrogens increase the anterior pituitary *Gal* content up to 3000-fold, and protein levels also up to 500-fold, whereas gonadectomy decreases pituitary GAL content (Wynick *et al.*, 1993; Wynick *et al.*, 1998). Lactotroph specific *galanin* overexpression induces pituitary hyperplasia and hyperprolactinemia and mice lacking galanin expression show impaired secretion of PRL (Wynick *et al.*, 1998; Cai *et al.*, 1999). Galanin has also been shown to induce PRL secretion in humans (Invitti *et al.*, 1993). Further, galanin expression has been found in the normal pituitary gland and in pituitary adenomas (Bennet *et al.*, 1991; Grenback *et al.*, 2004). However, the molecular mechanism underlying galanin induced lactotroph proliferation has remained unclear, and it is

regarded rather as a factor induced by estrogens than a factor responsible for pituitary adenomas alone.

PRL and PRLR: First evidence of PRL signalling affecting lactotroph proliferation was obtained from the *Drd2* KO mouse model (Kelly *et al.*, 1997; Saiardi *et al.*, 1997). *Drd2* KO females and males presented with a similar estrogen levels and *Drd2* deficiency, and females exhibited clearly elevated PRL levels and increased pituitary size compared with male mice. Therefore, it was suggested that higher PRL levels in female mice might act as a mitogenic stimulus for lactotroph cells (Saiardi *et al.*, 1997). The same group showed expression of the functional PRLR in pituitary gland (Saiardi *et al.*, 1997). Further evidence of the importance of intact PRL signalling controlling lactotroph proliferation was obtained from *Prl* KO mice, which showed pituitary hyperplasia and tumorigenesis of pituitary lactotroph cells (Steger *et al.*, 1998; Cruz-Soto *et al.*, 2002). *Prolactin receptor* knockout (*Prlr* KO) mice develop lactotroph adenomas, and an elegant experiment with double knockout (*Prlr* KO × *Drd2* KO) mice revealed that PRL signalling participates in lactotroph proliferation both in DA dependent and independent manner (Schuff *et al.*, 2002). There were two solid facts for this suggestion. 1) *Prlr* KO mice presented more severe pituitary tumorigenesis than *Drd2* KO mice (Schuff *et al.*, 2002). 2) The double knockout males presented more severe tumorigenesis than either of the KO models alone (Schuff *et al.*, 2002). However, the clinical significance of intact PRL/PRLR signalling has not been demonstrated in pituitary adenomas.

2.3.3.6. Diagnosis and evaluation of pituitary adenomas

Symptoms leading to the diagnosis of pituitary adenomas can be due to hormone overproduction or local pressure effects including headache, visual disturbances, and the loss of hormone production (Melmed, 2006a). Because most pituitary adenomas are hormonally active, it is possible to measure PRL, GH and IGF-1, LH, FSH, Te and E2, ACTH and cortisol, TSH and thyroxin to obtain information of the type of pituitary adenomas. In the case of large tumors, the secretion of anterior pituitary hormone(s) is impaired indicating the need for hormone replacement especially in the case of thyroid and adrenal hormones. MRI is the most sensitive method to evaluate pituitary adenomas, and this imaging technique has improved the diagnosis of pituitary adenomas (Melmed, 2006a). In the case of aggressive and large tumors, CT may be used especially to detect the invasion of the tumor into the bone (Melmed, 2006a). Rarely, SPECT with radiolabeled somatostatin analogue or DA antagonist is used to detect respective receptors (Melmed, 2006a). In some cases, microadenomas are too small to be detected by MRI despite the increased hormone production. Many of these small hormonally active adenomas are ACTH-producing adenomas and diagnosis can be improved by bilateral inferior petrosal sinus sampling together with the CRH-stimulation tests (Morris *et al.*, 2006).

2.3.3.7. Treatment of pituitary adenomas

Many clinical criteria determine the treatment of choice in the case of pituitary adenomas. PRL producing adenomas are especially sensitive to medical therapy. They are efficiently treated by dopamine receptor 2 agonist including bromocriptine,

pergolide, quinagolide or cabergoline (Heaney and Melmed, 2004; Bronstein, 2006). Effective treatment for GH producing adenomas is available, and these include somatostatin analogues octreotide and lantreotide (Heaney and Melmed, 2004; Melmed, 2006b). Some TSH producing adenomas also respond to octreotide and lantreotide treatment (Heaney and Melmed, 2004). Unfortunately, there is no effective medical treatment for silent adenomas, gonadotroph adenoma and ACTH-producing adenomas. In case, where the medical treatment is ineffective, tumors recover after treatment, pituitary adenomas are large causing pressure symptoms, or tumors produce ACTH, LH, FSH or if they are silent, endonasal surgery is the treatment of choice (Heaney and Melmed, 2004). If the adenomas are large or recover after treatment, radiotherapy can be used together with the surgical treatment to improve the outcome (Heaney and Melmed, 2004). Because in many pituitary adenomas, the endonasal surgery is required, it is understandable that new insights for treating pituitary adenomas are needed. Currently, somastostatin-dopamine chimeric compounds are promising candidates as new therapeutic agents for treating pituitary tumors (Heaney and Melmed, 2004; Florio *et al.*, 2008).

2.4. Genetically modified mouse models for studying exaggerated actions of LH/hCG

bLH-CTP mice: Transgenic mice expressing the fusion protein of bovine *LHB* and hCG C-terminal peptide under bovine *Cga* promoter have been previously developed (Risma *et al.*, 1995). In this model, the expression of bLH-CTP occurs only in pituitary gonadotroph cells. TG mice presented with 10-fold LH levels (Risma *et al.*, 1995). TG male mice were sub-fertile, and presented smaller testis than WT males, but their LH and Te levels were normal (Risma *et al.*, 1995). However, TG females developed a substantial phenotype. In original article, the formation of ovarian granulosa cell tumors was described. Later, it was shown that the formation of ovarian tumors is dependent on the mouse strain used, and hCG injections were able to prevent tumor formation (Risma *et al.*, 1995; Keri *et al.*, 2000; Owens *et al.*, 2002). The molecular mechanism underlying the ovarian tumorigenesis in this model has remained unsolved (Owens *et al.*, 2002). Female bLH-CTP mice developed precocious puberty, were infertile due to anovulation, they had uterine defects, and they presented with miscarriages due to hormonal defects (Risma *et al.*, 1997; Mann *et al.*, 1999). These female mice were also obese and developed adrenal hyperfunction due to LHCGR induction in the adrenal glands (Kero *et al.*, 2000; Kero *et al.*, 2003). It has been shown that increased LH levels promote gonadal and adrenal tumorigenesis (Mikola *et al.*, 2003). Mammary gland tumors (Milliken *et al.*, 2002), and pituitary tumors were found in TG female mice (Mohammad *et al.*, 2003). Pituitary tumors were E2-dependent, and reexpression of *Nupr1 (p8*) contributed to pituitary tumorigenesis (Mohammad *et al.*, 2003; Mohammad *et al.*, 2004). However, *Nupr1* expression was found only in 20 % of the adenomas present in pituitary gland suggesting that the reexpression of *Nupr1* was a late event in the pituitary tumorigenesis rather than a trigger (Mohammad *et al.*, 2004).

hCG overexpressing mice: Simultaneously with our study, another group described hCG overexpressing mouse model. In this model, both human *CGA* and *CGB* subunits were driven by mouse *metallothionein 1* promoter, which is not active in the pituitary gland (Matzuk *et al.*, 2003). They described three models; one expressing CGB subunit only and the other two models expressing both subunits even with low or high quantities (Matzuk *et al.*, 2003). Neither male nor female mice expressing *CGB* subunit expressed intact hCG, but they were infertile, and the authors suggested that high amounts of free CGB interferes with normal LH binding and LHCGR signalling (Matzuk *et al.*, 2003). In contrast to this, mice expressing low amounts of both subunits presenting low levels of intact hCG were initially fertile, developed progressive infertility, but no other obvious phenotype (Matzuk *et al.*, 2003). However, mice presenting with high intact hCG levels present clearly elevated serum hCG. In male mice, this led to Leydig cell hyperplasia, increased Te production and infertility. Males also presented with aggressive behaviour and did not mate with the females (Matzuk *et al.*, 2003). In female mice, high hCG levels led to polycystic ovaries, ovarian thecomas and infertility (Matzuk *et al.*, 2003). The authors did not detect any abnormalities in the mammary and adrenal glands (Matzuk *et al.*, 2003).

We have previously characterized our hCGB expressing females mice (hCGβ) (Rulli *et al.*, 2002). These mice develop precocious puberty due to elevated estradiol levels (Rulli *et al.*, 2002). These females also developed PRL producing pituitary adenomas and mammary adenocarcinomas both of which were prevented by gonadectomy at two months of age (Rulli *et al.*, 2002). We have also further characterized the mammary gland tumorigenesis in these females and shown that gonadectomy at older ages (> 3 months of age) caused regression of mammary gland tumorigenesis, but some potentially premalignant lesions remained in the mammary glands (Kuorelahti *et al.*, 2007). Wnt5b, Wnt7b and some known WNT target genes were also slightly upregulated after hCG injection in gonadectomized, E2 and P treated TG female mice (Kuorelahti *et al.*, 2007). It is currently under the evaluation whether the effects of hCG are direct or mediated by some, yet unknown, secondary changes.

3. AIMS OF THE PRESENT STUDY

The normal physiological functions of LH, hCG and their cognate receptor LHCGR are well characterized. The role of LH/hCG/LHCGR in gonadal tumorigenesis has also been intensively studied using genetically modified mouse models, and the activation of LH/hCG/LHCGR has convincingly been linked to gonadal tumorigenesis in humans. Recently, we have shown that from the physiological point of view, the functional LH/LHCGR signalling is mandatory only for normal gonadal function. In the current literature, the role of elevated action of LH/hCG/LHCGR has been linked to the formation of many nongonadal neoplasms. We extended our previous studies and now address the effect of elevated LH/hCG/LHCGR signalling on gonadal and nongonadal phenotypes of hCG overexpressing mice.

The specific aims of the study were:

1. To analyse the developmental aspect of endocrine and neoplastic changes occurring in males expressing moderate and high amounts of hCG. The main goal is to find out whether high hCG levels are able to advance pubertal development of male mice, to cause a change in the fate of FLCs and to induce the formation of Leydig cell tumors without any contributing factors including aberrant oncogene expression as is the case in certain activating LHCGR mutation in humans.

2. To analyse the effects of elevated LH-action on tumorigenesis and physiological functions in aged males expressing moderate and high amounts of hCG. We want especially to know whether the chronic high levels of hCG is able to induce testicular tumors and other tumors in male male. We also pay attention on normal physiological function of hCG overexpressing male mice. With this model, we try to clarify the role of nongonadal LHCGR in tumorigenesis and in normal physiological functions of male mice.

3. To evaluate the tumorigenic properties of elevated LH/hCG action on female mice and clarify the mechanisms of pituitary tumorigenesis in hCG overexpressing female mice. Special attention is paid on the nongonadal phenotypes of female mice, in order to show whether the nonganadal LHCGR is playing important role in tumorigenesis and in normal physiological functions of female mice.

4. MATERIALS AND METHODS

4.1. hCG overexpressing animals

The 2.4-kb human *CGA* minigene was subcloned into the *Bam*HI site of pGEM-4Z vector (Promega, Madison, WI). Then, the 1.2-kb *Bgl*II-*Bam*HI human ubiquitin C promoter fragment was inserted upstream of the *CGA* minigene. The 3.6-kb ubiquitin C/hCGA fragment was released from the backbone vector and used for microinjection. The 2.8-kb ubiquitin C/h*CGB* transgene was constructed and used for TG mouse generation as described previously (Rulli *et al.*, 2002). The TG mice carrying the ubiquitin C/h*CGA* transgene (hCGα mice) were generated by a conventional microinjecting technique. The generation of ubiquitin C/h*CGB* TG mice (hCGβ) has been described previously (Rulli *et al.*, 2002). Genotyping was performed by PCR using the following primers: 1) common 5'-primer (CGCGCCCTCGTCGTGTC) and gene-specific 3'-primers *(CGA*: CCGGCTGGGAGAAGAATGG and *CGB*: AAGCGGGGGTCATCACAGGTC), and the products were analyzed by electrophoresis. For obtaining hCGα mice, males positive for this transgene were used for breeding. To maintain hCGβ colonies of mice, males carrying hCGB transgene were used for breeding. To obtain hCGαβ mice, female positive for h*CGA* transgene and male positive for h*CGB* transgene were used for breeding. The animals were housed in a pathogen-free environment under controlled conditions of temperature and light. They were provided with tap water and commercial mouse chow *ad libitum (al)*. All mice were handled in accordance with the institutional animal care policies of the University of Turku.

4.2. Tissue and serum collection and treatment of the mice

All mice were sacrificed by cervical dislocation or by excessive carbon dioxide followed by cervical dislocation. For measurement of PRL, mice were sacrificed with minimal stress and between 8-11 am. Blood was collected by cardiac puncture and serum was separated by centrifugation. In some studies, blood was collected one day prior the specific treatments and serum samples were collected from the vena saphena between 11 am.-1pm. Tissues were collected and even snap-frozen in liquid nitrogen or fixed with 4% paraformaldehyde or Bouen's solution. Serum and snap-frozen tissues were stored at -70 °C until needed.

To study the fate of FLCs, bromodeoxyuridine (BrdU) was injected ip. between 3-5 days of age (50 mg/kg body weight, twice per day, 6 injection altogether). The testes were collected at the age of 10 days and 60 days. Both WT and hCGαβ males were treated by flutamide (200mg/pellet, 60-day release, IRA, Sarasota, FL) starting at the age of 21 days. Mice were sacrificed at the age of 60-70 days.

During the operations, anaesthesia was induced by 2% tribromoethanol. Gonadectomy was performed through the small incision made in the back skin of the animals, the ovaries were localized, small incision was made into the peritoneum, and the ovaries were pulled out from the peritoneal cavity and dissected by using thermal coagulation of the blood

vessels. The uterus was replaced back to the peritoneal cavity, the peritoneum was closed by one suture, a hormone pellet or tube was placed under the skin (if necessary) and the skin was closed by sutures. To place the hormone tubes or pellets in the mice, a small incision (5-8 mm) was made in the back skin of the mice and silicon tubes and pellets were implanted under the skin and the incision was closed by one suture.

Hormones used and the study set-ups for the pituitary studies are seen in the original publication III. In the hormone antagonist (HA) study, wild-type (WT) and TG females were treated by sham operation, gonadectomy (G), tamoxifen (T, Sigma-Aldrich Co., St. Louis, MO), mifepristone (M, Sigma-Aldrich), bromocriptine (B, 5mg/pellet, 90-day release, catalog number NC-231), or their various combinations. In the hormone replacement (HR) study, WT and TG females were gonadectomized and estradiol (E2) and progesterone (P) were administered. E2 was administered by commercial pellets, 0.25 mg/pellet, 90-day release (catalog number NE-121, IRA), and P (Fluka Chemie GmBH, Buchs, Switzerland) using 2 cm long silastic tubes.

In the diet experiments, WT and hCGβ-mice received normal mouse chow SDS RM1 (control chow), or SDS AIN60 60% (treatment chow). AIN60 60% was designed according to a previous publication (Hursting *et al.*, 1994), and it was calculated to contain 60% of the calorie intake of mice on a normal diet. However, the amount of protein and fat was normal, and lower calorie consumption was due to lower carbohydrate intake. To determine weekly consumption of AIN60 60%, mice were housed individually in clean cages, chow was weighed and the residual chow was weighed after one week of the diet. Weekly consumption of the chow was used to calculate the daily intake for the mice receiving the 60 % diet. Before starting special diet, all mice received SDS RM1 chow *ad libitum* (*al)*. The study set-up is seen in table 6. To determine the proliferative activity of the pituitary cells, 6-8 mice in each treatment group were injected with BrdU (150 mg/kg body weight, once per day, 72 h, 48 h, and 24 h prior to sacrifice) and sacrificed as mentioned above.

Table 6. *Experimental protocol for diet experiment.*

Trial	***Treatment***	***Age (0-2 mo) chow***	***Age (2-6 mo) chow***	***Age (6-7 mo) chow***	***Age (for sacrifice)***
Prevent (prev)	*Sham*	*RM1(al)*	*RM1 (al)*		*6 mo*
	Sham	*RM1(al)*	*AIN60 (al)*		*6 mo*
	CRD	*RM1(al)*	*AIN60 (CRD)*		*6 mo*
Treatment (treat)	*Sham*	*RM1(al)*	*RM1(al)*	*RM1 (al)*	*7 mo*
	Sham	*RM1(al)*	*RM1(al)*	*AIN60 (al)*	*7 mo*
	CRD	*RM1(al)*	*RM1(al)*	*AIN60 (CRD)*	*7 mo*

Sham; non-treated, RM1; mouse chow, AIN60; mouse chow, al; ad libitum, CRD; calori restricted diet, mo; months.

4.3. Fertility studies and determination of pubertal status

Wild-type, hCGα, hCGβ, or hCGαβ male mice were bred individually starting at the age of 6–8 wk of age with randomly cycling wt female mice. The frequency of births and number of offspring born were recorded. Six-week-old WT females were

superovulated by PMSG and hCG, and immediately mated individually with WT, hCGβ, or hCGαβ male mice. Vaginal plugs were monitored on the following morning to confirm mating. Balanopreputial separation, an external sign of the onset of male puberty in rodents (Korenbrot *et al.*, 1977), was monitored daily beginning at day 21 postpartum.

4.4. Hormone measurements

Serum hCGα was measured by an immunofluorometric assay technique (IFMA; Delfia, Wallac Oy, Turku, Finland). Microtitration wells coated with streptavidin and a biotinylated monoclonal antibody against the α-subunit were used with a europium-labelled mouse monoclonal antibody against the α-subunit from the human FSH Delfia kit. The reference preparation from the NIDDK, NIH (Bethesda, MD), αhCG CR-119, was used as standard. The detection limit was 50 ng/l. Serum hCGβ-levels were measured by IFMA as described previously (Rulli *et al.*, 2002). The detection limit was 40 µg/l. Serum levels of dimeric hCG were measured by IFMA using the Delfia hCG kit (Delfia, Wallac, Turku, Finland) according to the manufacturer's instructions; the detection limit was 0.5 IU/l. FSH levels were measured by IFMA (Delfia, Wallac, Turku, Finland) as described previously (van Casteren *et al.*, 2000); the sensitivity of the assay was 50 ng/l.

The bioactivity of circulating hCG was determined by the mouse interstitial cell *in vitro* bioassay as described previously (Ding and Huhtaniemi, 1989). The sensitivity of the bioassay was 0.5 IU/l. For measuring intratesticular Te and P, the testis was homogenized in PBS. Intratesticular and serum Te and P and serum E2 were measured after diethyl ether extraction. Te was measured by conventional RIA (Huhtaniemi *et al.*, 1985a), P by RIA or FIA (Delfia, Perkin Elmer-Wallac, Turku, Finland) and E by FIA (Delfia, Perkin Elmer-Wallac, Turku, Finland). Intratesticular inhibin B was measured with a serum inhibin B kit (Oxford Bio-Innovation Ltd., Oxford, UK) for the human according to the manufacturer's protocol. Before assay, frozen testis samples were weighed, homogenized in PBS, centrifuged at 13,000 rpm for 10 min, and supernatants were used for protein and inhibin B measurements. Prolactin (PRL) was measured by RIA, as described previously, using a mouse PRL antibody and mouse reference preparation AFP-6476C, provided by NIDDK (Bergendahl *et al.*, 1989). Serum corticosterone was measured by RIA (ICN Biomedicals, Inc., Costa Mesa, CA) according to the manufacturer's instructions. Serum thyroid stimulating hormone (TSH) was measured by RIA using rat TSH antibody and rat TSH reference preparation NIDDK-rTSH-RP-3 kindly provided by NIDDK. Serum Le was measured by mouse leptin RIA kit (Linco Research, Inc., St. Charles, MO, USA) according to the manufacturer's instructions.

4.5. Histological analysis

Paraffin sections were stained with Harris' haematoxylin–eosin and used for light microscopy. Histological sections were photographed using the IM1000 microscope (Leica, Heerbrugg, Switzerland). The LC areas (original publication I) were

determined by using Leica IM 1000, version 1.02 software (Leica Microsystems AG, Heerbrugg, Switzerland). The whole testicular sections and LCs were manually outlined, areas were determined and the volume density of LCs and the largest LC islets were calculated by relating the total or largest continuous LC area to the area of the whole section. The index of total LC volume per testis was calculated by multiplying the volume density by total testis mass, and the density of tissue was regarded as constant, 1 mg/mm^3. Three to eight testicular sections from three animals per group were analyzed.

4.6. Immunohistochemical analyses

Briefly, 5 micrometer thick sections were deparaffinized, microwaved for 15 minutes in citrate buffer (10 mM, pH 6.0), and endogenous peroxidase were blocked by 3 % H_2O_2. The background was blocked by appropriate 10 % normal serum, and the sections were incubated at 4°C overnight with primary antibody (seen in original publications I-III). The sections were incubated with secondary antibody for 1 h, and staining was visualized using Vectastain Elite kit (Vector Laboratoires Inc., Burlingame, CA, USA) according to the manufacturer's instructions. The PowerVision+ IHC detection system for mouse and rabbit primary antibodies were used according to manufacturer's instructions to detect RB-positive cells (ImmunoVision, Brisbane, CA, USA), and the M.O.M.^{tm} kit (Vector Laboratoires) was used according to the manufacturer's instructions to detect CCND1 and CDK4 positive cells. To detect BrdU positive cells, the BrdU *In-Situ* Detection Kit (BD Biosciences Pharmingen, San Diego, CA) was used according to the manufacturer's instructions.

The proliferation index was determined by counting the KI67 positive nuclei and adjusting the result for measurement area (mm^2). Areas were determined by using Leica IM 1000, version 1.02 software (Leica Microsystems AG, Heerbrugg, Switzerland). At least 3 samples per group and 2 sections per sample were calculated. From the pituitary nodules, KI67 positive cells were calculated also as percentage of all cells in the nodular area and also from surrounding area. For this, samples were counterstained by haematoxylin, and 10 fields seen with 40 x magnification was calculated per each nodules. The results were also calculated as number of positive cell per measurement area (mm^2) in order to adjust the results with other groups.

4.7. LH/hCG binding assay

Testicular LH/hCG receptor binding was measured as previously described (Huhtaniemi *et al.*, 1986). hCG (NIH CR-125; 13,000 IU/mg) was radioiodinated by lactoperoxidase method (Huhtaniemi *et al.*, 1986). Briefly, one testis was homogenized in Dulbecco's PBS and 0.1% BSA and testicular homogenate were incubated in triplicate in the presence of saturating concentrations of [^{125}I]iodo-hCG (150,000 cpm/tube; ~3 ng). Nonspecific binding was assessed in the presence of 50 IU unlabeled hCG (Pregnyl, Organon, Oss, The Netherlands). After overnight incubation at room temperature, the homogenates were washed with 4 ml ice-cold Dulbecco's PBS and 0.1% BSA and centrifuged at 2000 g for 20 min. Pellets were counted in a γ-

spectrometer. Protein concentrations were measured using the Bradford method, and specific hCG-binding was expressed as a percentage of the control, based on counts per minute per milligram of protein.

4.8. Eletron microscopy

Pieces of testes cut into pieces of approximately 1 mm^3, were first fixed in 5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.16 mol/L s-collidine buffer (pH 7.4) and postfixed in potassium ferrocyanide-osmium tetroxide. Then pieces were embedded in epoxy resin (Glycidether 100, Merck, Darmstadt, Germany) and cut into sections as described earlier (Sundstrom *et al.*, 1999). For light microscopic survey, 1-mm-thick sections were stained with 0.5% toluidine blue. The sections for electron microscopy were stained with 5% uranyl acetate and 5% lead citrate in Ultrostainer (Leica Corp., Wien, Austria) and examined in JEM 1200 EX transmission electron microscope (JEOL, Tokyo, Japan).

4.9. RNA isolation and DNAse treatment

Total tissue and cellular RNA was extracted by a Rneasy mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Tissues were homogenized by using a rotor stator homogenator and cultured cells by using QiaShredders (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Prior to quantitative RT–PCR, all samples were treated by deoxyribonuclease I (Invitrogen, Carlsbad, CA, USA).

4.10. RT-PCR and Quantitative RT-PCR analysis

The expression patterns of the genes presented in Table 7 were determined. The names of the genes, gene bank accession codes, primer pairs, and annealing temperatures are listed in Table 7. Quantitative RT–PCR analysis was carried out by using the DNA Engine Opticon System (MJ Research Inc., Waltham, MA, USA) with continuous fluorescence detection. RT–PCR reactions were carried out using the Quantitect SYBR Green RT-PCR Kit (Qiagen, Valencia, CA, USA) (original publications I, III) according to the manufacturer's instructions. RNA (20-100 ng) was used in all reactions. Standards and samples were run in duplicate or triplicate, and three to seven individual samples were analyzed per experimental group. The level of cytoplasmic b-actin mRNA was used to normalize the expression of the other mRNA species.

4.11. Primary pituitary cell culture

Primary pituitary cell cultures were performed as described earlier with minor modification (Pastorcic *et al.*, 1995). Briefly, 3-5 pituitary glands of 4 month old hCGβ were collected and minced and washed in ice-cold PBS (without calcium and

magnesium) containing 1 g/L D-glucose (Sigma-Aldrich Co., St. Louis, MO) and 200 μM ascorbic acid (Merck, Darmstadt, Germany). Tissue fragments were enzymatically dissociated in the previous buffer containing 2mg/mL collagenase (Sigma-Aldrich Co., St. Louis, MO), 60 U/ml DNase 1 (Epicentre, Madison, WI) and 1mg/ml BSA (Sigma-Aldrich Co., St. Louis, MO) for 40 minutes in a shaker at 37°C and fragments were dispersed into a single cells by trituration. Cells were plated at 20.000/well in Growth factor reduced Matrigel matrix (BD biosciences, Bedford, MI) coated 24-well plates in DMEM/F12 medium (Sigma-Aldrich Co., St. Louis, MO) containing penicillin, streptomycin, 200 μM ascorbic acid, 1mg/ml BSA and 10% FCS for 24 hours. Then cells were washed twice with PBS and incubated 24 hours in DMEM/F12 medium containing penicillin, streptomycin and 5 % dextran coated charcoal treated FCS in the presence of E2 (Sigma Chemical Co., St. Louis, MO), P (Fluka Chemie GmBH, Buchs, Switzerland), or combination. RNA was collected immediately after the experiments by using Rneasy mini kit (Qiagen).

4.12. Statistical analysis

Statistical analyses were performed using the SigmaStat 2.03 (I-II) or 3.1 (III) software (SPSS Inc., Chicago, IL) or SAS System software version 9.1.3 SP2 (SAS Institute, Gary, NC). One-way ANOVA was used for multiple comparisons followed by Student-Newman-Keuls'(I-II), Holm-Sidak's or Dunn's (III) post hoc tests. The Student's t test (I-III) was used to analyze differences between two groups. The Friedman test followed by Dunn's multiple comparison test was used to analyze the results of the cell culture experiment (III). In the case of pituitary weights and PRL levels in HA and HR experiments (III), statistical analyses were performed by two-way ANOVA and analysis of covariance. Natural log-transformation was used when necessary. For correlation analyses, Pearson product moment correlation was used. Significance was set at $p < 0.05$, and all results are presented as mean ± SEM except for correlation analysis, where individual values are shown.

Table 7. *The genes, GeneBank accession numbers, primer sequences, and annealing temperatures for the genes whose transcripts were measured by quantitative RT-PCR.*

Gene	Gene bank	Primer 5'	Primer 3'	T
Actb	NM_007393	cgtgggccgccctaggcacca	ttggccttagggttcagggg	54
Thbs	NM_011581	gcaggatgtacgtggccaaa	gagatctgtggtgatgtgag	54
Hsd3b6	NM_013821	ggaggagatcagggtcctgg	tctaggatggtctgcctggg	59
Ptgds	NM_008963	agtggtagcccctccacag	gagtggatgctgcccgagtg	61
Lhcgr	NM_013582	ttgccgaagaaagaacagaat	agccaaatcaacaccctaag	54
Star	NM_011485	cagggagaggtggctatgca	ccgtgtcttttccaatcctctg	56
Cyp11a1	NM_019779	agatcccttccccctggcgacaatg	cgcatgagaagagtatcgacgcatc	64
Hsd3b1	NM_008293	caggagcaggagggtttggt	gtggccattcaggacgat	54
Cyp17a1	NM_007809	ggccccagatggtgactct	ggactccccgtcgtatgtaa	54
Hsd17b3	NM_008291	atcaatgggacaatgggcag	tctaagccttcaaggtgttc	55
Eif4h	NM_033561	ctttgctctctctcaagtga	ttcccttgtgtctgtctga	54
Rpl19	NM_009078	ctgaaggtcaaagggaatgtg	ggacagagtcttgatgatctc	55
Prl	NM_011164	ctcaggccatcttggagaag	tcggagagaagtctggcagt	57
Gh	NM_008117	tcctgtggacagatcactgc	aatgtaggcacgctcgaact	62
Pomc	NM_008895	gcaccagctccacacatcta	catctttgtccccagagagc	60
Tshb	NM_009432	cgggatatcaatggcaaact	tctgacagcctcgtgtatgc	58
Fshb	NM_008045	ccgagctgggtccttataca	tcagctttcccagaagaga	60
Drd2s	NM_010077	caaaatctacatggttctccgcaag	cggcgggcagcatccttgagtg	60
Drd2l	NM_010077	caaaatctacatggttctccgcaag	cgggcggcagcatccattctc	60
Esr1	NM_007956	gtgcttcaacattctccctcctc	ccgtgtgcaatgactatgcc	56
Esr2	NM_010157	cttggtcacgtacccttac	gtatcgcgtcactttccttt	55
Cyp19a1	NM_007810	ggccaaatagcgcaagatgttctt	aatgaggggcccaattcccaga	61
Pgr	NM_008829	gatgaagcatctggctgtca	gggttctcataactcggac	56
Pgr (B)	NM_008829	tcgtctgtagtctcgcctataccg	cggagggagtcaacaacgagt	56
Ar	NM_013476	ctgcctccgaagtgtggtat	gccagaagcttcatctccac	59
Lepr	NM_101704	aggaatcgttctgcaaatcca	tatgccaggttaagtgcagctatc	60
Gal	NM_010253	gtgaccctgtcagccactct	ggtctcctttcctccacctc	62
Pttg1	NM_013917	agttgccgaaaagcctatga	ccattcaaggggagaagtga	57
Fgf2	NM_008006	cagcggcatcacctcgcttc	ccggttggcacacactccc	54
Bmp4	NM_007554	cttctacagatgtttgggct	gatgttctccagatgttctt	53
Nog	NM_008711	tgtggtcacagaccttctgc	atggggtactggatgggaat	60
Myc	NM_010849	ggaagaaattcgagctgcttc	cttgatgaaggtctcgtcgtc	57
Ccnd1	NM_007631	tctcttgctaccgcacaac	ttcctccacttccccctc	59
Ccnd2	NM_009829	tcccgcagtgttcctatttc	ccaagaaacggtccaggtaa	59
Ccnd3	NM_007632	agacctttggccctctgt	gtccacttcagtgcctgtga	59
Ccne1	NM_007633	tccagaaaaaggaaggcaaa	cagggctgactgctatcctc	56
Fos	NM_010234	ggggcaaagtagagcagcta	tgcaacgcagacttctcatc	61
Egr1	NM_007913	ccacccaacatcagttctcc	ttgctcagcagcatcatctc	58
Ets1	NM_011808	tccagacagacaccttgcag	ggtgaggcggtcacaactat	61
Elk1	NM_007922	ctgctcccacacatacctt	gagaggccatccacactgat	60
Hras1	NM_008284	atggcatcccctacattgaa	acagcacacatttgcagctc	57
E2f1	NM_007891	actgtgactttggggacctg	cagagggtatggatcgtgct	60
E2f3	NM_010093	ggaaggcatccacctcatta	gtccagggtacagctttgg	60
Hmga2	NM_178057	acccagaggaagacccaaag	cagtctcctgagcaggcttc	58
Pten	NM_008960	gattacagacccgtggcact	gggtcctgaattggaggaat	59
Nupr1	NM_019738	gagaagctgctgccaatacc	tgtggtctggccttatctcc	58
Cdkn1a	NM_007669	gtacttcctctgccctgctg	aatctgtcaggctggtctgc	58
Cdkn1b	NM_009875	agggccaacagaacagaaga	ccagatggggtgtcagtttt	59
Cdkn2a	NM_009877	ctttgtgtaccgctgggaac	ctgaggccggatttagctct	57
Cdkn2b	NM_007671	aatggatttgggagaactgc	tgacagcaaaaccagttcca	56
Paqr7	NM_027995	cgctgcatcagaactggtgtt	cacacgttcacagcctcgtt	60
Paqr8	NM_028829	gccctgtccccgagaagtac	gaaggcgtggaagatttgatg	60
Paqr5	NM_028748	ctcacctgccagagcgactag	ggatgacgcacacatgaaaca	60

T; annealing temperature (°C).

5. RESULTS

5.1. Transgenic mouse models used in this study (I, II, III)

To evaluate the effects of elevated LH/hCG levels on gonadal and nongonadal tumorigenesis, two different hCG overexpressing mouse models were used.

Moderatately elevated hCG levels: hCGβ mice express human *CGB* subunit under universal promoter also directing the expression of the gene into the pituitary gland. There, CGB couples to mouse own CGA subunit naturally present in thyrotrope and gonadotrope cell lineages. Thus, the intact hCG molecules produced in this model are chimeric hormones composed of mouse CGA and human CGB, and the LH-bioactivities were 3-fold and 30-fold increased in hCGβ males and females, respectively.

Highly elevated hCG levels: In another model, transgenic mice expressed both human *CGA* and *CGB* subunits (hCGαβ mice) under a universal promoter directing the expression of these genes into almost all tissues. Therefore, the coupling of CGA and CGB subunits to the dimeric active hCG molecules occurs everywhere leading to extremely high levels of hCG. LH-bioactivity was more than 1000-fold increased in hCGαβ males in comparison to WT males.

5.2. Testicular phenotype of hCG overexpressing males (I, II)

5.2.1. Leydig cell adenomas in young hCG overexpressing males (I)

We evaluated the testicular phenotype of 5, 10, 21, and 60 –day-old WT and hCGαβ mice males. Testis weights were equal in WT and hCGαβ males at 5 and 10 days of age, but hCGαβ males presented almost 50% smaller testis at the ages of 21 and 60 days as compared with WT mice. However, in the histological evaluation, large Leydig cell (LC) nodules were observed in 5, 10, and 21 day-old hCGαβ males. At the age of 10 days in hCGαβ males, these nodules were clearly larger than the diameter of seminiferous tubules and some mitotic figures were also observed at this age (Figure 8) indicating the presence of LC adenoma (Clegg *et al.*, 1997). However, at the age of 60 days, only slight hypertrophy of the LCs were seen, and the index of total LC volume was similar between WT and hCGαβ males at this age. To answer the question whether the LC adenomas observed were derived from fetal or adult type of LCs, the mice were injected with BrdU between the ages of 3-5 days, and BrdU labelling was assessed at the ages of 10 and 60 days. At the age of 10 days, numerous BrdU positive LCs were observed in hCGαβ males, while in WT males only few positive LCs were present (Figure 8). In adult mice, only few BrdU positive LCs were observed both in WT and hCGαβ males (Figure 8). In line with these observations, at the age of 10 days, a fetal LC marker, *thrombospondin 2* (*Thbs2*), were upregulated and adult LC markers, *hydroxy-steroid dehydrogenase-6* (*Hsd3b6*) and *prostaglandin D synthase* (*Ptgds*), were downregulated in hCGαβ males as compared with adult males. Serum and testicular Te production were highly increased in hCGαβ males at all ages studied

indicating the presence of functional LCs. The histological evaluation of hCGβ testis did not reveal visible abnormalities in all age-groups studied as compared with age-matched WT males.

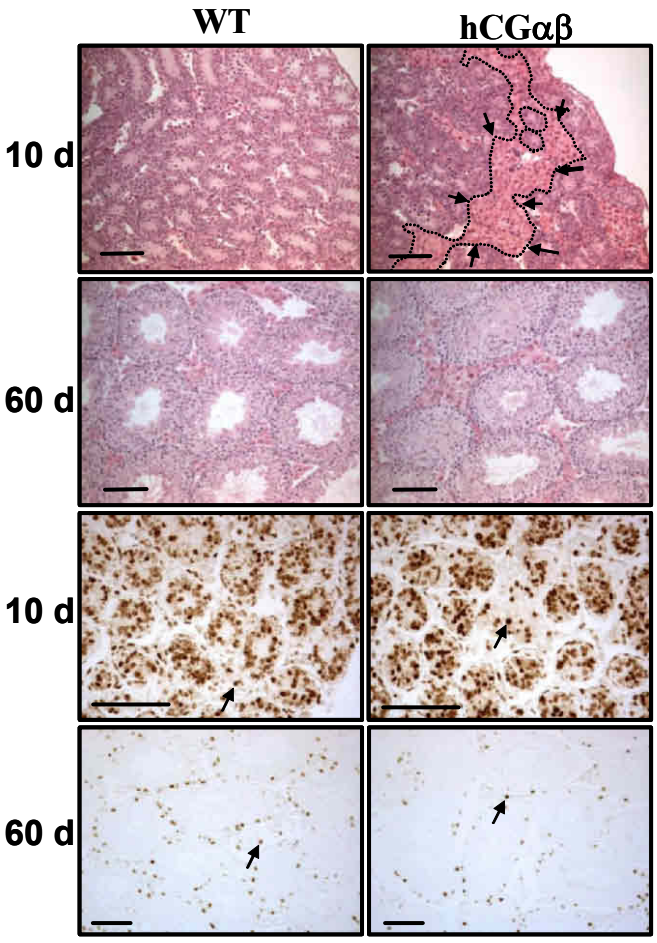


Figure 8. *Testicular histology and BrdU labelling of 10- and 60-day (d) old WT and hCGαβ males. Histology shows the presence of LC adenomas indicated by the dotted line and arrows in 10-day-old hCGαβ males. In the adult male, only slightly hypertrophied LCs are seen. For evaluating LC fate and proliferation, mice were treated with BrdU between the ages of 3 to 5 days, and BrdU labelling was assessed at the ages of 10 and 60 days. Several FLCs show positive BrdU labelling (arrows) in 10-day-old hCGαβ, but only few positive cells are seen in WT mice. In adult males, some BrdU positive LCs (arrows) are seen in both WT and hCGαβ males. Results suggest that hCG induce the proliferation of FLCs. The BrdU staining present in adult males might also reflect that SLCs proliferate during the injection period or that some FLCs remain dormant in the adult testis. The bar is 100 µm.*

5.2.2. Slight hypertrophy of Leydig cells in adult hCG overexpressing males (II)

We also evaluated possible testicular tumorigenesis in old hCGαβ males. In 6-month-old mice, hCG overexpression led to decreased testicular weight both in hCGβ and hCGαβ males compared with WT males, and the reduction was 25% and 50%, respectively. To our surprise, we did not detect testicular tumorigenesis even in 9-month-old hCGαβ males. In histological analysis, there seemed to be slight Leydig cell hypertrophy and hyperplasia present in hCGαβ males, but no signs of LC adenomas were observed. We did not evaluate the index of total LC volume at this age, but the overall view was similar as in 60-day-old hCGαβ males, which presented the equal index of total LC volume compared with WT males. Therefore, we think that the relative hypertrophy/hyperplasia observed in adult hCGαβ males is due to decreased

length of the seminiferous tubule leading to decreased testicular weight, decreased diameter of testicular section and to the concentrated accumulation of LCs.

5.2.3. Adult Leydig cells are protected against high LH/hCG levels (I, II)

The potential role of hCG in inducing LC tumors was different between the FLC and ALC population. We showed that *Lhcgr* expression were highest in 10-day-old hCGαβ compared with any other group. The testicular binding assay showed reduced hCG binding especially in adult hCGαβ males, but also in hCGβ males. However, testicular and serum Te levels were almost equal in all hCGαβ age-groups. We also analysed the expression pattern of dynamins and arrestins, but did not find significant differences in their expression between different age-groups (data not shown). Currently, we are studying the expression of some G-protein coupled receptor kinases including *G-protein coupled receptor kinase 4* (*Grk4*) and *adrenergic receptor kinase beta 2* (*Adrbk2*, or Grk3). These kinases showed decreased expression in 10-day-old WT and hCGαβ males compared with adult males of the same genotype (Figure 9, unpublished results). The GRK4 localized especially in ALCs showing a dotted staining pattern enhanced by highly elevated hCG levels (Figure 9, unpublished result).

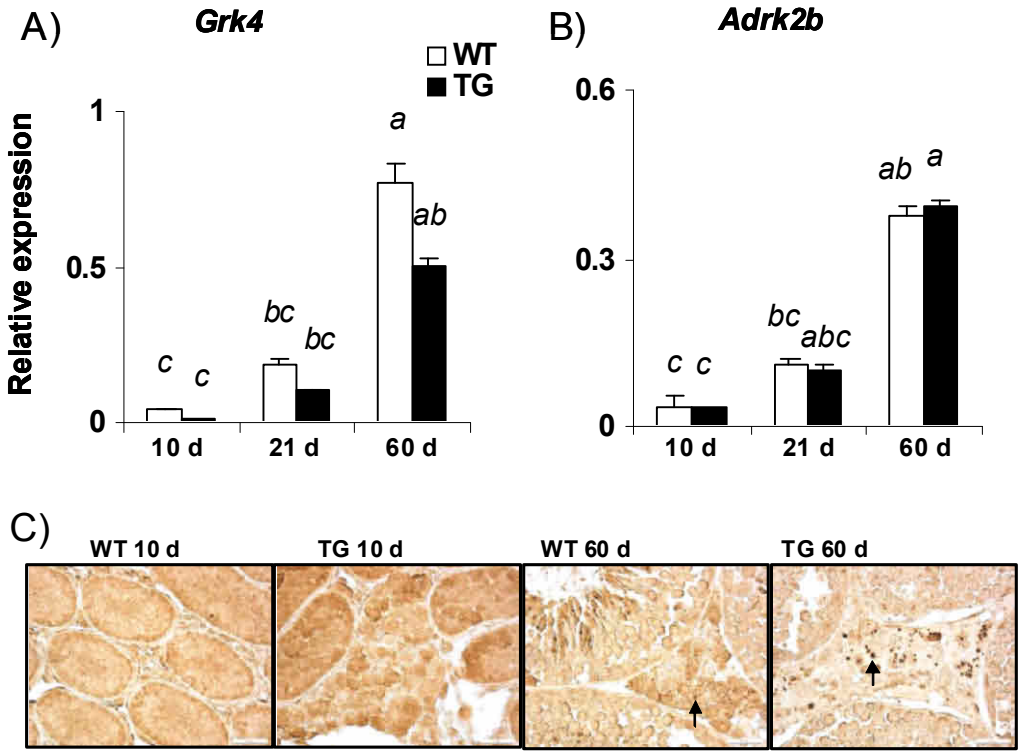


Figure 9. *The testicular expression pattern of a) Grk4 and b) Adrk2b genes, and c) localization of GRK4 protein in WT and hCGαβ male mice. The results shown are mean ± SEM of 3-5 mice per group. Different letters indicate significant differences between mice groups. White columns present WT and black columns TG males. Ages (days, d) are given in the figures. In figure c) arrows indicate GRK4 positive staining. WT; wild-type mice, TG; hCGαβ mice.*

In FLCs, we did not observe dotted staining pattern of GRK4. Dots in the ALCs might correspond to endosomes participating in the desensitization of LHCGR in response to elevated hCG levels.

5.2.4. Spermatogenesis and infertility of adult hCG overexpressing males (I, II)

We evaluated the progression and quality of spermatogenesis by histology. During the pubertal development of mice, we did not detect any advancement in spermatogenesis in 5, 10, 21, 28 and 60 day-old hCGαβ males compared with WT males. Moreover, in adult hCGαβ mice spermatogenesis seemed qualitatively similar to the WT males. In 6-month-old and older hCGαβ males, progressive degeneration was observed. First, some vacuoles were observed in the basal compartment of the seminiferous tubules, and the degeneration of some tubules was evident. In 8-9-month-old males, tubular degeneration was increased, interfering with spermatogenesis in those tubules most severely affected. It is likely that degeneration of seminiferous tubules is caused by back-pressure effects due to functional/anatomical abnormalities in epididymides, vas deferens or prostate leading to obstruction at the level of urethra (see section 5.3.2).

Because the spermatogenesis seemed normal, we mated adult hCGαβ males with normal and superovulated WT females, but hCGαβ males were not able to produce offsprings. We did not observe any vaginal plugs suggesting that hCGαβ males were infertile due to abnormal mating behaviour. This might be due to the aggressive behaviour of hCGαβ males, since the female mice mated with the males were often injured and occasionally even died due to aggressive behaviour of male mice.

5.3. Nongonadal phenotype of hCG overexpressing males (I, II)

5.3.1. No signs of precocious puberty in hCG overexpressing males (I)

We also analyzed the beginning of puberty in WT and hCGαβ males by observing the occurrence of balanopreputial separation (BPS). To our surprise, the highly elevated Te in hCGαβ males was not able to induce precocious puberty, since the BPS occurred at day 34.2 ± 1.0 and 34.6 ± 1.0 in WT and hCGαβ males, respectively. Results are in line with the similar progression of spermatogenesis in hCGαβ and WT males. However, flutamide treatment starting at the age of 21 days postponed or completely prevented BPS during the 40-day follow-up period both in WT and hCGαβ males. Results suggest that the beginning of puberty in male mice is 1) androgen dependent and 2) some other factor(s) not regulated by hCG synergize with androgens to induce the beginning of puberty.

5.3.2. Enlarged prostate and seminal vesicles in hCG overexpressing males (II)

As a sign of infravesical (urethral) obstruction, enlarged kidneys were observed in 6-month-old hCGαβ males. In renal parenchyma, lumens of tubules were distended and cells were enlarged. Renal pelvis, ureters and urinary bladder were distended. The ventral prostate was clearly enlarged and associated with enlarged glands with a hyperplastic, multilayered epithelium. Seminal vesicles were also enlarged with a flattened epithelium, impaired mucosal folding and distended glandular lumina. Distal parts of the *vasa*

deferentia were distended and filled with sperm, and in 8-9-month-old males, as increased amount of connective tissue and smooth muscle cells were observed in cauda epididymis. Many sperm granulomas were also observed in the tubular lumen of the epididymis. Due to all these changes, we carefully analyzed the urethra to find anatomical obstruction, but we only found slight hyperplasia of the posterior wall of urethra. The lack of visible obstruction in the urethra led us to suspect that there might be a functional obstruction responsible for infravesical obstruction. Some hCGαβ males were also treated with flutamide, an androgen receptor antagonist. Flutamide treatment decreased the size of the prostate and seminal vesicles indicating that the phenotype observed in hCGαβ males is due to elevated androgens and other related steroid hormones, not due to hCG itself (Ahtianen et al., unpublished observation, data not shown).

5.3.3. hCG does not induce nongonadal tumorigenesis in hCG overexpressing males (I, II)

One of the most important finding in this experiment was the lack of tumorigenesis in nongonadal tissues. Most of the hCGαβ males died before 9 months of age due to urethral obstruction and possibly associated infection. Despite the FLC adenomas and slight hyperplasia of the prostate, we did not detected any other tumors in these mice during 7 years of the study. Although the number of 8-9-month-old hCGαβ mice analysed is small (<20), we conclude that nongonadal tumors are rare in hCGαβ males indicating that elevated LH/hCG action is not a major cause of cancers in males.

5.4. Ovarian phenotype of hCG overexpressing females

Both hCGβ and hCGαβ females were infertile. Moderate hCG levels in hCGβ females induced luteinization of the ovaries and in some mice, luteoomas were also present in the ovaries. These females presented high serum levels of P and Te (Rulli *et al.*, 2002). However, E2 levels were increased only during the pre- and peripubertal period, but remained normal in adulthood (Rulli *et al.*, 2002). Because PRL has been shown to induce luteinization of the ovaries in mice, we treated the mice with bromocriptine, a DA agonist, to decrease their PRL levels and studied the ovarian histology. In histological analysis, it was evident that bromocriptine treatment and low PRL decreased luteinization (Figure 10, unpublished results) and serum P levels (III) in bromocriptine treated hCGβ females compared with untreated hCGβ females. However, P levels remained 3-fold higher than in WT females (III). Results indicate that hCG, through elevated PRL, indirectly induces the formation of luteomas.

In hCGαβ females, large ovarian tumors were seen, and the histological evaluation of the tumors revealed the presence of the tissues derived from all three embryonal layers indicating that tumors were teratomas (Figure 10, unpublished results). Some oocytes from 3-4-week-old hCGαβ females undergo spontaneous activation and development until the blastocyst stage suggesting that ovarian teratomas originate from parthenogenically activated oocytes (Rulli et al., unpublished results, data not shown). In the ovaries of 5-7-week-old hCGαβ females, cellular structures resembling the trophoblastic giant cells were seen indicating that oocyte activation inside the ovaries is the cause of teratomas (Figure 10, unpublished results).

5.5. Pituitary adenomas in hCG overexpressing females (III)

In our original publication, we described the development of prolactinomas in hCGβ females (Rulli *et al.*, 2002). In this study, we further analyzed the hormonal factors and mechanisms underlying the formation of PAs in hCGβ females. In line with hCGβ females, hCGαβ females also developed pituitary adenomas, but due to their rapidly growing ovarian teratomas, PAs were not further analyzed.

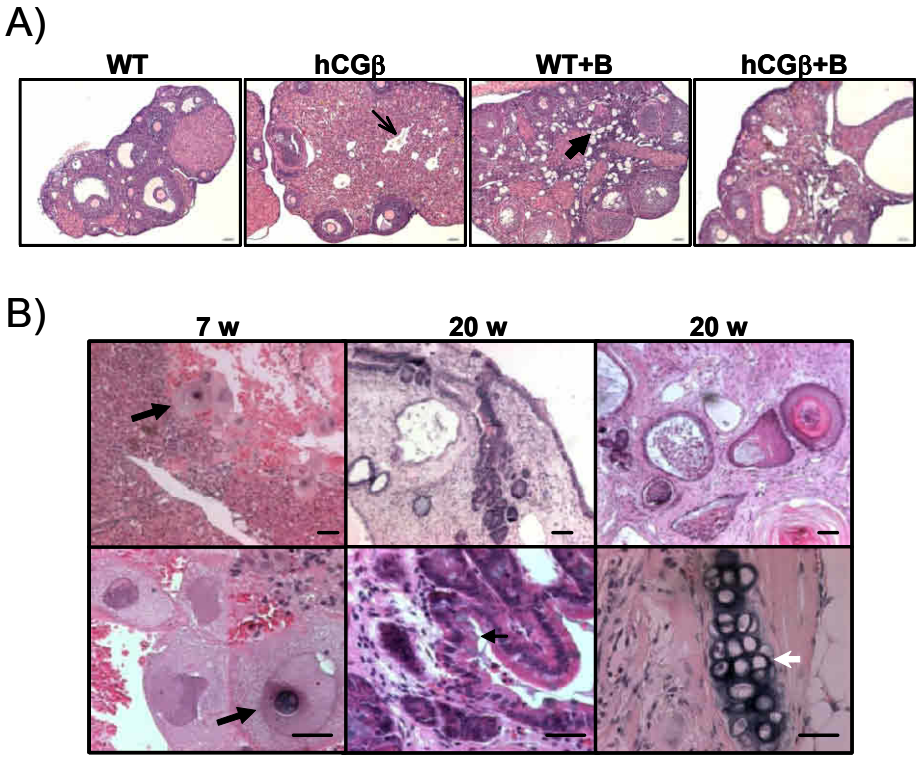


Figure 10. *Histological analysis of a) the sham and bromocriptine treated WT and hCGβ ovaries, and b) ovarian teratomas seen in hCGαβ females. In a), small arrows show the hemorrhagic cysts found in TG ovaries. Large arrows indicate the atrophied follicles in WT+B ovaries. The genotypes and treatment are given above the panel. The scale bar is 100 μm. In b), the left micrographs present 7-week-old hCGαβ female ovaries. Large arrows indicate trophoblastic giant cells suggesting parthenogenetic activation of oocytes inside the ovaries. The middle and right micrographs show representative micrographs of ovarian teratomas. The small arrow indicates goblet cells and white arrow shows cartilage. The bar is 100 μm in the upper panels, and 50 μm in the lower panels.*

5.5.1. Abnormal histology and exceptionally high KI-67 index in pituitary adenomas (III)

In our original publication describing the hCGβ females, we did not analyze the time-course of pituitary tumorigenesis (Rulli *et al.*, 2002).

Histological evaluation of the pituitary glands revealed that 4-month-old hCGβ females presented with enlarged anterior lobes and signs of increased vascularization. However, the cellular architecture remained rather normal, but some mitotic figures

and some large vacuolized cells were seen as a sign of secretory activity at this age. At the age of 4 months, laminin, a marker for the presence of PAs (Kuchenbauer *et al.*, 2003), showed an almost normal staining pattern. In the middle portions of the glands, normal pituitary tissue was seen. KI-67 positive cells were distributed uniformly throughout the pituitary gland.

At the age of 6 months, the anterior pituitary glands of hCGβ females were clearly enlarged with increased number of mitotic figures, extravasated blood cells, prominent vasculature, and atypical cellular architecture with large, vacuolized cells with prominent nuclei indicating high secretory activity. Small nodules containing morphologically identical cells covered most of the lateral lobes of the anterior pituitary gland. In the middle part of the gland, normal pituitary tissue was also seen. Laminin immunostaining revealed unstained nodules in all pituitaries studied, indicating the presence of adenomas (Kuchenbauer *et al.*, 2003). KI-67 labelling showed that already in 6-month-old hCGβ females, positive cells were accumulated in small foci. Hence, the transition from hyperplastic to adenomatous pituitary growth occurred between 4-6 months of age.

At the age of 12 months, clearly visible macroscopic nodules of adenomas were seen in the hCGβ pituitary glands and the weight of pituitary glands was ~ 100mg (2-3mg in WT mice). These nodules were surrounded by a thin pseudocapsule consisting of cells of the adjacent tissue being compressed by the adenomatous nodule. The cells in the nodules were vacuolized with prominent nuclei and large numbers of mitotic figures. At this age, all the normal pituitary tissue had disappeared. In 12-month-old TG females, large nodules of macroprolactinomas were seen with numerous KI-67 positive cells.

The density of proliferating cells per mm^2 (DPC) in the macroadenomas was higher than in surrounding pituitary parenchyma, and the number of KI-67 positive cells increased with age. All hCGβ groups showed a higher DPC value compared with the pooled WT group. The mean proliferation index (PI) in the nodules of macroadenomas was 23.6 %, and the highest PI was 40%. The PI of this magnitude suggested complete loss of cell-cycle regulation. However, the PAs were not regarded as malignant, since they did not invade or metastasize.

5.5.2. Aberrant cell-cycle regulation in pituitary adenomas (III)

We found several aberrations in the cell-cycle regulation in 6 and 12-month-old hCGβ females presenting PAs. First, the expression of an important cell-cycle regulator, *Cyclin D1* (*Ccnd1*), was increased. Second, its downstream target, *E2F transcription factor 1* (*E2f1*) was similarly upregulated in pituitary adenomas. Third, a novel architectural factor, *high mobility group AT-hook 2* (*Hmga2*) enhancing the transcriptional activity of E2F1 was highly upregulated in pituitary adenomas.

At the protein level, CCND1 and its partner CDK4 showed clear nuclear localization in pituitary adenomas, but rarely in WT pituitary glands. The nuclear localization of important cell-cycle regulator, tumor suppressor RB1, was also diminished in pituitary adenomas but showed intense nuclear staining in WT mice. We also performed a primary pituitary cell culture to show the effect of E2 and P on *Ccnd1*

expression. We found that *Ccnd1* was upregulated by an E2+P combination but not by either of the hormones alone.

5.5.3. Gonadal dependency of pituitary adenomas (III)

We used hormone antagonist (HA) and hormone replacement (HR) strategies to identify the hormonal factors responsible for pituitary tumorigenesis. In the HA study, bromocriptine (B) (DA agonist) and mifepristone (antiprogestin) were equally efficient at inhibiting pituitary tumorigenesis, but the pituitary weights in these groups still remained larger than in WT females, indicating that blocking one pathway is not enough to abolish the development of PAs. In contrast, both gonadectomy and tamoxifen treatment prevented the enlargement of the pituitary glands completely, indicating that the ovaries and at least physiological E2 levels are needed for pituitary tumorigenesis. However, short term tamoxifen treatment (1 week – 2 months) only delayed the formation of PAs indicating that prepubertal E2 peak and precocious puberty seen in hCGβ females are not responsible for PAs.

In the HR study, E2 replacement after gonadectomy (G) was not able to recover pituitary tumorigenesis. A slight increase in pituitary weight was observed in WT and hCGβ females after this treatment. PRL secretion was more prominently induced by E2. However, when P was combined to the treatment, the weight of the pituitary gland was increased in both WT and hCGβ females as compared with E2 treated females. An E2 + P combination increased the KI-67 labelling index especially in hCGβ females compared with E2 treated females (E2: 17 ± 3 cells/mm^2 vs. E2+P:37 ± 6 cells/mm^2). More CCND1 positive nuclei were also seen in E2+P treated female pituitaries compared with E2 treated females. Furthermore, the pituitary weights did not present significant differences in E2+P treated hCGβ females compared with sham treated hCGβ females. The pituitary weights in G and G+P treated mice were equal both in WT and hCGβ females, and the weights were smaller in all these groups compared with sham treated WT females, although a significant difference was not observed.

Because the results indicated that P induces PAs in combination with physiological E2 levels, we also analysed the expression pattern of *Ccnd*1, *E2f1* and *Hmga2* genes from mice in the HA study. The expression levels of all these genes were normalized in G treated mice. Also, mifepristone treatment decreased the expression levels of all these genes almost to the WT level. Furthermore, mifepristone treatment decreased the nuclear CCND1 staining as compared with sham treated hCGβ females.

5.5.4. Obesity is associated with the formation of pituitary adenomas

As we have previously shown, hCGβ females are obese (Rulli *et al.*, 2002). In the HA-study, we found a strong correlation between obesity and pituitary weights, and between serum leptin levels and pituitary weights (Figure 11).

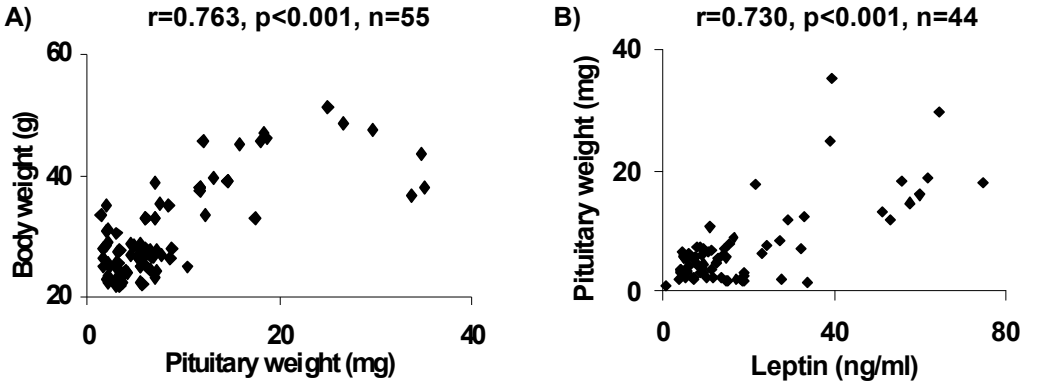


Figure 11. *Correlation analysis of a) body weight and pituitary weight and b) pituitary weight and serum leptin levels of mice in HA study. Correlation coefficient (Pearson product moment), significance, and number of mice are presented above each panel.*

Because obesity is a risk factor for many cancers, and there is also some but not strong clinical evidence, that obesity is associated with PAs, we evaluated the effect of a calorie restricted diet (CRD) in the formation of PAs in hCGβ females. Both WT and hCGβ females underwent short term diet between the ages of 6-7 months (when PAs already exist), and the purpose of the study was to treat PAs (treat = treatment trial = short diet). The long term diet started at 2 months of age (before PAs appear) and continued until the age of 6 months. The purpose of the study was to prevent pituitary tumorigenesis (prev = prevention trial = long diet). The body weights at the beginning and at the end of the study are shown in Table 8. As seen in the table, both short term and long term diet led to clearly decreased body weights in hCGβ females. Pituitary weights are shown in figure 13. In WT mice, both short and long diets decreased pituitary weight.

Table 8. *Body weights in the diet experiment.*

Prevention trial	***Treatment***	***Weight (g) at 2 mo***	***Weight (g) at 6 mo***
	WT sham	$21.3 \pm 1.1^{ns(a)}$	$25.5 \pm 1.3^{b,\ *}$
	TG sham	$25.1 \pm 0.8^{ns(b)}$	$32.0 \pm 1.1^{a,\ *}$
	WT prevent	22.5 ± 1.0^{ns}	$24.2 \pm 1.3^{b,\ ns}$
	TG prevent	24.4 ± 1.0^{ns}	$18.5 \pm 0.7^{c,\ *}$
Treatment trial	***Treatment***	***Weight (g) at 6 mo***	***Weight (g) at 7 mo***
	WT sham	24.6 ± 0.9^{a}	$24.7 \pm 0.4^{b,\ ns}$
	TG sham	42.9 ± 1.3^{b}	$40.4 \pm 1.4^{a,\ *}$
	WT treat	25.3 ± 0.9^{a}	$20.6 \pm 1.3^{c,\ *}$
	TG treat	42.0 ± 2.0^{b}	$26.3 \pm 0.8^{b,\ *}$

Results show mean ± SEM of 6-16 mice per groups. Different letters indicate significant difference in each experiment at a given time point; analyses over the experiments are not shown. Letters in brackets (Prevention trial, Weight at 2 mo) indicate that significant differences were observed between WT and TG group, when two WT and TG groups were pooled (p=0.004). Asterisks denote a significant difference in the change of body weights during the experiment (p<0.05). ns; not significant, g; gram, mo; months.*

Treatmen trial: In hCGβ females, CRD treatment did not affect pituitary weight (Fig. 13). However, the BrdU labelling index was 46% reduced in $hCG\beta_{treat}$ females compared with control hCGβ females (Fig. 12). We did not observe significant differences in the expression of the *Ccnd1*, *E2f1* and *Hmga2* genes between control and $hCG\beta_{treat}$ females (data not shown). However, the nuclear localization of CCND1 and CDK4 was clearly reduced in $hCG\beta_{treat}$ females compared with control hCGβ females (Fig 14). In this study, we also measured gonadal steroid hormones, which have previously been shown to be involved in pituitary tumorigenesis. Serum P levels were equal between control and $hCG\beta_{treat}$ females, but serum Te and E2 were approximately 2-fold increased in $hCG\beta_{treat}$ females as compared with control mice (Fig. 13). As expected, serum Le levels were 8-fold higher in control than in $hCG\beta_{treat}$ females (Fig. 13).

Prevention trial: The long diet clearly prevented pituitary tumorigenesis in hCGβ females. As seen in Figure 13, pituitary weights in $hCG\beta_{prev}$ females were more than 50% reduced as compared with control hCGβ females. Furthermore, BrdU labelling index was 90% reduced in $hCG\beta_{prev}$ females as compared with control mice, and the labelling index of $hCG\beta_{prev}$ females was even smaller than in WT mice (Fig. 12). An approximately 50% decrease was observed in the pituitary expression of *Ccnd1* and *E2f1* genes but not in the Hmga2 gene in $hCG\beta_{prev}$ females as compared with control females (data not shown). We also found clearly reduced nuclear staining of CCND1 and CDK4 in $hCG\beta_{prev}$ females as compared with control females (Fig. 14). Serum Le, P and E2 levels were clearly reduced and serum Te increased in $hCG\beta_{prev}$ females as compared with control females (Fig. 13).

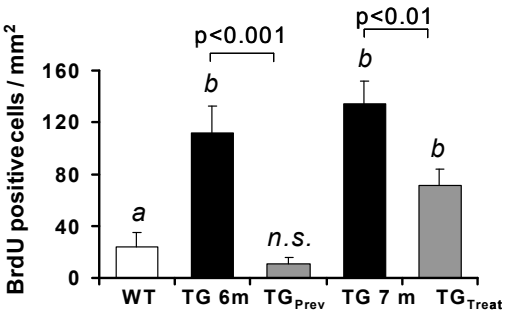


Figure 12. *Pituitary BrdU positive cells per unit area in the pituitary gland. Results show mean ± SEM of 5-6 mice per group. Two sections per mouse were calculated. Significant differences between age-matched TG controls and TG_{Prev} and TG_{Treat} are shown in figures (Student's t-test). Different letters above the bars indicate a significant difference as compared with WT pituitaries (ANOVA, all groups analysed together). Prev; prevention trial, Treat; treatment trial)*

5.5.5. hCG overexpressing females are obese without adrenal hyperfunction

As we have shown previously, hCGβ females are obese, but the aetiology of this obesity has remained unclear (Rulli *et al.*, 2002). In the HA study (see above), hCGβ females presented clearly elevated body weights as compared with any other group, and G, bromocriptine, mifepristone and tamoxifen treatment prevented the weight gain of these mice (data not shown). The body weights between G, tamoxifen, mifepristone and tamoxifen+mifepristone treated WT and hCGβ females were also equal (data not shown) indicating that hCG induced ovarian stimulation is responsible for obesity in

hCGβ females. In the HR study, E2, P or a combination of these hormones were not able to recover weight gain in hCGβ females (data not shown).

Because in another model, LH was able to induce functional LHCGR in the adrenal gland, stimulate corticosterone production and thus cause obesity (Kero *et al.*, 2000), we analyzed the adrenal phenotype of these mice. Briefly, the adrenal glands of hCGβ females were not hyperplastic/hypertrophied, and their adrenal weight was equal to age-matched littermates (WT: 9.7 ± 0.3 mg vs. hCGβ: 10.0 ± 0.3 mg). However, histological evaluation revealed an enlarged innermost zone of the adrenal cortex in all hCGβ females studied. In the HR study, E2 and E2+P were also able to induce the appearance of this zone in WT mice (Fig. 15). G alone was not able to induce this zone in WT mice, although their LH levels were clearly elevated compared with WT females. The LH levels of G+E2 treated WT females were also elevated compared with WT mice, but in E2+P treated females LH levels were clearly lower than in WT females (Fig. 15). Finally, we measured the corticosterone levels of 6-month-old WT and hCGβ females. We paid close attention on the sample collection, and these mice were sacrificed at 7.30-8.00 am. with cervical dislocation without any delay after touching the cage, and only two mice were sacrificed per day. Serum corticosterone levels were 25 ± 4 ng/ml in WT females and 44 ± 10 ng/ml in hCGβ females, and the body weights were 29.6 ± 0.7 g and 39.3 ± 1.9 g, respectively. In another study set-up, 4.5-month-old WT and hCGβ females were sacrificed as described above, and in this study corticosterone levels and body weights were 127 ± 45 ng/ml and 24.8 ± 0.7 g in WT females and 70 ± 13 ng/ml and 34.6 ± 1.8 g in hCGβ females. It seems that morning corticosterone levels are equal in WT and hCGβ females, and obesity seen in hCGβ females is not caused by elevated corticosterone production.

The adrenal phenotype of hCGαβ has not been thoroughly analyzed, but the macroscopical evaluation revealed, that the adrenals of hCGαβ females are smaller compared with WT females.

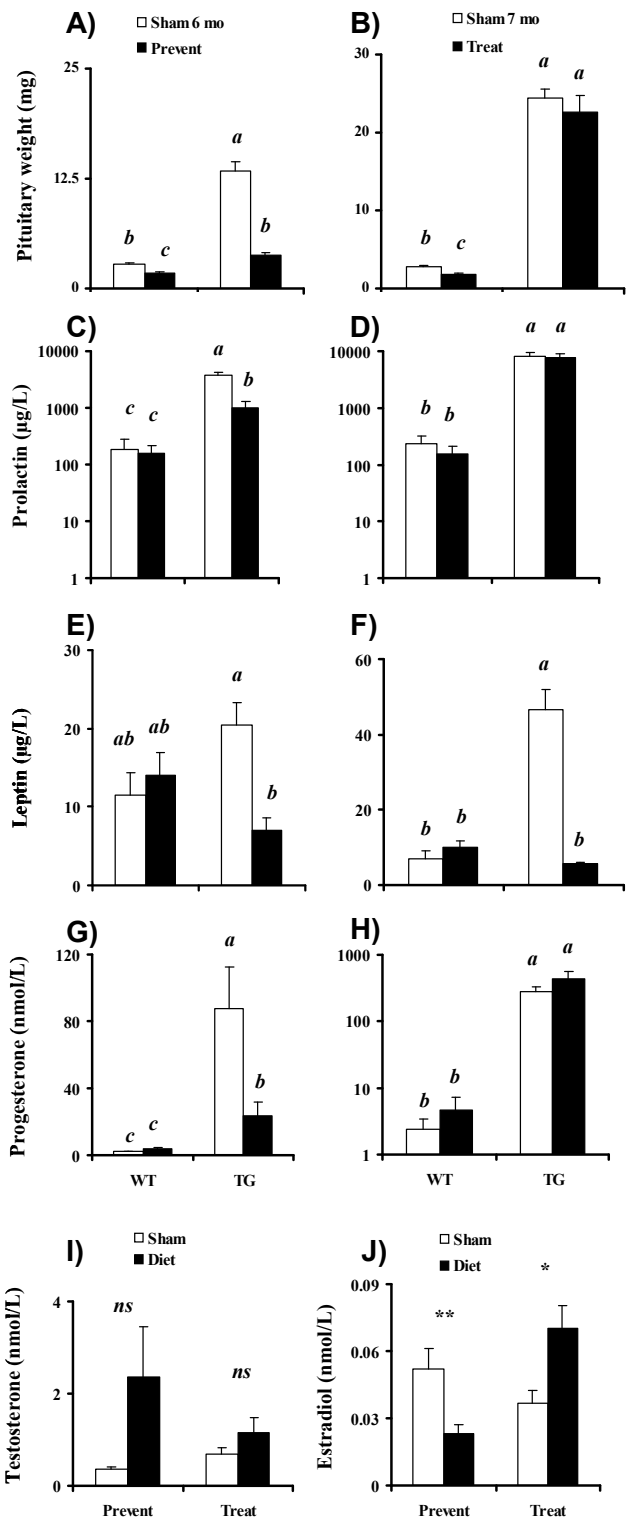


Figure 13. *Pituitary weights and serum hormone levels of mice in the diet experiment. Panels* ***a)***, ***c)***, ***e)***, *and* ***g)*** *show the results of the prevention trial and* ***b)***, ***d)***, ***f)***, *and* ***h)*** *show the results from the treatment trial, and white colums represent sham treated mice (ad libitum fed) and black colums present the mice on CRD. The parameters measured are seen in the left panels. Both WT and TG mice are included in the panels* ***a-h)***, *and genotypes are presented below the panels* ***g)*** *and* ***h)***. *Panels* ***i)*** *and* ***j)*** *present the serum testosterone and estradiol levels as indicated in each panel. Serums of TG mice in prevention and treatment trials were measured, and the trials are given below panels* ***i-j)***. *In the case of E2, equal amounts of serum from two mice were pooled, if needed. Each value was used only once. Each of the figures* ***a-j)*** *shows mean ± SEM of 5-16 mice per group. Statistical analysis was performed by using ANOVA with the Holm-Sidak post-test in figures* ***a-h)***. *In figures* ***i)*** *and* ***j)***, *the t-test was used to evaluate the statistical difference between sham and diet treated mice in each trial. Different letters in each figure* ***a-h)*** *denote significant difference between treatment groups, and statistical significance was set at level* $p < 0.05$. *Asterisks show a significant difference between groups, and ns denotes no significant difference, when the T-test was used (* $p<0.05$, ** $p<0.01$).*

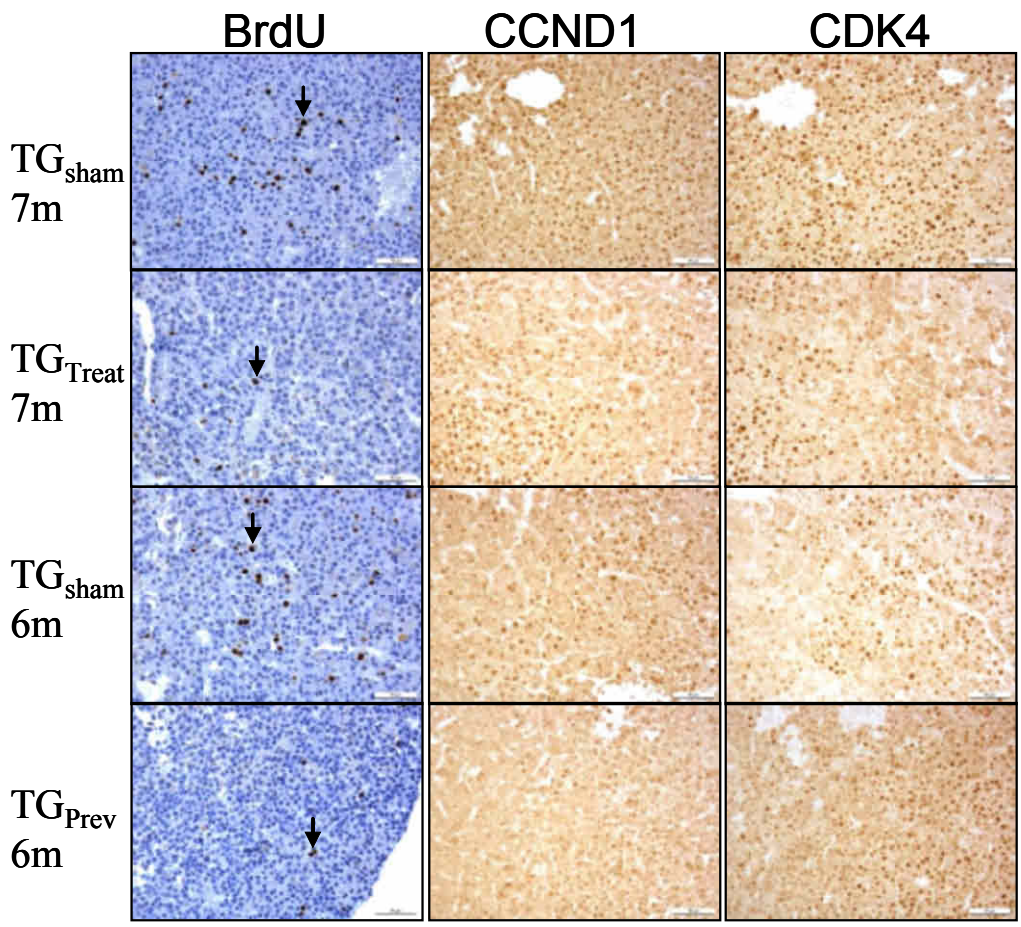


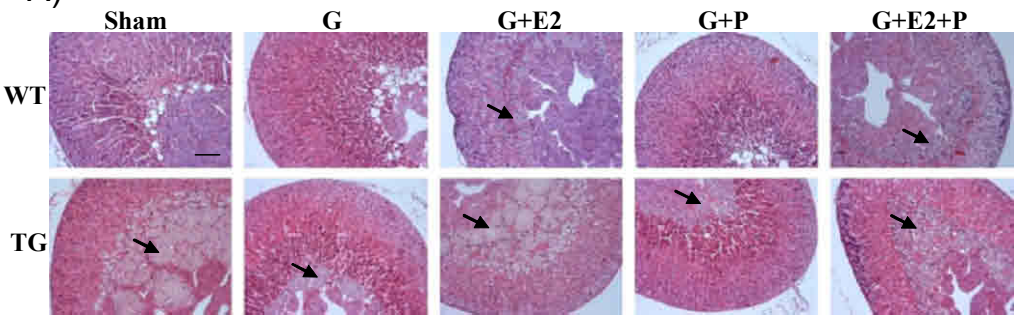
Figure 14. *Immunohistochemical evaluation of pituitary BrdU, CCND1, and CDK4 of TG mice in the diet experiment. The left panels show BrdU, the middle panels present CCND1, and the right panels show CDK4 immunohistochemistry. Different treatment groups are annotated on the left. Representative micrographs taken from the same specimen and the same area are shown. Arrows indicate positive nuclear staining of BrdU. Dark, intense nuclei are positive for CCND1 and CDK4. It was evident, that the pituitary glands of TG mice showed the highest density of BrdU positive nucleus. Positive nuclei were scattered throughout the whole anterior pituitary gland in TG_{sham} females. All TG_{sham} mice showed also histologically evident nodules, in which the density of BrdU positive cells was clearly higher as compared with surrounding parenchyma. In contrast, TG_{treat} females presented decreased density of BrdU-positive nucleus as compared with TG_{sham} females, and the effect of the CR diet was even more pronounced in the TG_{prev} females, in which only few BrdU positive cells were seen. In line with BrdU staining, both CCND1 and CDK4 showed strong nuclear staining throughout the anterior pituitary gland of TG_{sham} females, and the staining was also enhanced in those nodules presenting increased number of BrdU positive cells. In TG_{treat} females, large areas of faint nuclear staining/no nuclear staining of CCND1 and CDK4 were seen. Nuclear staining of CCND1 and CDK4 co-localized to the same areas as the BrdU-positive cells in TG_{treat} females. Similar, but even more decreased staining patterns of CCND1 and CDK4 were seen in TG_{prev} females. BrdU-positive nuclei were found in one or two small clusters in TG_{prev} females, and BrdU staining co-localized with the same areas as the nuclear staining of CCND1 and CDK4, whereas the parenchyma outside these clusters presented with only faint/no nuclear staining.*

5.6. Nongonadal phenotype of hCG overexpressing females

5.6.1. Gonadal dependency of nongonadal tumorigenesis in hCG overexpressing females

Thus far, the only nongonadal tumors which have been shown to appear in hCG overexpressing females are pituitary and mammary gland tumors. Mammary gland tumors have been shown to be dependent on gonadal function (Rulli *et al.*, 2002; Rulli and Huhtaniemi, 2005). In this study, the only mammary gland tumors were observed in old hCGβ females. The pituitary adenomas were strictly dependent on gonadal function and their hormonal secretion in hCGβ females. We also carefully analyzed many other tissues including brain, adrenals and uterus in gonadectomized hCGβ females, but no signs of tumorigenesis were observed.

A)



B)

Treatment	*Sham*	*G*	*G+E2*	*G+P*	*G+E2+P*
LH (ng/ml)	0.47 ± 0.13^{d}	3.19 ± 0.55^{b}	2.10 ± 0.23^{bc}	3.71 ± 0.41^{a}	0.12 ± 0.07^{e}

Figure 15. *Histological analysis of the adrenal glands and serum LH levels of mice in the HR-study. In panel A), representative adrenal micrographs of given genotypes and treatments are seen. Arrows indicate lipid droplet in the innermost zone of the adrenal cortex. Notice that these droplets are found in all TG females, and in WT mice treated with G+E2 and G+E2+P. In panel B), LH levels (ng/ml) of WT mice are shown. Results are mean ± SEM of 6-12 mice per group. Different letters indicate significant difference ($p < 0.05$) between groups. Bar = 100 µm. TG; hCGβ females, G; gonadectomy, E2; estradiol, P; progesterone.*

6. DISCUSSION

6.1. hCG is a weak promoter of tumorigenesis in male mice

The hCG overexpressing mice developed in this study and in another study (Matzuk *et al.*, 2003) are the first male models for highly elevated LH-action, because the bLH-CTP males did not show elevated LH levels (Risma *et al.*, 1995). The adult males in both hCG overexpressing models showed LC hyperplasia, increased Te production, enlarged accessory sex organs, and aggressive behaviour, but no tumorigenesis of any tissue was seen in adult mice (Matzuk *et al.*, 2003).

The most notable feature in our model was the development of hormonally active FLC adenomas in young males, which disappeared later in life. The normal expression of a FLC marker and very low expression of ALC markers in 10-day-old hCGαβ indicated that adenomas are derived from FLCs. We also traced the fate of LCs in mice treated with BrdU at 3-5 days of age. It was convincingly shown that LC adenomas had many BrdU positive cells at 10 days of age, but the number of positive LCs was low in adult hCGαβ males, and did not differ from adult WT males, indicating that adenomas originate from FLCs. The current study also showed that the regression of FLCs occur normally despite the high hCG levels suggesting that low LH level during infancy and childhood is not the factor causing the regression of FLCs.

The formation of FLC adenomas in response to high hCG levels is in line with previous studies showing that enhanced LH-action due to chronic administration of hCG, hCG producing neoplasms, flutamide (or other compounds decreasing the bioactivity of Te and increasing LH levels), or estrogens (increasing the number of LHCGR) cause the proliferation of LCs or formation of LCTs suggesting that LH/hCG is able to drive cell cycle progression in LCs (Christensen and Peacock, 1980; Huseby, 1980; Navickis *et al.*, 1981; Asa *et al.*, 1984; Prahalada *et al.*, 1994; Clegg *et al.*, 1997; Cook *et al.*, 1999). Several activating mutations in human LHCGR have been found, and the boys having these mutations develop precocious puberty and testotoxicosis (Themmen and Huhtaniemi, 2000). One particular mutation of LHCGR (D578H) has been shown to induce LC adenomas in boys (Liu *et al.*, 1999; Richter-Unruh *et al.*, 2002), but whether these adenomas originate from FLCs or ALCs is not known. Some FLCs exist in humans after the birth suggesting that the LC adenomas might originate from FLCs in the boys having this mutated receptor (Habert *et al.*, 2001). The mouse model described in this study is a close phenocopy of boys having activating mutation of LHCGR (D578H) (Liu *et al.*, 1999; Richter-Unruh *et al.*, 2002), although in our model tumorigenesis is driven by ligand induced activation of LHCGR signalling and in human model ligand-independent, constitutive LHCGR signalling. In both cases, enhanced LHCGR signalling induces Leydig cell adenomas and high testosterone production. It is possible, that one reason for the tumorigenic property of the mutated receptor is impaired desensitization. It has been shown that the ligand induced internalization of this mutated receptor is faster than that of the WT receptor

suggesting that enhanced signalling of mutated receptor is not due to the impaired desensitization/internalization (Min and Ascoli, 2000).

The current study raised the question of why the high hCG level is not able to induce LCTs in adult males or even hyperplasia of ALCs. Some old studies have shown that chronically elevated hCG level is able to induce LC hyperplasia in adult males (Christensen and Peacock, 1980; Asa *et al.*, 1984), and elevated LH levels in combination with androgen action blockers are able to induce LCTs (Prahalada *et al.*, 1994; Clegg *et al.*, 1997; Cook *et al.*, 1999). The formation of LCTs is also induced by oncogene/lack of tumor suppressor gene, and could be inhibited pharmacologically by decreasing LH levels or promoted by concomitant expression of elevated LH (Kumar *et al.*, 1996; Kananen *et al.*, 1997; Mikola *et al.*, 2003). Results suggest that elevated LH/hCG action is only weakly if at all oncogenic in adult males even in gonads, and that LH/hCG requires other oncogenic hits or at least blockade of androgen action to induce LCTs in adult males.

Why then are the high hCG levels able to induce FLC tumors? We have paid close attention to the effects of LH/hCG in FLCs or ALCs. As we have previously shown, there exist many important functional differences between fetal and adult LCs. In contrast to ALCs, FLCs lack the LHCGR donwregulation and the steroidogenic desensitization in response to high LH/hCG levels (Huhtaniemi *et al.*, 1981; Huhtaniemi *et al.*, 1982b; Huhtaniemi *et al.*, 1985b; Pakarinen *et al.*, 1990). The pysiological importance of this is not known, but the impaired desensitization might have protective functions for Te synthesis and masculinization of male fetuses.

At the moment, there are several factors known to be involved in LHCGR desensitization and donwregulation including arrestins, dynamins and G-protein receptor coupled kinases (Ascoli *et al.*, 2002). We currently extended our previous studies and identified the decreased expression of two important G-protein receptor coupled kinases (*Grk4, Adrbk2*) in young males compared with adult males. Because the testicular and serum Te levels were similar in adult and young hCGαβ males despite decreased number of LHCGRs in adult males, we suggest that cAMP signalling is not impaired in adult hCGαβ males. It has been shown that the maximal steroidogenic response in LCs occurs, when only 0.8 % of LHCGR receptors are occupied (Huhtaniemi *et al.*, 1982a). The high hCG levels in TG males corresponds more than 1000-fold higher LH-bioactivity as compared with WT males, and levels far exceed the normal physiological levels seen in LH pulses of WT males. The high hCG levels could thus still occupy and activate sufficient amount of receptors for inducing steroidogenesis despite of the desensitization and downregulation of LHCGR seen in TG males. However, the increased desensitization of LHCGR in hCGαβ males could more prominently decrease the other LHCGR signalling pathways including MAPK and PLC pathways (Ascoli *et al.*, 2002). Because primary LC proliferation after LHCGR activation is mediated by transactivation of EGFR and ERK1/2 pathway (Shiraishi and Ascoli, 2007, 2008), it is possible that desensitization impairs especially the transactivation. The PLC pathway is also interesting because in the case of activating LHCGR mutation causing LC adenoma, basal PLC pathway activity was increased (Liu *et al.*, 1999).

Based on our results, we suggest that the difference of hCG induced testicular tumorigenesis between FLCs and ALCs is due to the lack of desensitization in FLCs. Because the LHCGR gene and the promoter is the same in FLCs and ALCs, we suggest that the lack of desensitization is the property of FLCs itself, and it might

involve also other GPCRs than LHCGR as well. The impaired desensitization of LHCGR in FLCs might be caused by the low or absent expression of *Grk4* and *Adrbk2* leading to enhanced signalling possibly through the PLC or ERK pathways (Figure 16). The factors inducing GRKs in ALCs are not known, but it is tempting to suggest that Te itself might control the expression of GRKs being an autoregulatory mechanism for controlling androgen production and protecting the males and testosterone synthesis for high and low LH levels. If androgens regulate GRKs, the formation of LCTs in rodents treated by flutamide or other agents decreasing Te bioactivity (Clegg *et al.*, 1997) could be caused by simultaneous upregulation of LH and decreased desentization due to inhibited expression of GRKs. Furthermore, the high Te production in our model could then protect LCs for transformation, and thus prevent the LC tumorigenesis. Administration of androgen receptor blockers or combining hCG transgenic mice with the LC specific *AR* knockout model could elucidate the importance of Te autoregulation on LCs. Further studies are also pending to clarify the mechanism of impaired desensitization in FLCs.

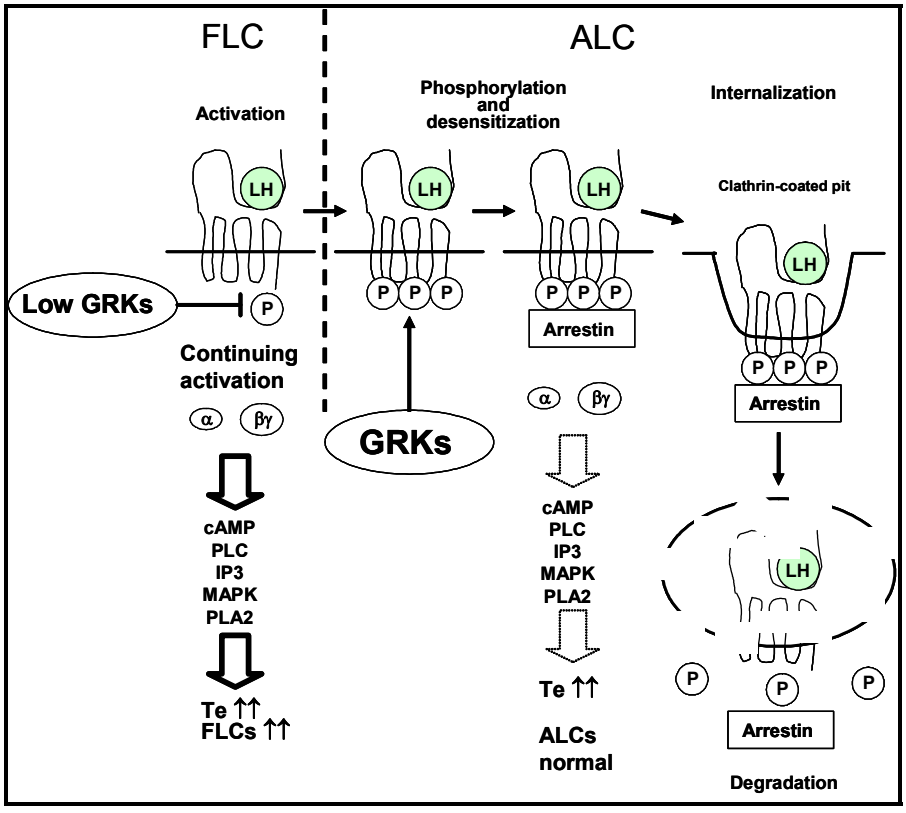


Figure 16. *Sedcism for the lack of LHCGR desensitization and formation of FLC adenomas in hCGαβ males. The amount of GRKs is low in FLCs leading to impaired LHCGR desensitization and continuous signalling. In ALC, LHCGR is extensively phosphorylated by GRKs, signalling is terminated and LHCGR is degraded. However, the cAMP pathway is at least slightly intact, since Te levels in adult hCGαβ males are elevated. The intracellular expression pattern of different GRKs might have a role in signal divergence leading to more pronounced desensitization of certain signalling pathways.*

One interesting finding in this study was that the timing of the puberty in hCG overexpressing males was equal as compared with WT mice suggesting that the pace of the puberty in male mice is already maximal, and could not be enhanced by androgen excess. Thus, it seems that mouse is not good model for studying male precocious puberty.

6.2. Nongonadal hCG action in males – does it exist?

Currently, the expression of *LHCGR* has been found in many tissues in male rodents and humans. These tissues include the prostate, seminal vesicles, sperm and kidneys (Reiter *et al.*, 1995; Tao *et al.*, 1995; Tao *et al.*, 1998b; Eblen *et al.*, 2001; Apaja *et al.*, 2005). In hCGαβ males, the phenotype of these tissues was also affected. We found hyperplastic lesions in the prostate, and the sizes of the seminal vesicles were enlarged. Both of these tissues have been shown to be androgen dependent, since in Lhcgr KO males these accessory sex organs were rudimentary, but recovered after Te replacement (Pakarainen *et al.*, 2005a). We also treated the hCGαβ males with flutamide, which led to decrease in prostate and seminal vesicle weights indicating the androgen dependency of these tissues. However, more thorough analysis of these tissues is still needed.

The changes observed in the urinary tract including a distended urinary vesicle and enlarged kidneys seemed to be secondary changes due to urethral wall thickening and obstruction of unknown origin. Distension of distal parts of *vasa deferentia* was also clear. Furthermore, in old males inflammatory reactions of *epididymides* were seen, and together with these changes, bending of the sperm tails was observed. However, in young hCGαβ males without clear signs of infravesical obstruction, sperm was morphologically normal. The degenerative lesions in old hCGαβ male testis associated with the abnormal epididymides, and clear signs of infavesical obstruction. In *Lhcgr* KO males treated with Te, but not with dihydrotestosterone (DHT), inflammatory reaction was seen in the prostate and vas deferens (Pakarainen *et al.*, 2005a; Savolainen *et al.*, 2007). The mechanism for Te induced inflammation and hyperplasia was the aromatization of Te to E2 and the activation of ESR1 by E2 (Savolainen *et al.*, 2007). The protective mechanism of DHT seemed to be the activation of ESR2 by DHT metabolite 5α-androstane-3β,17βdiol (3βAdiol) (Savolainen *et al.*, 2007). It is possible that increased Te production in hCGαβ males leads to increased aromatization of Te and thus increased activation of ESR1. We also tried to detect the expression of *Lhcgr* from kidneys by RT-PCR with no success, indicating that hCG does not directly mediate the effects in the kidneys.

Based on the results we obtained, it is highly unlikely that increased LH action has any direct, nongonadal physiological or pathophysiological effects on male mice. However, it is possible that in the presence of additional factors, increased LH action can modulate the phenotype.

6.3. Nongonadal hCG action in females – does it exist?

In this study, we showed that hCG induces ovarian tumors. These results are in line with previous LH results (Risma *et al.*, 1995; Wise *et al.*, 1996). Contrary to these

results, the pituitary tumors showed gonadal dependency and lack of expression of *Lhcgr*. The mammary gland tumors in hCGβ analyzed previously were also ovarian dependent (Rulli *et al.*, 2002), although low *Lhcgr* expression was seen in the mammary glands, and some genes analysed showed response to hCG injections (Kuorelahti *et al.*, 2007). Because the gonadectomized mice did not develop mammary gland tumors in this model, it is clear that hCG is not responsible for mammary gland tumors. hCG rather modulates the effects of other factors inducing the tumors.

In this study, we also showed that obesity in hCGβ mice is ovarian dependent, because G treated hCGβ females were as lean as WT females with similar treatment. One possible mechanism for obesity is the high PRL levels, since the PRLR KO females showed reduced body weight as compared with WT females (Freemark *et al.*, 2001). Because PRL is a lactational hormone, it is understandable that likewise during pregnancy, elevated PRL levels in hCGβ females prepare them for increased calorie consumption normally needed for lactation.

We also analyzed the adrenal phenotype of hCGβ females. In contrast to previous studies with quite similar transgenic mice showing adrenal hyperfunction and tumorigenesis (Kero *et al.*, 2000; Mikola *et al.*, 2003), we were not able to detect any signs of increased adrenal steroidogenesis or tumorigenesis in hCGβ females. The only phenotypic difference in hCGβ females as compared with WT females was the appearance of slight hyperplasia of the innermost zone of the adrenal cortex as also found in other models, but in contrast to these models, the other cortical zones were not affected in hCGβ females (Kero *et al.*, 2000; Li *et al.*, 2001). We also showed that this hyperplasia is not dependent of LH/hCG, since WT mice treated with E2 and E2+P had the hyperplasia, and the LH levels in the latter group was far below the WT levels. Moreover, gonadectomy of WT females did not induce hyperplasia of this innermost zone. The previous results and current results convincingly indicate that the appearance of hyperplasia of the innermost zone of adrenal cortex is driven by increased E2 or PRL. However, the post-gonadectomy response in the adrenal glands has been shown to be strictly dependent of mouse-strain used, and the FVB/N strain currently used was one of the low-response strains (Bielinska *et al.*, 2003). Several models have also shown that high LH induce *Lhcgr* expression in adrenal gland, and in some strains this induced Lhcgr expression could promote adrenal tumorigenesis (Bernichtein *et al.*, 2008a). Several studies have shown that human adrenal gland tumors also express *LHCGR* (Lacroix *et al.*, 2001; Saner-Amigh *et al.*, 2006), and several clinical reports of LH-dependent hypercortisolism (Cushing's syndrome) exist (Lacroix *et al.*, 1999; Feelders *et al.*, 2003). At the moment, it seems that the adrenal gland expresses LHCGR, and in certain conditions it could promote adrenal tumorigenesis and hyperfunction. However, the clinical impact of elevated LH levels in normal adrenal physiology is not known, and to show the effect of elevated LH in adrenal function of postmenopausal women needs more properly controlled studies.

Briefly, in the current study increased hCG levels were able to directly induce gonadal tumors, but all the other phenotypes observed in hCG overexpressing females can be explained by ovarian hyperstimulation or elevated PRL levels. Thus, the direct nongonadal physiological and pathophysiological effect of elevated LH/hCG action seems unlikely in female mice. However, it is possible that elevated LH/hCG synergizes with other factors to induce non-gonadal tumorigenesis. The role of

elevated LH/hCG action in the adrenal gland remained unclear, because the mouse-strain we used was shown to be low-response mouse-strain in the respect of adrenal LHCGR, and further studies using different mouse-strain(s) are needed to confirm the adrenal effects of hCG.

6.4. Progesterone and obesity – the new candidates for pituitary tumor inducers

In the current study, we showed that both P and obesity are risk factors for formation of PAs. The role of P in the development of pituitary adenomas have been shown in some older studies, but the effects have mainly been inhibitory (Poel, 1966; Haug and Gautvik, 1976; Winneker and Parsons, 1981; Lamberts *et al.*, 1985; Lamberts *et al.*, 1987; Caronti *et al.*, 1993; Piroli *et al.*, 1998; Piroli *et al.*, 2001). However, mifepristone treatment has been shown to inhibit the growth of transplantable pituitary tumors (Lamberts *et al.*, 1985; Sakamoto *et al.*, 1987). One rather interesting detail in these studies is the fact that the stimulatory role of P and the inhibitory role of mifepristone in pituitary tumorigenesis have been shown in non-gonadectomized models and the inhibitory role of progestins mainly in gonadectomized models. In these experiments, serum E2 and P have not been measured, but it can be speculated that the nongonadectomized progestin treated mice should have normal/low normal E2 levels. The P levels in gonadectomized and nongonadectomized mifepristone treated mice should also be low and normal, respectively. Briefly, it seems that adequate E2 in nongonadectomized P treated mice is required for stimulatory action of P, and mifepristone shows inhibitory effects, when an inhibitable amount of P is present. These older results support our results showing that the stimulatory action of P requires at least a physiological E2 concentration. Mifepristone treatment also significantly reduces the size of PA in hCGβ females, further suggesting the importance of P in pituitary tumorigenesis. The results are in line with a recent large scale clinical study showing the increased risk of pituitary and central nervous system cancers in women using or having used oral contraception (OC), although we need to keep in mind that pituitary tumors and central nervous system tumors were analysed as combined group, not separately (Hannaford *et al.*, 2007).

In this study, we also showed that obesity participates in the formation of PAs. Although a short term diet was not able to decrease pituitary weight, the number of proliferative cells was almost 50% reduced compared to mice on a normal diet. The results obtained from a long diet were even more convincing; pituitary weight was clearly reduced with only a marginal number of proliferating cells compared with control hCGβ females. These facts suggest that obesity is promoting pituitary tumorigenesis. Obesity is a risk factor for many cancers, and its role has been highlighted especially in breast cancer (Berrigan *et al.*, 2002; Hu *et al.*, 2002; Gonzalez *et al.*, 2006; Renehan *et al.*, 2008). Obesity is also associated with pituitary adenomas, and leptin receptors are expressed in pituitary adenomas (Greenman *et al.*, 1998; Shimon *et al.*, 1998) in line with our results. We showed the correlation of body weight and serum Le levels with pituitary weights in the HA-study, but the characterization of the mechanism is currently pending. Le signals through the JAK2 and ERK1/2

pathway, the main regulator of *Ccnd1* expression (Bjorbaek *et al.*, 1997; Fruhbeck, 2006). Thus, it is possible that Le sustains ERK1/2 with E2, P and lack of DA, and this sustained ERK1/2 is able to stabilize MYC, leading to upregulation of *Ccnd1* (Lavoie *et al.*, 1996; Weber *et al.*, 1997; Roovers *et al.*, 1999). Results from breast cancer cells support this theory (Gonzalez *et al.*, 2006).

In this study we showed that both obesity and P are able to induce PAs in mice. Considering the importance of P in all physiological functions of the female, as well as one component of numerous medications (including contraceptive and HRT regimens), it is very important that its potential role in the formation of pituitary tumours becomes recognized. Appropriately controlled clinical trial is needed to clarify the role of obesity and P in pituitary tumorigenesis, because antiprogestins could be used as a potential therapeutic agent for DA agonist resistant prolactinomas.

6.5. The hormonally induced CCND1/CDK4/RB/E2F1 pathway in pituitary adenomas and its clinical relevance

In this study, we showed that PAs in hCGβ females had increased expression and nuclear localization of CCND1/CDK4 leading to impaired nuclear localization of RB1. This same mechanism is the major regulator of the cell-cycle at the G1-S phase transition (Massague, 2004). The induction of the CCND1/CDK4/RB pathway has been shown to cause many cancers including breast cancers, and its overexpression has been found in the majority of human breast cancers (Butt *et al.*, 2005). We also described in the current study, that P is the actual ovarian derived hormone upregulating the *Ccnd1* expression in primary pituitary cells. However, the effects mediated by P seemed to require at least normal physiological E2 levels, because P alone even *in vivo* or *in vitro* was not able to upregulate *Ccnd1*. Primary pituitary cells were harvested from 4.5-month-old hCGβ females. We selected the mice of this age due to *in vivo* experiment showing that in 4.5-month-old hCGβ females clear adenomatous foci are not usually present in pituitary glands and that the *Ccnd1* expression is only slightly increased. It is however possible, that the transformation of pituitary cells has already occurred at this age, and thus the upregulation of *Ccnd1* by P is not necessary a primary event leading to pituitary adenomas. Due to technical difficulties, we have not stimulated WT pituitary cells with E2 and P. This experiment might reveal, whether the *Ccnd1* is induced by E2 and P in non-tumorous pituitary cells leading to cell growth and transfromation. Our *in vivo* experiment with gonadectomized and E2 and P replaced mice suggests, that the effect of E2 and P require prolonged administration and thus the primary cell stimulations might not reveal *Ccnd1* upregulation.

The possible mechanism of *Ccnd1* upregulation in our model might be the activation of the ERK1/2 pathway, since activated ERK1/2 has been shown to be required for sustained *Ccnd1* expression (Lavoie *et al.*, 1996; Weber *et al.*, 1997; Roovers and Assoian, 2000). It is also important to notice that both E2 and P have been shown to activate ERK1/2 in breast cancer models, and the crosstalk of these two steroids occurs through the same pathway (Migliaccio *et al.*, 1998; Ballare *et al.*, 2003; Butt *et al.*, 2005; Faivre *et al.*, 2005; Skildum *et al.*, 2005). The fact that bromocriptine

inhibited the *Ccnd1* expression *in vivo* supports the role of ERK1/2 in *Ccnd1* induction, since DRD2 activation has been shown to inhibit ERK1/2 (Liu *et al.*, 2002). However, not only pituitary *Ccnd1* expression but also body weight was much lower in E2+P treated than in sham treated hCGβ females. Because the obese hCGβ females presented elevated Le levels, and Le has also been shown to activate the ERK1/2 pathway and stimulate PRL synthesis in many species including rats and fish (tilapia) (Gonzalez *et al.*, 1999; Fruhbeck, 2006; Tipsmark *et al.*, 2008), one possible explanation for *Ccnd1* induction in hCGβ females is the combined effect of E2, P, low dopaminergic tone and obesity through ERK1/2. The proposed mechanism of pituitary tumorigenesis in hCGβ females is presented in Figure 17.

As already mentioned in section *2.3.3.5*, pituitary adenomas appear in mice and men in association with impaired G_1-S phase cell-cycle regulation. However in mice, many of these pituitary tumors tend to arise from the intermediate lobe (Jacks *et al.*, 1992; Fero *et al.*, 1996; Nakayama *et al.*, 1996; Franklin *et al.*, 1998), but in man they arise more often from the anterior lobe (Woloschak *et al.*, 1996; Jordan *et al.*, 2000; Simpson *et al.*, 2000; Turner *et al.*, 2000; Korbonits *et al.*, 2002; Simpson *et al.*, 2004; Morris *et al.*, 2005; Scheithauer *et al.*, 2006; Boikos and Stratakis, 2007b). The cells in the intermediate lobe of pituitary glands could have higher basal activity of cell-cycle inducers as compared with cells in the anterior lobe, and thus the lack of cell-cycle inhibitors induces tumors, especially in the intermediate lobe. If the cell-cycle is induced through hormonal regulation as in our model, tumorigenesis is limited in those cells expressing the respective receptors. Also, the presence of PRL-producing pituitary carcinomas in mice expressing the cyclin-dependent kinase resistant form of CDK4 also suggests that the tumorigenic effects of cell-cycle iducers and the lack of cell-cycle inhibitors are not the same.

There are no reports on the hormonal status of those patients having pituitary adenomas with abnormal CCND1 expression, but it is possible that the hormones presented in this study might be involved in the regulation of CCND1, also in human PAs. The higher prevalence of PAs and the higher levels of E2 and P in women as compared with men suggest the combined effect of these two hormones as inducers of the PAs possibly through CCND1 induction. We preliminarily analysed the CCND1 expression by IHC from 6 human prolactinomas (all women), and found that 2/6 showed nuclear CCND1 staining. Although it is too early to say that 1/3 of prolactinomas could be explained by CCND1 overexpression, the magnitude of 30% is far above the magnitudes of currently described mechanism underlying PAs. This demands further and thorough evaluation of the role of CCND1 in PAs.

6.6. Therapeutic approaches to prevent and to treat nongonadal cancers through LH/hCG/LHCGR

The current study and the previous studies from our laboratory suggest that direct LH/hCG/LHCGR mediated events do not play a critical role in normal physiological and pathophysiological functions of nongonadal tissues (Pakarainen *et al.*, 2005a; Pakarainen *et al.*, 2005b). However, it is obvious that some nongonadal cancers express LHCGR, although the LHCGR expression in not the molecular event

underlying the formation of these cancers. The expression of LHCGRs in cancers could be used as a target to direct pharmacological intervention into the cancer tissue as has been recently shown and also reviewed (Ziecik *et al.*, 2007; Vuorenoja *et al.*, 2008). If found to be safe, efficient and cost effective, this kind of cancer therapy could lead to better survival rates when used together with conventional therapies, when the cancers in the patients selected for treatment appropriately express the LHCGR receptors.

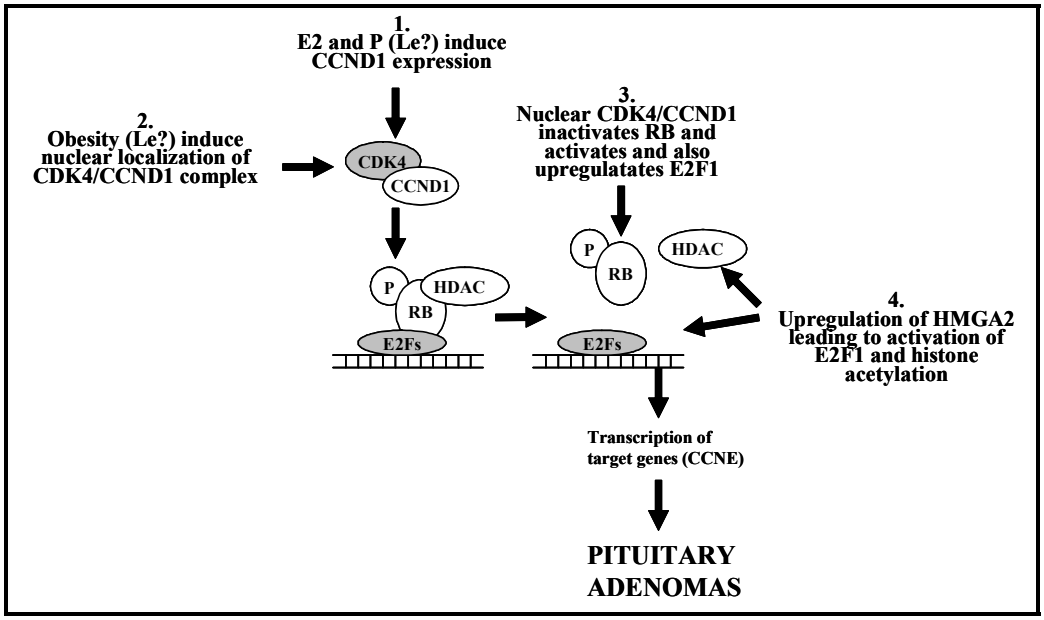


Figure 17. *Suggested mechanisms for pituitary tumorigenesis in hCGβ females. 1) Moderately elevated hCG levels stimulate ovarian steroidogenesis and mainly the production of P. However, at least physiological E2 levels are needed to induce pituitary Ccnd1. Leptin might also be involved in the process, since pituitary Ccnd1 expression was not increased in E2+P treated females having a lean phenotype compared to hCGβ females with similar hormone levels but increased obesity. 2) Obesity, possibly through elevated leptin levels, increases nuclear localization of the CCND1/CDK4 complex. Increased nuclear localization of CCND1 is thought to occur by decreasing its nuclear export, but whether it is the case in hCGβ females, is not known. 3) Increased expression and enhanced nuclear localization of CCND1 leads to phsphorylation and thus inactivation of known tumor supressor RB. Phosphorylated RB dissociates from E2F1 causing its activation and transcription of target genes. 4) Activity of E2F1 is further enhanced by upregulation of the Hmga2 gene. HMGA2 is known to replace HDAC1 from RB. This leads to acetylation of histones, chromatin opening, and revealing the sites for active transcription. HDAC1 acetylates E2F1 enhancing its stability and activity leading to highly increased transcription of target genes and finally rapidly growing pituitary adenomas.*

7. SUMMARY AND CONCLUSIONS

The importance of LH and LHCGR in the regulation of gonadal development and function is well characterized. The similarities between *LHB* and *LHCGR* deficient men and mice have given new insights into the molecular mechanisms of LH/LHCGR in reproductive function. Although these models have convincingly shown that the gonads are the only physiologically relevant site for the action of LH/LHCGR, they have failed to clarify the possible pathophysiological role of increased LH/hCG action. Therefore, in the current study we used two different mouse models presenting moderate and extremely high LH-action through expression of potent LH analogue hCG to clarify the pathophysiological role of elevated LH action. The main conclusions of the current study are the following:

1. Highly elevated LH action is able to induce functional fetal Leydig adenomas in young males, but is not able to impair the regression of FLC, because the adenomas disappeared between 3-4 weeks of age. The model described in this study is a phenocopy of human activating LHCGR mutation, and facilitates the evaluation of the molecular mechanism underlying Leydig cell tumorigenesis. Our model is a good tool to develop pharmacological compounds for treating LC adenomas and replace the currently used therapy, orchiectomy.

2. Adult males are protected against elevated LH action due to enhanced desensitization and downregulation of LHCGR. Even highly elevated LH action was not able to induce tumorigenesis in male mice. The differences in the expression pattern of G protein-coupled receptor kinases explain the difference of the LH action between FLC and ALC.

3. The nongonadal effects of highly elevated LH/hCG levels were not detected in hCG overexpressing males. The phenotype of the prostate and seminal vesicles were Te dependent, since flutamide treatment decreased the size of both of the tissues. It seems unlikely that even pharmacological hCG levels could induce the nongonadal phenotype, if androgen action is blocked. Thus, nongonadal pathophysiological actions of LH seem highly unlikely in males.

4. Direct LH action induces gonadal but not nongonadal tumors in female mice. The luteoomas and teratomas were seen in response to moderate and highly elevated LH action, respectively. Gonadectomy abolished the physiologically significant nongonadal phenotype in hCG overexpressing females. A slight adrenal phenotype remained after gonadectomy, but a similar adrenal phenotype was seen in E2 treated WT females indicating that hCG stimulated gonadal function before gonadectomy was responsible for the adrenal phenotype seen in hCG overexpressing females. Thus, elevated LH action does not directly induce a nongonadal phenotype in female mice.

5. P and obesity promote the formation of pituitary adenomas in female mice. Elevated P levels together with physiological E2 levels, upregulated pituitary *Ccnd1* expression and led to development of PAs. Caloric restricted diet was shown to decrease pituitary cell proliferation and prevent the formation of PAs by decreasing pituitary *Ccnd1* expression possibly through decreased Le levels. Thus, we found a potential mechanism and two independent factors inducing pituitary adenomas, and the same mechanism and factors are also associated with human PAs, highlighting the importance of our finding.

Thus we have shown that the transgenic expression of even one hormone could affect the function of several endocrine systems. It has been said that this kind of transgenic approach leading to pharmacological hormone concentrations is not the best possible model for analysing human diseases, in which hormonal changes are usually modest. However, small changes in humans could affect tens of years before the disease manifests itself. To get equal "hormonal energy" in mice, whose life-span is maximally 2 % of that in humans, we need to apply high hormone concentrations. Thus, the pharmacological hormone concentration in our model corresponded to the human model with lower but longer hormone exposure. As also shown in the current study, the genetically modified mouse models, if used and analysed carefully, are good tools to find new mechanisms and therapeutic approaches for human diseases.

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