TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 858

MEDICA - ODONTOLOGICA

TARGETED ABLATION OF GONADOTROPIN DEPENDENT ENDOCRINE TUMORS IN TRANSGENIC MICE THROUGH THEIR LUTEINIZING HORMONE RECEPTOR (LHR)

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TURUN YLIOPISTO
Turku 2009

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ISBN 978-951-29-3961-9 (PRINT)
ISBN 978-951-29-3962-6 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2009

To Ari-Matti

ABSTRACT

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Targeted ablation of gonadotropin dependent endocrine tumors in transgenic mice through their luteinizing hormone receptor (LHR)
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Gonadal somatic cell and adrenocortical endocrine tumors are rare. The incidence of adrenocortical carcinomas is only 1-2/1000000 a year. However, they are aggressive, especially in adulthood and currently surgery is the only curative treatment. Cytotoxic agents are in use in advanced cancers, but side effects and multidrug resistance are often problems. Thus there is a need for novel curative treatment methods. In contrast, ovarian granulosa cell tumors and testicular Leydig cell tumors are usually benign, especially at a younger age.

The aim of the present thesis was to study a novel targeted treatment method through luteinizing hormone/chorionic gonadotropin receptor (LHCGR) in a transgenic mouse tumor model. The cytotoxic agent was lytic peptide Hecate-CGβ conjugate where 23 amino acid Hecate, a synthetic form of honeybee venom melittin, was conjugated to 15 amino acid fragment of human chorionic gonadotropin β subunit. Lytic peptides are known to act only on negatively charged cells, such as bacteria and cancer cells and hereby, due to hCGβ fragment, the conjugate is able to bind directly to LHCGR bearing cancer cells, saving the healthy ones. The experiments were carried out in inhibin-α-Simian Virus 40-T-antigen transgenic mice that are known to express LHCGR-bearing gonadal tumors, namely Leydig and granulosa cell tumors by 100% penetrance. If the mice are gonadectomized prepubertally they form adrenocortical tumors instead. Transgenic and wild type mice were treated for three consecutive weeks with control vehicle, Hecate or Hecate-CGβ conjugate. GnRH antagonist or estradiol was given to a group of mice with or without Hecate-CGβ conjugate to analyze the additive role of gonadotropin blockage in adrenocortical tumor treatment efficacy.

Hecate-CGβ conjugate was able to diminish the gonadal and adrenal tumor size effectively in males. No treatment related side effects were found. Gonadotropin blockage through GnRH antagonist was the best treatment in female adrenal tumors. The mode of cell death by Hecate-CGβ conjugate was proven to be through necrosis. LHCGR and GATA-4 were co-expressed in tumors, where the treatment down-regulated their expression simultaneously, suggesting their possible use as tumor markers.

In conclusion, the present thesis showed that Hecate-CGβ conjugate targets its action selectively through LHCGR and selectively kills the LHCGR bearing tumor cells. It works both in gonadal somatic and in ectopic LHCGR bearing adrenal tumors. These results establish a more general principle that receptors expressed ectopically in malignant cells can be exploited in targeted cytotoxic therapies without affecting the normal healthy cells.

Key words: Hecate, lytic peptide, targeted cancer therapy, LHCGR, adrenal, gonad, ectopic hormone receptor

TIIVISTELMÄ

Susanna Vuorenoja
Gonadotropiiniriippuvaisten hormonaalisten syöpien kohdennettu ablaatio LH-reseptorivälitteisesti
Biolääketieteen laitos, fysiologia ja Kliininen laitos, lastentaudit, lääketieteellinen tiedekunta,
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Hormonaaliset sukupuolirauhas- ja lisämunuaissyövät, erityisesti lisämunuaiskarsinoomat, ovat harvinaisia. Lisämunuaiskarsinoomien esiintyvyydeksi on raportoitu ainoastaan 1-2/1000000/vuosi. Ne ovat kuitenkin aggressiivisia ja erityisesti aikuisten lisämunuaiskarsinoomilla on huono ennuste, sillä ne havaitaan usein myöhäisessä vaiheessa. Leydiginsolusyövät ja munasarjojen granuloosasolusyövät ovat puolestaan useinmiten benignejä, erityisesti nuorilla. Tämän hetken tärkein hoitomuoto näillä syövillä on kasvainten kirurginen poisto. Solunsalpaajat ovat käytössä levinneissä muodoissa, mutta niiden sivuvaikutukset ja resistenssiongelmat heikentävät hoitotuloksia. Uusille hoitomuodoille on tämän vuoksi tarvetta.

Väitöskirjatyön tarkoituksena oli testata uutta, LH-reseptoreihin kohdennettua syövänhoitomuotoa siirtogeenisella hiirimallilla. Tutkimuksen kohteena oli lyyttinen peptidi Hecate-CGβ -konjugaatti, jossa on yhdistettynä 23 aminohapon jakso Hecate- ja 15 aminohapon jakso hCGβ-ketjua. Hecate on synteettinen muoto mehiläisen myrkystä ja se tunnistaa negatiivisesti varautuneet solut, joita ovat mm. bakteeri- ja syöpäsolut. hCG-jakson vuoksi yhdiste kiinnittyy ainoastaan niihin negatiivisesti varautuneisiin soluihin, joissa on LH-reseptori. Mekanismina on siis LH-reseptoria ilmentävien syöpäsolujen kohdennettu tuhoaminen, jonka vuoksi terveet solut säästyvät. Tautimallina käytettiin Inhibiini-α-SV40-T-antigeeni -siirtogeenisiä hiiriä, joilla esiintyy LH-reseptoria ilmentäviä Leydigin- ja granuloosasolukasvaimia. Kun hiiriltä poistetaan sukupuolirauhaset ennen puberteettia, niille syntyy lisämunuaiskasvaimia. Siirtogeenisiä hiiriä ja kontrollihiiriä lääkittiin lumelääkkeellä, Hecatella tai Hecate-CGβ -konjugaatilla kerran viikossa 3 viikon ajan. Osalle hiiristä annettiin myös GnRH-antagonistia tai estrogeenia, joiden avulla tutkittiin gonadotropiinierityksen eston vaikutusta lisämunuaissyöpien hoitotuloksiin.

Hecate-CGβ -konjugaatti pienensi selvästi niin sukupuolirauhasten kuin lisämunuaiskasvaintenkin kokoa uroksilla. Hoidolla ei havaittu mitään sivuvaikutuksia. Gonadotropiinierityksen esto osoittautui tehokkaimmaksi hoidoksi naaraiden lisämunuaiskasvaimissa. Hecate-CGβ -konjugaatin aiheuttama solukuolema oli nekroosin aiheuttama. LH-reseptorin ja GATA-4:n on todettu ilmentyvän kasvainsoluissa yhtä aikaa. Hoitojen myötä niiden ilmentyminen myös väheni yhtä aikaa. Tämän vuoksi kyseisiä geenituotteita voidaan jatkossa pitää käyttökelpoisina kasvainmerkkiaineina.

Väitöskirjatyössä osoitettiin Hecate-CGβ -konjugaatin tuhoava vaikutus LH-reseptoria ilmentäviin syöpäsoluihin ilman sivuvaikutuksia muihin kudoksiin. Hecate-CGβ -konjugaatti osoittautui tehokkaaksi niin sukupuolirauhassyöpien kuin ektooppisia LH-reseptoreja ilmentävien lisämunuaissyöpienkin hoidossa. Tulokset osoittavat myös yleisemmällä tasolla sukupuolirauhaskudoksen ulkopuolella ilmentyvien ektooppisten hormonireseptorien käyttökelpoisuuden sytostaattihoitojen kohdentamisessa syöpäsoluihin.

Avainsanat: Hecate, lyyttinen peptidi, kohdennettu syöpähoito, LH-reseptori, lisämunuainen, sukupuolirauhanen, ektooppinen hormonireseptori

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ABBREVIATIONS

AB	antibody
ACC	adrenocortical carcinoma
ACT	adrenocortical tumor
ACTH	adrenocorticotropic hormone
Ad4BP	adrenal 4 binding protein
AGCT	adult type granulosa cell tumor
AGP	adrenogonadal primordium
AIMAH	ACHT-independent macronodular adrenal hyperplasia
ANOVA	analysis of variance
BCA	bicinchoninic acid
CAH	congenital adrenal hyperplasia
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
cGβ	chorionic gonadotropin β-chain
CKDN1A	cyclin-dependent kinase inhibitor 1A
CKDN1C	cyclin-dependent kinase inhibitor 1C; p57kip2
CRH	corticotropin releasing hormone
CRHR	corticotropin releasing hormone receptor
cRNA	RNA derived from cDNA
CT	computed tomography
CTP	C-terminal peptide
CVAH	congenital virilizing adrenal hyperplasia
CYP11B	P450c11; CYP11B1; cytochrome P450, family 11, subfamily B, polypeptide 1; steroid 11β-hydroxylase
CYP17	P450c17; CYP17A1; cytochrome P450, family 17, subfamily A, polypeptide 1; steroid 17α-hydroxylase/17,20-lyase
CYP19	P450arom; CYP19A1; cytochrome P450, family 19, subfamily A, polypeptide 1; aromatase
CYP21	P450c21; CYP21A2; cytochrome P450, family 21, subfamily A, polypeptide 2; CYP21B; CAH1; steroid 21-hydroxylase

DAX-1	dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X-chromosome
DDT	dichlorodiphenyltrichloroethane
DES	diethylstilbestrol
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DMBA	dimethylbenzanthracene
DNA	deoxyribonucleic acid
E2	estradiol
ECL	enhanced chemiluminescense
ELISA	enzyme-linked immunosorbent assay
ER	estrogen receptor
ERCC1	excicion repair cross-complementation group 1
EtOH	ethanol
F	fluorine
FACS	fluorescent-activated cell sorting
FCS	fetal calf serum
FDG	fluorodeoxyglucose
FMK	fluoromethylketone
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
GATA-4	GATA-binding protein 4
GATA-6	GATA-binding protein 6
GCT	granulosa cell tumor
GIP	gastric inhibitory peptide
GnRH	gonadotropin releasing hormone
GPCR	G protein-coupled receptor
GSP	Gs protein
17β-HSD	17β-hydroxysteroid dehydrogenase
3β-HSD	3β-hydroxysteroid dehydrogenase
H_20_2	hydrogen peroxide
hCG	human chorionic gonadotropin

HE	hematoxylin-eosin
HPG	hypogonadotropic
HRP	horse radish peroxidase
5-HT4	5-hydroxytryptamine 4 (serotonin)
IGF	insulin-like growth factor
IGF-IR	insulin-like growth factor receptor-1
IgG	immunoglobulin G
IMTO	iodometomidate
INHα	inhibin α-subunit
IVF	in vitro fertilization
JGCT	juvenile type granulosa cell tumor
KO	knockout
L19	L19 ribosomal protein
LCM	laser capture microdissection
LCT	Leydig cell tumor
LH	luteinizing hormone
LHCGR	luteinizing hormone/chorionic gonadotropin receptor
LHRH	luteinizing hormone releasing hormone
LOH	loss of heterozygocity
LPC	laser pressure catapulting
MDR	multidrug resistance
MEN1	multiple endocrine neoplasia 1
MMP2	matrix metalloproteinase 2
MIS	Müllerian inhibitory substance
MRI	magnetic resonance imaging
MTO	metomidate
MTT	dimethylthiatzol-tetrazolium bromide
NaCl	natrium chloride
NHL	non-Hodgkins lymphoma
o,p'DDD	1,1 dichloro-2 (O-chlorophenyl) ethane; mitotane
P450aldo	aldosterone synthase; cytochrome P450; CYP11B2
P450c11	11-hydroxylase; cytochrome P450, family 11

P450c21	21-hydroxylase; cytochrome P450, family 21
P450scc	cholesterol side chain cleavage enzyme; cytochrome P450, cholesterol side-chain cleavage
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PET	positron emission tomography
PI	propidium iodide
PRKAR1A	protein kinase, cAMP-dependent, regulatory, type 1, alpha
PSA	prostate specific antigen
PVDF	polyvinylidene fluoride
RIA	radioimmunoassay
RNA	ribonucleic acid
RT-PCR	reverse-transcription PCR
qRT-PCR	quantitative real-time RT-PCR
SDS	sodium docedyl sulfate
SEM	standard error of the mean
SF-1	steroidogenic factor 1
SPECT	single-photon emission CT
SV	simian virus
TAG	T-antigen
TBS	tris buffered saline
TP53	tumor protein p53
TG	transgenic
TMM	telomere maintenance mechanism
TSH	thyroid stimulating hormone
TSHR	thyroid stimulating hormone receptor
WHO	World Health Organization
WT	wild type
X-ray	roentgen ray
ZF	zona fasciculata
ZG	zona glomerularis
ZR	zona reticularis

LIST OF ORIGINAL PUBLICATIONS

The thesis is based on the following original publications, which are referred to in the text by Roman numerals (I-III). Some unpublished data is also included.

I Bodek G, **Vierre S**, Rivero-Muller A, Hansel W, Huhtaniemi I, Ziecik AJ and Rahman NA (2005). A novel targeted therapy of Leydig and granulosa cell tumors through their luteinizing hormone receptor using a Hecate-chorionic gonadotropin β conjugate in transgenic mice. Neoplasia 7:497-508.

II **Vuorenoja S**, Rivero-Muller A, Ziecik A, Huhtaniemi I, Toppari J, Rahman NA (2008). Targeted therapy for adrenocortical tumors in transgenic mice through their LH receptor by Hecate-human chorionic gonadotropin β conjugate. Endocr Relat Cancer 15:635-648.

III **Vuorenoja S**, Mohanty BP, Arola J, Huhtaniemi I, Toppari J, Rahman NA (2009). Hecate-CGβ conjugate and gonadotropin suppression shows two distinct mechanisms of action in the treatment of adrenocortical tumors in transgenic mice expressing Simian Virus 40 T antigen under inhibin α promoter. Endocr Relat Cancer 16:549-564.

1. INTRODUCTION

Gonadal somatic cell and adrenocortical tumors are usually endocrinologically active tumors that are potentially a fatal group of malignancies, as early diagnosis and prevention remains difficult. Only 1 % of all male tumors are of testicular origin, but they are the most common tumors in young men (Kinkade, 1999). Leydig cell tumors account for only up to 3% of testicular tumors. They are mostly benign at a younger age (Kim *et al.*, 1985), but malignant forms in older men can be aggressive. At the moment there is no well-established chemotherapeutic cure for this group of cancer. Granulosa cell tumors account only for 1-2% of all the ovarian tumors (Savage *et al.*, 1998). Similar to testicular tumors, most are benign in young patients. The adult form of the tumor, however, has a tendency of late recurrency and there is a group of tumors with early relapse and poor prognosis (Malmstrom *et al.*, 1994, Koukourakis *et al.*, 2008).

Adrenocortical carcinomas (ACC) are rare and aggressive malignancies with an incidence of only 1-2/1000000 a year (Allolio & Fassnacht, 2006). They are often diagnosed late and are usually resistant to chemotherapy, which makes prognosis often poor (Schulick & Brennan, 1999a). The only effective treatment for ACCs is complete adrenalectomy with a survival rate of 38-62%. With partial adrenalectomies the survival rate remains poorer, with a reported level of 0-9% (Schulick & Brennan, 1999a). Chemotherapy with mitotane is suggested for advanced stages of ACC where the surgery is no longer curative (Ahlman *et al.*, 2001). Adjuvant mitotane treatment is currently also recommended for all tumors after surgery (Terzolo *et al.*, 2007). However, the outcome of mitotane chemotherapy remains quite poor due to the severe gastrointestinal and neurological side-effects and treatment-induced adrenal insufficiency (Ng & Libertino, 2003). Benign adrenocortical adenomas are more frequent than ACCs. The incidentally found adrenocortical tumors, that are mainly either silent or hormone-secreting adenomas, are one of the most commonly found type of human tumors with a prevalence of 4% (Mansmann *et al.*, 2004, Young, 2007).

The chemotherapeutic agents used at present also affect the healthy cells as a major side effect due to the action of these agents on all rapidly dividing cells. Thus, studies at present are aimed at inventing targeted therapies that would only be aimed at the cancer cells, saving the healthy ones. For such selective targeted therapy a possibility could be to target the action through ectopic receptors expressed in cancer cells. In the present thesis, the role of luteinizing hormone/chorionic gonadotropin hormone receptor (LHCGR), that is known to be present in gonads but also in many non-gonadal organs such as adrenals, is studied for this matter.

Most of the cytotoxic agents mediate their action through apoptosis which is an energy-dependent mode of cell death with cell shrinkage and nuclear fragmentation.

However, many cancer cells develop resistance to apoptosis, which can make the use of conventional cytotoxic agents problematic (Martinez-Lorenzo *et al.*, 1998). In necrosis, often followed by a cellular injury, cells burst due to a rapid and cell specific membrane permeabilization.

Hecate is a 23 amino acid amphipathic, positively charged lytic peptide, a synthetic analogue of melittin, which is the principal toxic component of natural honeybee venom. In order to specifically target the action of this lytic peptide, Hecate was conjugated to a 15 amino acid segment (81-95) of the β subunit of human chorionic gonadotropin (hCG) (Leuschner *et al.*, 2001). This β chain possesses high receptor affinity towards luteinizing hormone receptors and it selectively destroys tumor cells expressing the LHCGR (Leuschner *et al.*, 2001, Bodek *et al.*, 2003, Bodek *et al.*, 2005b), but spares the normal healthy cells. In the present studies inhibin α-subunit/Simian Virus 40-T-antigen (Inhα/Tag) transgenic (TG) mouse model, which provides a good experimental model for the study of gonadal somatic cell and adrenocortical tumorigenesis, was used (Kananen *et al.*, 1995, Kananen *et al.*, 1996a, Kananen *et al.*, 1996b). The TG mice presented with gonadal tumors by 100% penetrance at the age of 5-8 months (Kananen *et al.*, 1995, Kananen *et al.*, 1996a), but when gonadectomized prepubertally they developed adrenocortical tumors also with 100% penetrance by the same age (Kananen *et al.*, 1996b).

The main objective of the present thesis study was to discover novel therapeutic possibilities for the treatment of LHCGR possessing endocrine tumors, such as gonadal somatic cell or adrenocortical tumors and also to study the molecular mechanisms underlying the treatment effects.

2. REVIEW OF THE LITERATURE

2.1. Common origin of the gonads and the adrenal gland

The two adrenal glands are located retroperitoneally above the upper part of the kidney. They consist of the adrenal cortex and medulla. The adrenal cortex is a major source of steroid hormones and is responsible for the regulation of glucose and electrolyte balance and for stress response. The adrenal medulla secretes e.g. epinephrine and norepinephrine, hormones responsible for the responses of the symphatetic nervous system.

The adrenal cortex and somatic parts of the gonads share a common origin. They arise during embryonic development from the same progenitor cells of the urogenital ridge called the adreno-gonadal primordium (AGP) (Morohashi, 1997, Keegan & Hammer, 2002). This primordium separates into distinct primordia for adrenals and gonads, which later on are separated into fetal adrenal and bipotential gonads (Fig. 1). The precise mechanism of this separation still remains unclear.

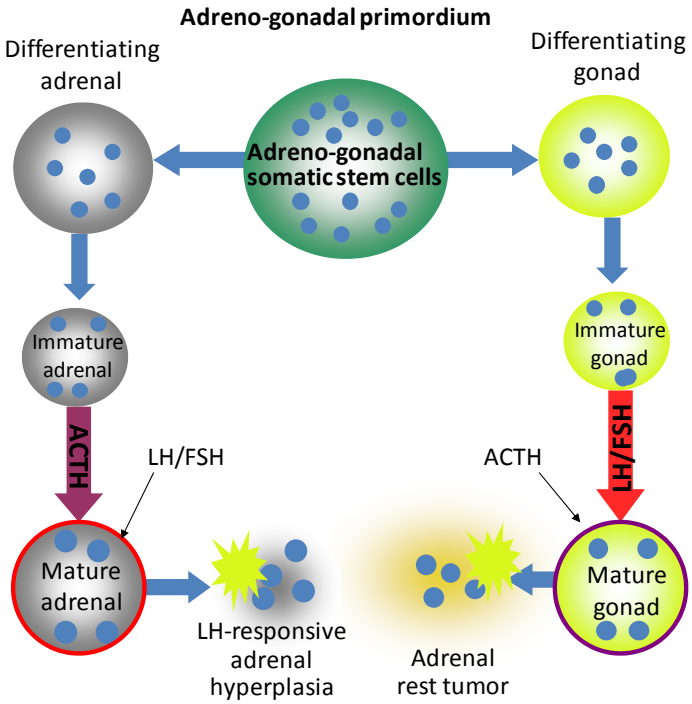


Figure 1. Both the adrenals and gonads originate from the common adreno-gonadal primordium (AGP), which contains common stem cells that are equally distributed in the differentiating gonads and adrenals. The stem cell population is thought to be one of the predisposing factors also for later gonadotropin dependent tumorigenesis in the adrenals or ACTH dependent adrenal rest tumor development in gonads. Figure modified from Bernichtein *et al*. (2008a).

The developing adrenal in human consists of an inner fetal zone and an outer definitive zone, both of which have characteristics of steroid-producing cells. The fetal zone secretes mainly dehydroepiandrosterone sulfate (DHEA-S) that will be converted to estrogens by the placenta. Cortisol is mainly secreted from the definitive zone (Mesiano & Jaffe, 1997). The fetal zone regresses through apoptosis during the first three postnatal months whereas during the age of 10-20 years the definitive zone will evolve and stabilize into the adult zones, mineralocorticoid-secreting zona glomerulosa (ZG), glucocorticoid-secreting zona fasciculata (ZF) and androgen-secreting zona reticularis (ZR) (Keegan & Hammer, 2002) (Fig. 2). These last two zones (ZF and ZR) also secrete glucocorticoids and androgens in an overlapping pattern. The steroid hormones are synthesized from cholesterol through actions of many cytochrome P450 enzymes (Fig. 3). Adrenocorticotropic hormone (ACTH) causes glucocorticoid and androgen production from the adrenals (Mesiano & Jaffe, 1997), while aldosterone is produced under the control of the renin-angiotensin system, mainly through angiotensin II. ACTH is secreted under the influence of corticotrophin releasing hormone (CRH) and vasopressin (VP) that is known to regulate the actions of CRH (Abou-Samra *et al.*, 1987, Bilezikjian & Vale, 1987) (Fig. 4).

The development and zonation of the adrenal in the mouse differs somewhat from human. The postnatal murine adrenal contains the X-zone, a layer next to the medulla that forms during the first weeks of life and enlarges until three weeks of age (Howard-Miller, 1928, Keegan & Hammer, 2002). It originates from the fetal zone analogous to the human fetal zone (Zubair *et al.*, 2006). The X-zone regresses in males after puberty and in females after the first pregnancy (Howard-Miller, 1928, Holmes & Dickson, 1971). Activin may play a role in the regression process of the X-zone (Beuschlein *et al.*, 2003). However, if mice are gonadectomized prepubertally the X-zone persists, probably due to the elevated LH (Jones, 1949, Deacon *et al.*, 1986, Kananen *et al.*, 1996a, Beuschlein *et al.*, 2003). The role of the X-zone still remains unclear (Keegan & Hammer, 2002). Although the X-zone has shown LH responsiveness, no evidence of androgen production has been found in it (Jones, 1949). In adult mice only ZG and ZF are present. CYP 17 is not expressed in adult mice adrenal glands and no androgens are produced (Dunn, 1970). Further, the main glucocorticoid produced by mice is corticosterone in comparison to cortisol in human.

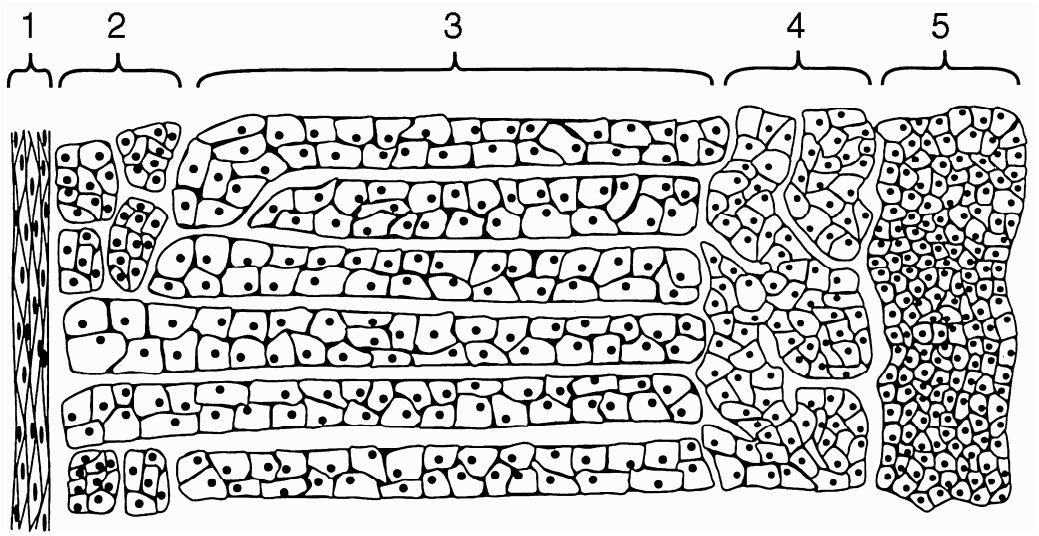


Figure 2. Zonation of the adrenal gland: (1) capsule, (2) zona glomerulosa, (3) zona fasciculata, (4) zona reticularis, (5) medulla. Modified from Goodman (1998).

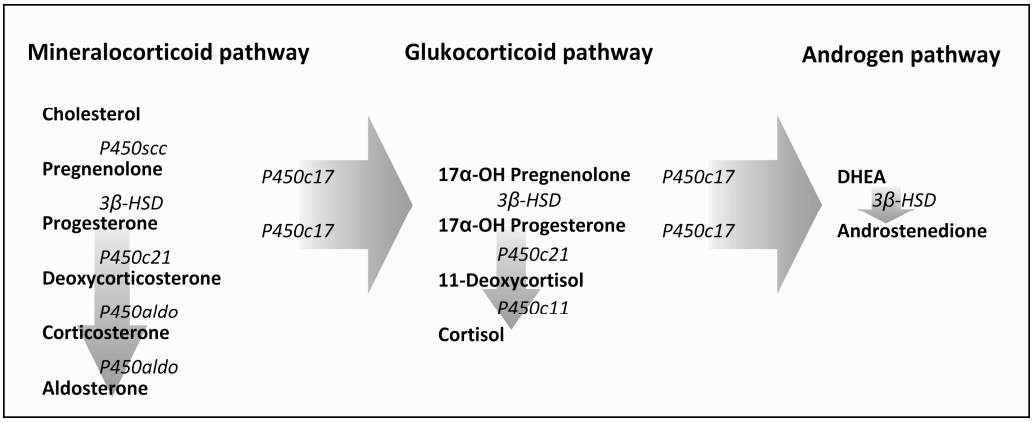


Figure 3. Enzymes needed for steroid production in adrenals. P450scc, cholesterol side chain cleavage enzyme; 3β-HSD, 3β-hydroxysteroid dehydrogenase; P450c17, enzyme containing 17α-hydroxylase and 17,20-lyase activities; P450c21, 21-hydroxylase; P450aldo, aldosterone synthase; P450c11, 11β-hydroxylase; DHEA, dehydroepiandrosterone.

The adrenals and gonads share common properties, i.e. the negative feedback regulation of the hypothalamus-pituitary-axis. They also both produce steroid hormones and during fetal life there is reciprocal hormone secretion. Gonad-specific CYP19 and CYP17 are expressed in fetal mouse adrenal glands (Toda *et al.*, 1994, Keeney *et al.*, 1995, Morohashi, 1997) and adrenal-specific CYP11B1 and CYP21 are expressed in mouse fetal testis (Val *et al.*, 2006). In mouse the fetal testis also produce testosterone by ACTH stimulation (O'Shaughnessy *et al.*, 2003). In the case of tumors this reciprocal secretion of gonadal and adrenal steroid hormones can be found again, e.g. in adrenal

tumorigenesis where the persistent high gonadotropin expression induces adrenal tumors with gonad-like appearance (Looyenga & Hammer, 2006) (Fig. 1). This phenomenon is thought to be possible through a stem cell pool/a group of developmentally plastic cells that respond to high LH by adopting a gonad-like appearance (Looyenga & Hammer, 2006, Huhtaniemi *et al.*, 2009).

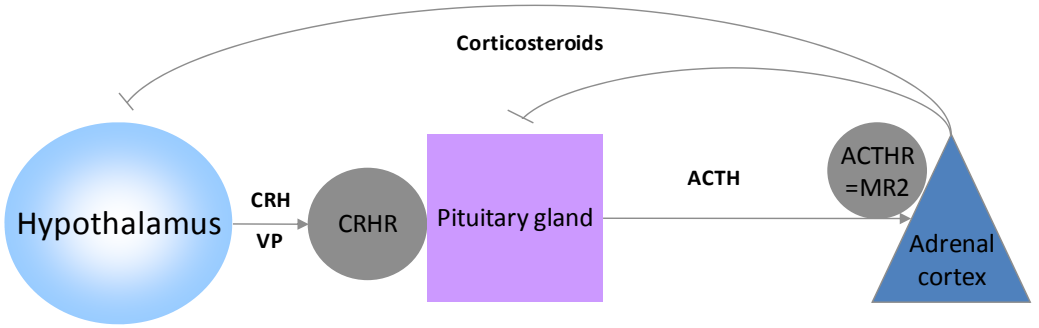


Figure 4. Negative feedback system of adrenals (CRH, corticotropin-releasing hormone; CRHR, corticotrophin-releasing hormone receptor; VP, vasopressin; ACTH, adrenocorticotropic hormone; MR2, melanocortin 2-receptor).

2.2. Gonadal tumors

2.2.1. Testicular Leydig cell tumors

Testicular tumors account only for 1% of all tumors in males, but they are the most common tumors in men between 15 to 34 years of age in many countries (Kinkade, 1999). According to the Finnish Cancer Registry, the frequency of all new testicular cancers during the years 2003-2007 in Finland was 116 per year, of which 83 occurred in the age group of 15 to 39 (www.cancerregistry.fi). The survival rate of early stage disease is 100% and in advanced disease, when the tumor is macroscopically detectable beyond the testis, the survival rate after the chemotherapy remains at 80% (Jones & Vasey, 2003). Out of all testicular tumors, 95% are of germ cell origin and in this group the most common tumors are seminomas. Stromal tumors, including Sertoli, granulosa cell and Leydig cell tumors (LCT), are rare in adults but in children they account for up to 40% of cases (Rushton & Belman, 1993, Rajpert-De Meyts *et al.*, 2006). Stromal tumors express inhibin α, which distinguishes them efficiently from germ cell tumors (Iczkowski *et al.*, 1998, Toppari *et al.*, 1998). LCTs, that account only for about 3% of all testicular tumors (Kim *et al.*, 1985), affect men in all ages, most commonly between 5-10 or 30-35 years of age (Kim *et al.*, 1985, Dilworth *et al.*, 1991, Rushton & Belman, 1993). In the pediatric population, LCTs represent 8% of all testicular tumors (Giebink & Ruymann, 1974).

The symptoms in children often manifest with testicular swelling and pseudoprecocious puberty (growth spurt, penile enlargement, pubic hair development) due to secretion

of androgens. Gynecomastia is also often present, especially in adults. The clinical diagnosis is made by testicular palpation or by ultrasonography, where LCT appears as a hypoechoic homogenous mass with high vascularity (Maizlin *et al.*, 2004). In histology the distinctive patterns of LCT are abundant lipofuscin and Reinke crystals (Al-Agha & Axiotis, 2007). Immunohistochemical markers useful in the diagnosis of LCT are especially calretinin (Augusto *et al.*, 2002) and inhibin α (Iczkowski *et al.*, 1998).

Leydig cell tumors are usually benign, especially in children. This was supported by a recent Finnish study concerning all pediatric testicular cancers (Taskinen *et al.*, 2008). The tumor is usually also unilateral, solid and encapsulated (Kim *et al.*, 1985). These facts make the treatment of choice to be orchiectomy or removal of the encapsulated tumor (Rushton & Belman, 1993, Chandak *et al.*, 2003). After surgery, the endocrine symptoms usually recede. The malignant forms of LCTs appear more often in older age (Kim *et al.*, 1985), and are often manifested by larger tumor size, infiltration of the adjacent parenchyma, necrosis and vascular invasion. In contrast to benign tumors, that often secrete both estrogens and androgens, the malignant forms may lack endocrine manifestations (Kim *et al.*, 1985). The treatment of malignant tumors requires a more invasive operation also including retroperitoneal lymph node excision (Kim *et al.*, 1985). Thus far there is no proof about the efficacy of any cytostatics in treating LCTs (Bertram *et al.*, 1991), whereas in germ cell tumors they are very effective (Rajpert-De Meyts *et al.*, 2006).

Congenital virilizing adrenal hyperplasia (CVAH), a syndrome of androgen overproduction of the adrenals and elevated ACTH, often due to 21-hydroxylase (CYP21) deficiency is often characterized by family history, small testes in males and accelerated growth. If the disease is poorly controlled, adrenal rest tumors can develop in the testes similar to Nelson's syndrome where the bilateral removal of the adrenals causes pituitary hyperplasia and consequent ACTH overproduction (Hamwi *et al.*, 1963). The signs and symptoms of CVAH-related adrenal rest tumors resemble those of Leydig cell tumors with testicular swelling and sexual precocity. In comparison to Leydig cell tumors, patients with adrenal rest tumors usually have bilateral testicular enlargement and elevated 17-hydroxyprogesterone due to ACTH stimulation. Blood hormone measurements, as well as dexamethasone suppression tests, are used to differentiate between these two illnesses. The treatment for adrenal rest tumors in CVAH is adequate corticosteroid treatment, in comparison to surgery in Leydig cell tumors (Hamwi *et al.*, 1963, Rutgers *et al.*, 1988, Rich & Keating, 2000). Adrenal and ovarian Leydig cell tumors can also occur (Pollock *et al.*, 1986, Diab *et al.*, 2008).

2.2.2. Ovarian granulosa cell tumors

Ovarian cancers are an aggressive type of cancer since they give symptoms only in the advanced stage with a 5-year survival rate of only about 30% (Cho & Shih, 2009). Most (90%) of these malignant tumors are of epithelial origin. The overall indicence

of ovarian tumors in Finland in 2003-2007 was 467 with 61% in the age group of 55-79 (www.cancerregistry.fi). Granulosa cell tumors (GCT) belong to a group of sex-cord stromal tumors affecting women of all age groups, with peak incidences in young women and around 50-55 years of age (Calaminus *et al.*, 1997, Gittleman *et al.*, 2003). They represent 1-2% of all ovarian tumors (Calaminus *et al.*, 1997), but in females under 20 years of age the proportion is higher with 10% of ovarian tumors being of granulosa cell origin (Merras-Salmio *et al.*, 2002). GCTs are divided into adult type (adult type granulosa cell tumor, AGCT, 95% of cases) and juvenile type (juvenile type granulosa cell tumor, JGCT, 5% of the cases) (Calaminus *et al.*, 1997). These tumors present with abdominal pain, and usually with high levels of estrogen with subsequent uterine bleeding and menstrual abnormalities (Cronje *et al.*, 1998). The high estrogen levels have been reported to induce endometrial hyperplasia and even endometrial carcinoma in the case of GCT (Malmstrom *et al.*, 1994).

JGCT is mainly detected in adolescents (Scully, 1988), whereas only 5% of cases are prepubertal (Young *et al.*, 1984). This tumor type is usually benign and rarely any relapses are detected after surgical resection. If relapses are to occur, they are normally evident during the first 3 years (Merras-Salmio *et al.*, 2002). Because of the secretion of estradiol, most of the prepubertal patients show signs of isosexual precocious puberty. Due to the hormonal activity, these types of ovarian tumors can be detected in the early phase of disease which makes the survival rates good; most of the granulosa cell tumors are stage I tumors and the survival rate is 90% (Merras-Salmio *et al.*, 2002). In addition to estradiol, Ca-125 and inhibin are used for preoperative laboratory evaluation in children (Merras-Salmio *et al.*, 2002). *Gsp* (Gs protein) oncogene, the mutationally activated gene for encoding $G\alpha_s$ (Jamieson & Fuller, 2008), and GATA-4 (Anttonen *et al.*, 2005) are currently evaluated as novel prognostic markers for JGCT.

AGCT in stage I, similar to JGCT, is also known to be benign with survival rates of 60-90% (Koukourakis *et al.*, 2008). Adult tumors are known to progress slowly with a tendency for late recurrency, normally just after 5-10 years (Miller *et al.*, 1997), although the reports exist showing recurrencies even after 20 years. 80-90 % of recurrent diseases are reported to be fatal (Jamieson & Fuller, 2008). In AGCT, inhibin α and Müllerian inhibitory substance (MIS) are considered as tumor markers and they become elevated many months prior to macroscopical recurrency (Koukourakis *et al.*, 2008). Imaging is normally carried out by ultrasonography, computed tomography (CT) or magnetic resonance imaging (MRI), where the granulosa cell tumors are most commonly detected as multiseptated cystic masses or unlobulated solid masses (Kim & Kim, 2002).

Normally, the curative treatment for GCTs is unilateral salpingo-oophorectomy in non-metastasized cases in children and young women. Hysterectomy can be done simultaneously in older women (Cronje *et al.*, 1998). Ascites cytology is routinely taken during the

operation (Merras-Salmio *et al.*, 2002, Koukourakis *et al.*, 2008). When there is a high mitotic index, large tumor size or ruptured tumor, the prognosis is worse and extensive surgery in indicated (Koukourakis *et al.*, 2008). In these cases, either adjuvant radiation therapy (Wolf *et al.*, 1999) or postoperative chemotherapy (Homesley *et al.*, 1999) is used. There are a small number of tumors with malignant behavior (e.g. metastases) with early relapses and poor prognosis. In this group, as well as in recurrent disease, chemotherapy is indicated (Zambetti *et al.*, 1990, Calaminus *et al.*, 1997, Koukourakis *et al.*, 2008). However, the problem with current chemotherapeutic agents is the myelotoxicity (Zambetti *et al.*, 1990, Homesley *et al.*, 1999). There have been trials on the use of GnRH antagonists in the treatment of GCTs (Martikainen *et al.*, 1989, Fishman *et al.*, 1996). Other hormonal treatments such as aromatase inhibitors and tyrosine kinase inhibitors for aggressive cases have recently been studied and reviewed (Jamieson & Fuller, 2008), but further studies are needed before any conclusions on their efficacy can be drawn.

2.3. Adrenal tumors

2.3.1. Common features of the tumors

Adrenal tumors are rare tumors which arise either from the adrenal cortex (adrenocortical adenoma/carcinoma) or from the medulla (pheochromocytoma, neuroblastoma). Most of the tumors in the adrenal cortex are incidentalomas, incidentally found adrenal tumors, often discovered in the radiological examination done for some other reason than evaluation of the adrenal glands. Their prevalence is around 4% and they are considered to be one of the most prevalent human tumors (Mantero *et al.*, 2000, Mansmann *et al.*, 2004). Over 80% of these incidentalomas are benign, asymptomatic adenomas, which usually need only a follow-up (Mantero *et al.*, 2000). However, they can be hormone producing adenomas, pheochromocytomas, adrenocortical carcinomas or metastases of carcinomas and thus both imaging and hormonal evaluation are needed after the diagnosis (Young 2007). Carcinomas in this group have been found to account for around 10% of cases (Mantero *et al.*, 2000, Mansmann *et al.*, 2004).

Adrenocortical carcinomas (ACC) are rare and aggressive malignancies with poor prognosis, because they are often diagnosed late and are usually resistant to chemotherapy (Schulick & Brennan, 1999a). In the USA, the annual incidence of the adrenocortical tumors is 1-2 per million (Allolio & Fassnacht, 2006), whereas in childhood it is 0.2-0.3 per million (Rodriguez-Galindo *et al.*, 2005). In Europe, 400-1500 new cases are reported every year (Berruti *et al.*, 2008). The disease peaks in children under the age of 5 years or over the age of 10 years and in adults in their fourth or fifth decade of life (Wajchenberg *et al.*, 2000, Dackiw *et al.*, 2001, Wieneke *et al.*, 2003, Allolio & Fassnacht, 2006). They are slightly more common in females (58,6%) than in males (41,4%) (Wooten & King, 1993, Schulick & Brennan, 1999a). The survival rate of

adrenocortical carcinoma is, according to many studies, only 15-35% (Ahlman *et al.*, 2001). Most common metastases occur in the lungs, liver and peritoneum (Schulick & Brennan, 1999a). In adults 50-60% of adrenocortical carcinomas are hormonally active, secreting glucocorticoids or androgens/estrogens and Cushingoid symptoms (truncal obesity, rounded face, buffalo hump, hirsutism, muscle and skin atrophy, osteoporosis, virilization) are the most common presentation (Schulick & Brennan, 1999b). Rare cases of oversecretion of mineralocorticoids can cause the clinical manifestation of hypertension and hypokalemia. However, in many cases the adult tumors are hormonally silent which causes delays in the diagnosis, thus leading to poor prognosis.

In pediatric patients, adrenocortical tumors (ACT) constitute only about 0.2% of all pediatric malignancies (Ribeiro & Figueiredo, 2004). In fact, only one new pediatric ACC have been reported in Finland during 2003-2007 (www.cancerregistry.fi). 80-90% of the childhood adrenal tumors have been reported to be ACCs (Ciftci *et al.*, 2001, Dehner, 2003). However, the prognosis in children is much better than in adults, probably because they might be structurally malignant but still benign in behavior (Dehner, 2003). As in infant neuroblastomas, even spontaneous recoveries of ACC in infancy have been reported (Kasat *et al.*, 2001). More than 90% of childhood ACCs are functional and virilization is the most common presentation in children (Sabbaga *et al.*, 1993), which is similar to gonadal tumor presentation. Symptoms usually reveal the disease early; normally the diagnosis is done at 5-8 months, which makes the prognosis favorable (Wieneke *et al.*, 2003, Rodriguez-Galindo *et al.*, 2005). Cushing syndrome, in contrast to adults, is very rare and cortisol-secreting tumors are considered to have worse prognosis than those with pure virilization (Ribeiro & Figueiredo, 2004). Again, feminizing tumors, that are known to indicate poor prognosis in adults, are not fatal in children (Wieneke *et al.*, 2003). The small tumor size, young patient age, and complete resection in the primary surgery are good prognostic factors (Ribeiro & Figueiredo, 2004, Rodriguez-Galindo *et al.*, 2005); even 100% 5-year survival in this group of patients has been reported (Lucon *et al.*, 2002). Patients under the age of 3 normally have a better prognosis than older children (Dehner, 2003).

2.3.2. Genes potentially involved in adrenal tumorigenesis

The etiology of adrenocortical carcinoma remains still unknown, even though recent studies point to many genetic alterations and syndromes behind ACTs. There are also publications suggesting that cigarette smoking in males (Chow *et al.*, 1996, Hsing *et al.*, 1996) and oral contraceptives in females (Hsing *et al.*, 1996) are risk factors for adrenocortical carcinoma. Adrenocortical tumors are associated with certain hereditary tumor syndromes, e.g. Li-Fraumeni (Li *et al.*, 1988). It is characterized by early onset and multiple tumors, mainly sarcomas, associated with germline tumor protein p53 (*TP53*) mutations (Wagner *et al.*, 1994). Overall, germline *TP53* mutation is a predisposing

factor in about 80% of pediatric ACCs (Rodriguez-Galindo *et al.*, 2005). In southern Brazil, the incidence of ACC in children is 10-15 times higher than anywhere else in the world and this has also been found to be caused by *TP53* mutation R337H, which is not, however, related to Li-Fraumeni -syndrome (Ribeiro *et al.*, 2001). Its malignant potential is low with no family history for cancers and it is considered as a predisposing factor only to childhood ACCs (Ribeiro & Figueiredo, 2004). Beckwith-Wiedemann syndrome is a congenital overgrowth syndrome with e.g. exomphalos, macroglossia, gigantism and neonate/childhood tumors (Engstrom *et al.*, 1988). In the syndrome, 20% of the neoplasms can be adrenocortical tumors, but also nephroblastomas, hepatoblastomas and rhabdomyosarcomas are found. The syndrome is caused by alterations in chromosome 11p15. LOH (loss of heterozygocity, loss of one of two alleles of a gene) or abnormal imprinting of 11p15.5 causes low *CDKN1C* (cyclin-dependent kinase inhibitor, $p57^{kip2}$), low *H19* and elevated *IGF-II* mRNA levels, which are all predisposing factors for adrenal tumors and are also involved in the induction of Beckwith-Wiedemann syndrome (Gicquel *et al.*, 1994a, Liu *et al.*, 1995, Gicquel *et al.*, 1997, Liu *et al.*, 1997). In fact, *IGF-II*, which is located in the chromosomal locus 11p15, is one of the most commonly overexpressed genes in ACC (Ilvesmaki *et al.*, 1993). However, in murine studies it was shown that although *IGF-II* overexpression is significant in ACC, it is not sufficient to induce tumorigenesis alone but needs the concomitant downregulation of $p57^{kip2}$ and *H19* for the development of ACC (Gabory *et al.*, 2006).

In Carney Complex (cardiac, endocrine, cutaneous, neural myxomatous tumors) (Carney *et al.*, 1986), multiple endocrine neoplasia (MEN1; parathyroid, pancreatic islet cell, anterior pituitary tumors) (Thakker, 1998), congenital adrenal hyperplasia (CAH, 21-hydroxylase deficiency causing overproduction of cortisol precursors and higher levels of androgens) (Merke & Bornstein, 2005) and McCune-Albright syndrome (polyostic fibrous dysplasia, café-au-lait spots, endocrine dysfunction, i.e. hyperthyroidism, gigantism, precocious puberty and Cushing's syndrome) (Weinstein *et al.*, 1991) there is also evidence of adrenal hyperplasia and adenomas. Carney complex is associated with mutations in the *PRKAR1A*-gene (Bertherat *et al.*, 2003), MEN1 with *MEN1* (Schulte *et al.*, 1999), CAH with *CYP21A2 (CYP21B)* (Merke & Bornstein, 2005) and McCune-Albright with *GNAS* (Fragoso *et al.*, 2003). However, most of the tumors are sporadic and only a small proportion of them are caused by hereditary defects. During the last 5 years, several microarray studies have been carried out in order to find novel genes involved in adrenal tumorigenesis, both in pediatric and adult patients. Table 1 and Table 2 summarize some of the main affected genes with adrenal tumorigenesis in children and adults, respectively, mainly from microarray reports. The genetical changes seen in microarrays in pediatric and adult adrenocortical tumors are quite similar, which may suggest the possibility of adrenal stem cells giving rise to the developing tumors (West *et al.*, 2007).

Table 1. Genetic susceptibility for adrenocortical tumors (especially adrenocortical carcinomas) in pediatric patients.

Upregulated		
Gene	*Gene name*	*Ref*
IGF-IR	insulin-like growth factor receptor-1	(West *et al.*, 2007, Almeida *et al.*, 2008)
IGF-II	insulin-like growth factor -2	(West *et al.*, 2007)
FGFR4	fibroblast growth factor receptor 4	(West *et al.*, 2007)
SF-1	steroidogenic factor -1	(Figueiredo *et al.*, 2005)
Downregulated		
Gene	*Gene name*	*Ref*
CDKN1C	cyclin-dependent kinase inhibitor 1c	(West *et al.*, 2007)
HSD3B2	3-beta hydroxysteroid dehydrogenase type 2	(West *et al.*, 2007)
KCNQ1	potassium voltage-gated channel, KQT-like subfamily, member 1	(West *et al.*, 2007)

Table 2. Genetic susceptibility for adrenocortical tumors (especially adrenocortical carcinomas) in adult patients.

Upregulated		
Gene	*Gene name*	*Ref*
ANG2	angiopoietin 2	(Giordano *et al.*, 2003)
CCNB1	cyclin B1	(Soon *et al.*, 2009)
FGFR1	fibroblast growth factor receptor 1	(Giordano *et al.*, 2003, de Fraipont *et al.*, 2005)
FGFR4	fibroblast growth factor receptor 4	(de Fraipont *et al.*, 2005)
GAPD	glyseraldehyde-3-phosphate dehydrogenase	(de Fraipont *et al.*, 2005)
IGF-II	insulin-like growth factor II	(Giordano *et al.*, 2003, de Fraipont *et al.*, 2005, Velazquez-Fernandez *et al.*, 2005, Slater *et al.*, 2006, Giordano *et al.*, 2009, Soon *et al.*, 2009)
LIT1	long QT internal transcript 1	(de Fraipont *et al.*, 2005)
MAD2L1	mitotic arrest deficient-like 1	(Soon *et al.*, 2009)
MMP2	matrix metalloproteinase 2	(Volante *et al.*, 2006)
MST1R	macrophage stimulating 1 receptor	(de Fraipont *et al.*, 2005)
SPP1	secreted phosphoprotein 1	(Giordano *et al.*, 2003, Giordano *et al.*, 2009)
STK15	serine-threonine kinase	(Giordano *et al.*, 2003)
TGFβ2	transforming growth factor β2	(de Fraipont *et al.*, 2005)
TOP2A	topoisomerase IIα	(Giordano *et al.*, 2003, Giordano *et al.*, 2009)
UFD1L	ubiquitin-fusion degradation 1 like	(Velazquez-Fernandez *et al.*, 2005)
USP4	ubiquitin-specific protease 4	(Velazquez-Fernandez *et al.*, 2005)
VEGF	vascular endothelial growth factor	(de Fraipont *et al.*, 2005)
Downregulated		
Gene	*Gene name*	*Ref.*
ABLIM	acting binding lim protein 1	(Soon *et al.*, 2009)
CgB (CHGB)	chromogranin B	(Slater *et al.*, 2006)
CDH2	cadherin 2, type 1, N-cadherin	(Velazquez-Fernandez *et al.*, 2005)
CXCL10	chemokine (C-X-C motif) ligand 10	(Velazquez-Fernandez *et al.*, 2005)
EGR-1	early growth response 1	(Slater *et al.*, 2006)
H19	imprinted maternally expressed transcript, non-protein coding	(Giordano *et al.*, 2009)
NAV3	neuron navigator 3	(Soon *et al.*, 2009)
RPRM	reprimo, TP53 dependent G2 arrest mediator candidate	(Soon *et al.*, 2009)
SEPT4	septin 4	(Soon *et al.*, 2009)

2.3.3. Diagnosis

It is difficult to distinguish between benign and malignant adrenocortical tumors. From the macroscopical point of view, adenomas are homogeneous, encapsulated, often less than 5 cm and weighing less than 50 g, whereas carcinomas are larger and heavier with a cyst type formation. ACC can also be diagnosed by detection of overproduction of hormones. Abundant co-secretion of androgens and glucocorticoids may suggest the higher malignant character of the tumor (Wajchenberg *et al.*, 2000, Allolio & Fassnacht, 2006). However, tumors are often found as incidentalomas during radiological examination (Luton *et al.*, 1990). The histological evaluation is based on Weiss criteria and its later modifications (Weiss, 1984, Aubert *et al.*, 2002). The nine criteria are the high grade of the tumor, high mitotic rate, atypical mitoses, low percentage of clear cell, diffuse growing and architecture, necrosis and capsular, sinusoidal and venous invasion. If two of the criteria are fulfilled, the tumor can be considered benign, whereas over four positive criteria are associated with tumor malignancy. For tumor staging, the TNM-classification of the WHO criteria modified from earlier established criteria is used (Macfarlane, 1958, Sullivan *et al.*, 1978) (Table 3). In children, it is preferable to use a separate staging system (Rodriguez-Galindo *et al.*, 2005) (Table 4).

For the ACC imaging, CT remains the first option, whereas tumor invasion and metastases are more clearly seen by MRI. 18fluorine-fluorodeoxyglucose-positron emission tomography (FDG-PET) (Becherer *et al*., 2001, Yun *et al.*, 2001, Tenenbaum *et al.*, 2004) and C-metomidate (MTO)-PET/ PET-CT are also useful methods. Especially the latter is becoming very popular in differentiating between hormonally active tumors due to its high affinity to steroidogenic enzymes, especially to CYP11B (Bergstrom *et al.*, 2000). However, the short half-life of metomidate requires on-site cyclotrone and is, therefore, not possible to use everywhere. Due to these restrictions, new modifications have appeared in the form of [123I]iodometomidate (IMTO) scintigraphy, a single-photon emission CT (SPECT), having the same approach as [11C]MTO-PET towards the steroidogenic tissue with a lower radiation dose and higher specific tracer uptake (Hahner *et al.*, 2008). There is also a proposal on the possible treatment efficacy of the [123I] IMTO-SPECT tracer due to its high tumor uptake (Hahner *et al.*, 2008), since radiation treatment has been shown to decrease the number of local recurrences (Fassnacht *et al.*, 2006). Bone gamma-imaging is done regurlarly to exclude bone metastases.

There are no existing specific markers for adrenocortical tumors for immunohistochemistry, but Ki-67 (Sasano *et al.*, 2001), p53 (TP53) (Arola *et al*., 2000) and cyclin-E (Tissier *et al*., 2004) are commonly used. p21 (CDKN1A) has also been found to be highly elevated in adrenocortical carcinomas (Stojadinovic et al., 2002), but its specificity has not proven to be high enough. Both Ki-67 and p53 levels have been proven to be higher in ACC than in adenomas (Arola *et al.*, 2000). Ki-67 expression in primary tumor, together with Weiss score, also seems to be a good

predictor of recurrence after tumor resection (Morimoto *et al*., 2008). IGF-II and MIB1 (mouse monoclonal antibody that recognizes a formalin fixation resistant epitope on the cell proliferation-associated antigen Ki-67) together have been shown to have a high specificity and sensitivity in recognizing adenoma and carcinoma (Schmitt *et al*., 2006, Soon *et al*., 2009). Adrenal 4 binding protein/steroidogenic factor 1 (Ad4BP/SF-1) (Sasano *et al.*, 2006, Kaneko *et al*., 2008) and dosage-sensitive sex reversal, adrenal hypoplasia congenita, critical region on the X chromosome gene (DAX-1) (Kaneko *et al*., 2008) were suggested as markers for ACC, where SF-1 is especially useful for the differentiation between ACC and metastasis of some other primary origin. In children histological markers are not always reliable.

Clonal analysis could also predict between benign and malignant tumors, since it has been shown that most of the ACCs are monoclonal in comparison to adrenocortical adenomas, that have been shown to be polyclonal (Beuschlein *et al.*, 1994, Gicquel *et al.*, 1994b). Since telomere maintenance mechanisms (TMM) are important for the malignant phenotype of ACC, the detection of TMM has been proposed to be of diagnostic value in order to distinguish between benign and malignant ACT (Else *et al.*, 2008). An LOH of 11p15 has also been found to be associated with transition to malignancy (Gicquel *et al.*, 1997).

Table 3. TNM-grading for adrenocortical cancers (WHO 2004) (Macfarlane, 1958, Sullivan *et al.*, 1978).

I	tumor <5 cm
II	tumor >5 cm, local carcinoma
III	tumor infiltration of neighboring structures, or positive lymph nodes
IV	infiltration of neighboring structures and positive lymph nodes or distant metastases

Table 4. Proposed staging of adrenocortical tumors in children (Rodriguez-Galindo *et al.*, 2005).

I	small tumors (<200cm3 or <100 g) **and** completely resected **and** normalization of hormone levels after surgery
II	large tumors (>200 cm3 or >100 g) **or** tumor spillage during surgery **and** normalization of hormone levels after surgery
III	unresectable tumors **or** residual disease after surgery **or** persistence of abnormal hormone levels after surgery
IV	metastatic disease

2.3.4. Treatment of adrenocortical tumors

Surgery is still the only curative form of ACT treatment (Schteingart *et al.*, 2005). Open surgery is mainly recommended, especially in malignant tumors. As a surgical method,

the laparoscopic approach is also currently used, but the risk of peritoneal cancer invasion during laparoscopy is very high (Cobb *et al.*, 2005). Laparoscopic methods are only recommended for benign tumors or exceptionally for malignant cases with careful patient selection (Kirshtein *et al.*, 2008). In a retrospective study the adjuvant irradiation of the tumor bed after the primary resection showed positive results and could perhaps be recommended to prevent the reoccurrence (Fassnacht *et al.*, 2006). Surgery should always aim at total primary resection, because with partial adrenalectomies the survival rate is 12 mo in comparison to 74 mo after total resection (Schulick & Brennan, 1999b). Even after radical resection there is a high recurrency rate and distance metastases occur in up to 85% of patients (Schulick & Brennan, 1999a). The high recurrency rate gives a reason for the use of adjuvant systemic therapies (Berruti *et al.*, 2008) and also emphasizes the need for new drugs to be tested as adjuvant therapy.

Chemotherapy with mitotane, 1,1 dichloro-2 (O-chlorophenyl) ethane (o,p'DDD), an analogue of insecticide dichlorodiphenyltrichloroethane (DDT), is indicated with advanced stages of the disease when the total removal of the tumor is not possible (Luton *et al.*, 1990, Wooten & King, 1993). However, the results often remain poor with only 30% survival (Wooten & King, 1993, Ng & Libertino, 2003). The results of the use of mitotane as adjuvant therapy shows somewhat improved survival rates (Terzolo *et al.*, 2007).

Mitotane has already been used for over 40 years for adrenocortical tumors (Bergenstal, 1960). It is known to be a very toxic drug with a narrow therapeutic index (Dackiw *et al.*, 2001, Schteingart *et al.*, 2005, Libe *et al.*, 2007). Mitotane has many gastrointestinal and neurological side-effects and also adrenal insufficiency is caused by the treatment. In addition to its action towards the ACC cells it also blocks cortisol and steroid synthesis by impairing 11β-hydroxylase (CYP11B) and cholesterol side chain cleavage (P450scc) (Libe *et al.*, 2007). Patients need replacement therapy, mainly with glucocorticoids which implicates the adrenolytic effect of mitotane to be mainly towards the zona fasciculata (Daffara *et al.*, 2008). Replacement with mineralocorticoids is also sometimes needed (Terzolo *et al.*, 2007). Central hypothyroidism (Rodriguez-Galindo *et al.*, 2005) and inhibition of testosterone secretion as well as altering cholesterol levels and liver toxicity has also been reported (Daffara *et al.*, 2008). However, mitotane is not effective in all patients, perhaps due to the difference in metabolizing activity of mitotane in cancer cells (Schteingart, 2007). To look at the safety and feasibility issue of mitotane, a prospective study with adjuvant mitotane was carried out recently (Daffara *et al.*, 2008). If the blood concentration of mitotane can be maintained in its threshold and safety range (14-20 mg/l), and close monitoring takes place as it has been proposed for clinical practice (Allolio & Fassnacht, 2006, Lee, 2007, Terzolo & Berruti, 2008), side effects could perhaps be lowered.

Despite many disadvantages, mitotane is still in use, since all other chemotherapeutics have been shown to be ineffective in ACC treatment due to high multidrug resistance

(MDR). MDR is caused by the MDR1-gene/P-glycoprotein produced by the adrenocortical tissue that works by pumping the chemotherapeutics out from the cells (Bates *et al.*, 1991, Flynn *et al.*, 1992). Amazingly, cell culture studies have shown that mitotane can reverse multidrug resistance (Bates *et al.*, 1991, Abraham *et al.*, 2002), which has led to the idea of a combination treatment of mitotane and chemotherapeutics.

Since the rarity of adrenocortical tumors, no consensus in their treatment have been existing and no extensive clinical studies have been carried out on them earlier. However, in 2003, the International Consensus Conference on Adrenal Cancer was held to discover more consistent treatment protocols for adrenocortical cancer throughout the world. Combinations of EDP (etoposide, doxorubicin, cisplatin) and mitotane or mitotane with streptozotocin were recommended (Schteingart *et al.*, 2005). Good results have been obtained e.g. from EDP with over 50% success for over 2 years, although accompanied by many side effects (neurotoxicity, lipid disorders) (Berruti *et al.*, 1998, Berruti *et al.*, 2005). A combination of mitotane and streptozotocin (Khan *et al.*, 2000) showed similar promising results. There are new studies underway for the ERCC1 (excision repair cross-complementation group 1), which is known to be a predictive factor for MDR (Allolio *et al.*, 2008). To monitor the effects of combination of conventional chemotherapeutics and mitotane in adrenocortical tumor treatment, the first international randomized control trial, FIRM-ACT, is ongoing. The results are expected to be available by 2011 (www.firm-act.org). Despite the improved results of chemotherapeutic treatment in ACC there is still a great need for new therapeutic approaches for the treatment of these tumors.

2.4. Gonadal and adrenal tumors in mice

A transgenic mouse line was established in our laboratory initially to study gonadal tumors. In this transgenic (TG) model, the mice harbor a 6 kb fragment of inhibin α-subunit promoter/Simian Virus 40 T-antigen (Inhα/Tag) fusion gene. These mice develop gonadal tumors, namely ovarian granulosa/theca cell and testicular Leydig cell tumors with 100% penetrance by the age of 5-8 months (Kananen *et al.*, 1995, Kananen *et al.*, 1996a). The cell lines derived from them (KK-1, BLT-1) retain the steroidogenic activity (Kananen *et al.*, 1995, Kananen *et al.*, 1996a). In order to discover whether the tumors in lung and liver, potentially due to the gonadal tumorigenesis are primary or secondary, prepubertal gonadectomies were carried out. It was noticed that the gonadectomy caused these mice to develop adrenocortical tumors with 100% penetrance by the age of 6-8 mo (Kananen *et al.*, 1996b, Rahman *et al.*, 2004). All the tumors seemed to originate from the X-zone and the tumors secreted high levels of progesterone. The corticosterone levels were low (Kananen *et al.*, 1996b). Adrenal tumorigenesis was first thought to be dependent on gonadal inhibin that was drastically reduced due to gonadectomy (Kananen *et al.*, 1996b). However, the tumors failed to appear by functional gonadectomy when the gonadotropin blockage was induced either by a

GnRH antagonist or by crossbreeding the mice to the gonadotropin-deficient *hpg* genetic background (Cattanach *et al.*, 1977, Kananen *et al.*, 1997). These experiments also proved that tumorigenesis and the tumor growth were gonadotropin dependent (Kananen *et al.*, 1997). While further characterizing these adrenal tumors it was noticed that both tumors and the derived cell line (Cα1) expressed a high level of LHCGR. The growth and steroidogenesis of these tumors were dependent on LH stimulation probably due to post-castration elevation of LH levels and the influence of oncogene SV40 T-antigen (Rilianawati *et al.*, 1998, Mikola *et al.*, 2003) (Fig. 5).

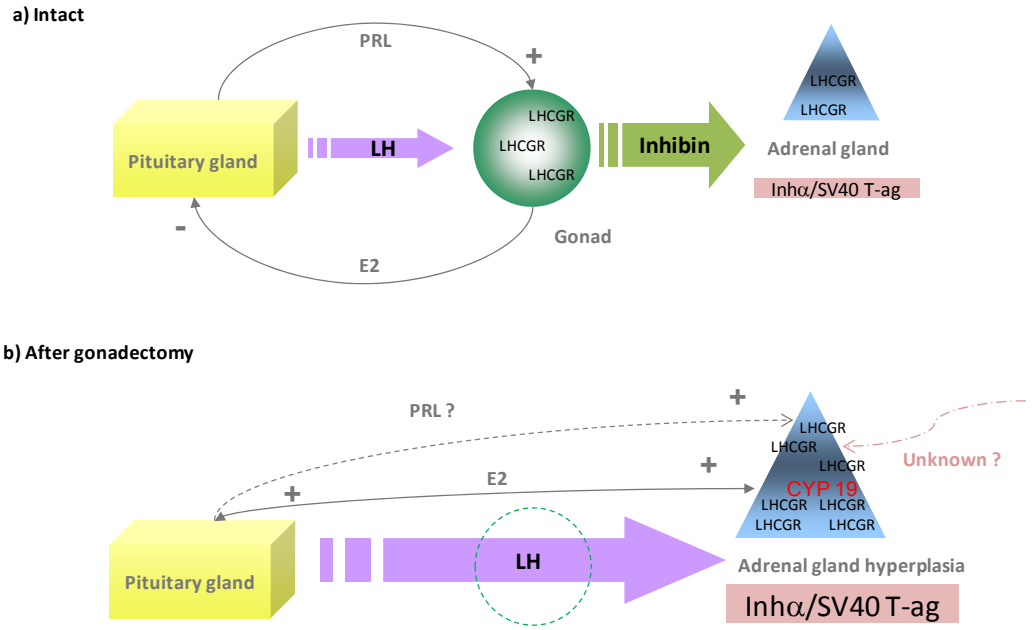


Figure 5. Proposed mechanisms of gonadotropin-induced tumorigenesis in the murine adrenal glands. In intact Inhα/Tag TG mice the gonadal inhibin suppresses adrenocortical tumorigenesis. After gonadectomy (the dotted green line) the chronically elevated LH levels upregulate the expression of adrenal LHCGR. This together with a lack of inhibin increases the effect of oncogene SV40-Tag and tumors will form. Another mechanism of post-gonadectomy tumorigenesis in murine models in general could be through other elevated hormone levels. After gonadectomy and upregulated LHCGR, aromatase (CYP19) is activated and E2 is synthetized. E2 itself and/ or increased prolactin might be involved in the expression of higher LHCGR. Due to the absence of gonads there is no feedback regulation and the continous adrenal stimulation causes tumor formations. Most probably there are also other factors predisposing to murine tumorigenesis, but they still remain unknown. Modified from Bernichtein *et al.* (2008a).

Inh -/- knockout mice develop gonadal tumors at an early age with a severe wasting syndrome followed by death. After prepubertal gonadectomy these inhibin knockout mice develop adrenal tumors by 99% penetrance (Matzuk et al., 1994). The earliest tumors were detected already at 4 mo of age. Based on this inhibin knockout study, the Inhα/Tag TG adrenal tumors were thought to depend on the loss of inhibins (Kananen *et al.*, 1996b). While cross-breeding inh -/- knockout mice with LH-overexpressing

mice (transgenic mice expressing the C-terminal peptide (CTP) of the human chorionic gonadotropin β subunit gene) (Risma *et al.*, 1995), very large adrenal tumors originating from the x-zone were detected at 4.5 months of age (Beuschlein *et al.*, 2003). This phenomenon further showed that high levels of LH promote adrenal tumor growth. The adrenal tumors of inh -/- knockout mice have recently been further studied. As shown earlier, the adrecortical tumors after gonadectomy resemble the gonads, particularly the ovaries in their molecular basis indicating the plasticity of the adrenal cortical cells to changes due to external stimuli (Looyenga & Hammer, 2006). In addition to the common feature of forming adrenal tumors, all the adrenal tumors in these transgenic murine lines express LHCGR and many also GATA-4 (Matzuk *et al.*, 1994, Kananen *et al.*, 1996b, Rilianawati *et al.*, 1998, Beuschlein *et al.*, 2003, Bielinska *et al.*, 2003, Bielinska *et al.*, 2004, Rahman *et al.*, 2004, Bielinska *et al.*, 2005).

Several inbred mouse strains, in addition to TG tumor models, also develop adrenal tumors after gonadectomy (Table 5). The first report on prepubertal gonadectomy causing nodular adrenocortical hyperplasia was reported in 1939 for a dilute female brown strain (DBA/2J) (Woolley *et al.*, 1939). In prepubertally gonadectomized DBA/2J mice, the tumors develop by six months of age and originate in the subcapsular region in contrast to X-zone originated Inhα/Tag TG adrenal tumors (Bielinska *et al.*, 2003). The tumor originates from spindle shaped A cells with abundant GATA-4 expression and later appearing, A cell derived, steroid producing B cells expressing abundant GATA-4 and steroidogenic factors, e.g. LHCGR and P450c17 (Bielinska *et al.*, 2003). Other mouse strains such as C3H and BALB/c, have been shown to develop adrenal adenomas (Woolley *et al.*, 1952) and in the CE mouse strain even adrenocortical carcinomas (Woolley *et al.*, 1943) after prepubertal gonadectomy. In CE/J mice the subcapsular tumors were found to form at the age of 5 months (Johnsen *et al.*, 2006). NIH Swiss mice were also reported to form adrenocortical tumors after ovariectomy at 15 mo of age (Strickland *et al.*, 1980). In inbred NU/J nude mice, tumor formation begins already at the age of 1-2 months and the tumorigenesis resembles that of DBA/2J with A and B cells and the subcapsular origin (Bielinska *et al.*, 2004, Bielinska *et al.*, 2005). The adrenal tumors in this mouse model can also be induced by gonadotropin elevation from xenografts of hCG-secreting CHO cells. In contrast to these models, C57Bl (Woolley *et al.*, 1952) and FVB/N (Bielinska *et al.*, 2003) mouse strains failed to form any adrenocortical neoplasias. One factor possibly affecting this phenomenon is the polymorphism in SF-1 encoding gene, SF-1^{S172}. The mice carrying this mutation (DBA/2J, C3H, CE/J) develop subcapsular adrenocortical tumors after gonadectomy and have lower steroidogenic capacity in comparison to a normal gene bearing C57Bl mice with high steroidogenic capacity (Bielinska *et al.*, 2003, Looyenga & Hammer, 2006). In addition to mice, neutered ferrets have been reported to form adrenocortical tumors (Peterson *et al.*, 2003, Peterson *et al.*, 2004).

Table 5. Animal models for adrenocortical tumorigenesis. Modified from Vuorenoja *et al.* (2007).

Animal model	DBA/2J mouse	CE/J mouse	ferret	Inh $^{-/-}$ KO mouse	Inh $^{-/-}$ -LH-CTP TG mouse	Inhα/Tag TG mouse	NU/J nude mouse
Gonadectomy needed	yes	yes	yes	yes	yes	yes	yes or xenograft
Age of the tumor formation	5-6 mo (A cells already at 2 mo)	5 mo		3-5 mo	4-5 mo	6-8 mo	1-2 mo
Histopathology	A and B cells in the subcapsular region	Densely packed cell nests in the subcapsular region	Tumors originating from zona reticularis (resembling those of DBA/2J mice), myxoid differentiation	Tumors derived from zona fasciculata and/ or X-zone	Tumors from X-zone	Tumors from X-zone (inner-most layers)	A and B cells in the subcapsular region (resembles DBA/2J mice)
LHCGR	In A and B cells	In the tumor area	In the tumor area	In the tumor area	In the tumor area	In the tumor area	In B cells
GATA-4	In A and B cells	Upregulated in the tumors	In the tumor area			In the tumor area	In A and B cells
Metastases			liver (lung, lymph node)	lung		liver	
Reference	(Bielinska et al., 2003)	(Johnsen et al., 2006)	(Peterson *et al.*, 2003, Peterson et al., 2004)	(Matzuk et al., 1994, Matzuk et al., 1996, Beuschlein et al., 2003)	(Beuschlein et al., 2003)	(Kananen et al., 1996b, Rilianawati et al., 1998, Rahman et al., 2004)	(Bielinska et al., 2004, Bielinska et al., 2005)

2.5. Luteinizing hormone receptor

2.5.1. LH and hCG

Gonadotropins secreted in humans are luteinizing hormone (LH), follicle-stimulating hormone (FSH) and human chorionic gonadotropin (hCG). They are heterodimeric glycoprotein hormones composed of α- and β-subunits (Pierce & Parsons, 1981). The α-subunit is common for all the gonadotropins as well as for thyroid-stimulating hormone (TSH). The β-subunit is hormone specific and the hormones bind accurately to their own receptors through it. LH and FSH are secreted from the anterior pituitary gland under the control of hypothalamus originated gonadotropin releasing hormone (GnRH). There is a negative feedback loop for the secretion of LH, FSH and GnRH regulated by gonadal sex steroids (Fig. 6). Gonadotropins are responsible for normal reproductive development and function. hCG is secreted from the placenta of pregnant women and is responsible for maintaining pregnancy by stimulating progesterone production by the corpus luteum gravidarum. In mouse there is no existing gene for chorionic gonadotropin. LH and hCG share a high homology in their beta-subunits; hCGβ differs from LH only by 24 additional amino acids in the carboxy terminal region and with four additional O-glycosylation sites (Gharib *et al.*, 1990). They also both act through the same receptor, luteinizing hormone/chorionic gonadotropin receptor (LHCGR). FSH acts through follicle stimulating hormone receptor (FSHR) and TSH through thyroid stimulating hormone receptor (TSHR). The receptors also share high structural homology.

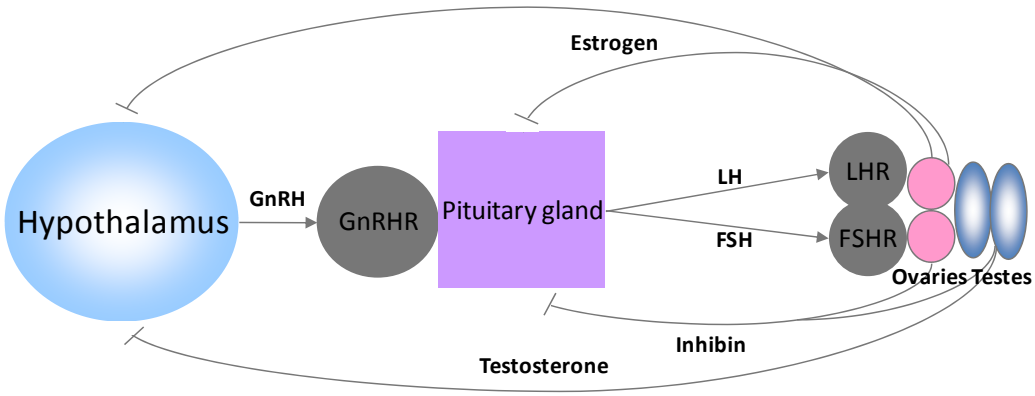


Figure 6. The negative feedback loop from the gonads to regulate gonadotropin secretion. Modified from Huhtaniemi *et al.* (2009).

2.5.2. LHCGR

LHCGR belongs to the glycoprotein hormone receptor subfamily of G protein-coupled seven-transmembrane domain receptors (GPCR) (McFarland *et al.*, 1989). Its gene is located on chromosome 2 in humans (Rousseau-Merck *et al.*, 1990) and on chromosome 7 in mouse. LHCGR consist of 11 exons and 10 introns in human. The first 10 exons are responsible for the extracellular part and ligand binding, whereas the long 11th exon encodes the seven-transmembrane and G protein-coupled intracellular domains (McFarland *et al.*, 1989). LHCGR protein consists of 701 amino acid residues and the molecular weight of the protein is approximately 75 kDA both in humans and in mice (McFarland *et al.*, 1989). The mature receptor is 10-20 kDA larger due to extensive glycosylation of the extracellular domain (Ascoli *et al.*, 2002).

Signal transduction of LHCGR takes place by ligand binding (LH/hCG) to the extracellular part of LHCGR, followed by conformational changes in the hormone-receptor complex leading to the activation of G-proteins. If the binding defective mutant LHCGR and signal-defective mutant LHCGR are co-trasfected, cAMP-production is induced. This suggests physical interactions between the two mutant receptors (Ji *et al.*, 2002, Lee C. *et al.*, 2002). In LHCGR KO mice, where no LHCGR is expressed endogenously, a normal reproductive phenotype can be rescued if two receptor mutants, one without ligand-binding and the other without signal transduction properties are together (Rivero-Muller *et al*., personal communication). This suggests dimerization/oligomerization of LHCGR upon ligand binding and a possible activation of other receptors in the close proximity of the activated receptor-ligand complex. It is likely that amplification of the signal transduction already occurs at the level of the hormone-receptor complex.

2.5.3. Signaling cascades

Most of the LHCGR-mediated intracellular events are mediated by the activation of the Gs/adenylyl cyclase/cAMP/PKA pathway, resulting in increases in cAMP production (Gudermann *et al.*, 1992). Another pathway stimulates phospholipase C (PLC) (Davis *et al.*, 1986, Nishizuka, 1988), resulting in formation of inositol phosphates and elevations in Ca^{2+} (Gudermann *et al.*, 1992). There is also evidence of the need for transactivation, i.e. crosstalk between GPCRs and epidermal growth factor receptors (EGFRs), for full activation of intracellular events (Prenzel *et al.*, 1999, Rozengurt, 2007, Evaul & Hammes, 2008). Pathways of mitogen activated protein kinases (MAPK) (Cameron *et al.*, 1996, Hirakawa *et al.*, 2002) and phospholipase A2 (PLA2) (Johnson *et al.*, 1991) are also activated through LHCGR.

2.5.4. Non-gonadal LHCGR

LHCGR is mainly found in the gonads. In the ovary, LHCGR is expressed in theca, granulosa, luteal and interstitial cells, whereas in the testis LHCGR is expressed in Leydig

cells. During the last 20 years there have been several studies reporting the non-gonadal expression of LHCGR in many non-tumorous tissues, as listed in Table 6. In addition to these, abundant LHCGR has been found in many types of cancer, i.e. in ovarian cancer (Simon *et al.*, 1983), prostate (Tao *et al.*, 1997a), mammary (Meduri *et al.*, 1997, Funaro *et al.*, 2003, Meduri *et al.*, 2003) and uterine cancers (Lei *et al.*, 1993b). Thus far, most evidence of functional non-gonadal ectopic LHCGR has been found in the adrenals.

Table 6. LHCGR found in human and rodent extragonadal tissues.

Tissue	*Species*	*References*
Adrenal gland	human	(Pabon *et al.*, 1996b, Couzinet *et al.*, 2001, Dall'Asta *et al.*, 2004, Costa *et al.*, 2009, Zwermann *et al.*, 2009)
	rat	(Apaja *et al.*, 2005)
	mouse	(Kero *et al.*, 2000, Bielinska *et al.*, 2003, Bernichtein *et al.*, 2008b)
Blood vessel	human	(Reshef *et al.*, 1990, Lei *et al.*, 1992, Toth *et al.*, 1994)
Brain	rat	(Lei *et al.*, 1993a, Apaja *et al.*, 2004)
Fetal tissues	human	(Abdallah *et al.*, 2004)
Kidney	rat	(Apaja *et al.*, 2005)
Lymphocytes	human	(Lin *et al.*, 1995)
Mammary gland	rat	(Lojun *et al.*, 1997, Tao *et al.*, 1997b, Funaro *et al.*, 2003)
Oviduct	human	(Lei *et al.*, 1993c, Han *et al.*, 1996)
	mouse	(Zhang *et al.*, 2001)
Placenta	human	(Reshef *et al.*, 1990)
Prostate	rat	(Reiter *et al.*, 1995, Tao *et al.*, 1995)
Seminal vesicle	rat	(Tao *et al.*, 1998)
Skin	human	(Pabon *et al.*, 1996a, Venencie *et al.*, 1999)
Sperm	human	(Eblen *et al.*, 2001)
Spinal cord	rat	(Rao *et al.*, 2003)
Umbilical cord	human	(Rao *et al.*, 1993)
Uterine cervix	human	(Lin *et al.*, 2003)
Uterus	human	(Reshef *et al.*, 1990, Han *et al.*, 1997)
	mouse	(Zhang *et al.*, 2001)

2.5.5. LHCGR in the adrenals

The human adrenal LHCGR receptor has not thus far been sequenced and the hormone-binding characteristics of the receptor have not been thoroughly explored. There are questions whether the receptor would have differences in either its amino acid sequence or in the glycosylation pattern in comparison to gonadal LHCGR (Carlson, 2007). Low levels of LHCGR expression have been found in normal human adrenal cortex by in situ hybridization, immunohistochemistry, reverse-transcription polymerase chain reaction (RT-PCR) and quantitative real-time RT-PCR (qRT-PCR) (Pabon *et al.*, 1996b, Couzinet *et al.*, 2001, Dall'Asta *et al.*, 2004, Costa *et al.*, 2009, Zwermann *et al.*, 2009). LHCGR is localized in the zona reticularis and the inner parts of zona fasciculata (Pabon *et al.*,

1996b). Human fetal adrenal cortex has also been shown to express LHCGR (Abdallah *et al.*, 2004) and hCG stimulates dehydroepiandrosterone sulfate synthesis of the fetal adrenal gland (Jaffe *et al.*, 1981). However, until now no physiological function for the expression of LHCGR in the normal adrenal gland has yet been found (Carlson, 2007). Further studies are needed to explore the possibility of activation of LHCGR in human adrenals in either physiological or pathological conditions with elevated LH (Rao *et al.*, 2004, Alevizaki *et al.*, 2006, Vuorenoja *et al.*, 2007).

The expression of ectopic LHCGR in the adrenals has often been associated with chronic gonadotropin overload, which can be seen in conditions with physiologically elevated gonadotropins, e.g. in pregnancy (Sheeler, 1994) or after menopause (Mijnhout *et al.*, 2004). This can cause ACTH-independent macronodular adrenocortical hyperplasia (AIMAH) and Cushingoid symptoms (Lacroix *et al.*, 1999, Bourdeau *et al.*, 2001, Miyamura *et al.*, 2002, Feelders *et al.*, 2003, Goodarzi *et al.*, 2003). The role of LHCGR in postmenopausal women has also been discussed with the finding of elevated cortisol levels concurrently with chronically elevated LH (Alevizaki *et al.*, 2006). However, ectopic LHCGRs are more often associated with pathological conditions such as LH-dependent adrenal adenomas producing aldosterone (Saner-Amigh *et al.*, 2006) or androgens (Werk *et al.*, 1973, Givens *et al.*, 1975, Larson *et al.*, 1976, Smith *et al.*, 1978, Takahashi *et al.*, 1978, de Lange *et al.*, 1980, Leinonen *et al.*, 1991). Adrenocortical carcinomas (Wy *et al.*, 2002, Barbosa *et al.*, 2004) with abundant LHCGR expression and estradiol production (Millington *et al.*, 1976) have been described.

The chronically elevated LH induces ectopic LHCGR in the mouse adrenal cortex and promotes tumor progression in gonadectomized Inhα/Tag TG mice as in many other TG mouse models (Kananen *et al.*, 1996b, Rilianawati *et al.*, 1998, Kero *et al.*, 2000, Beuschlein *et al.*, 2003, Bielinska *et al.*, 2003, Mikola *et al.*, 2003, Looyenga & Hammer, 2006). No LHCGR has been found in the wild type (WT) gonad-intact murine adrenal gland. However, wild type murine adrenal glands also expressed LHCGR, when they were subjected to postgonadectomy-induced chronically elevated LH levels (Kero *et al.*, 2000). LHCGR appearance after gonadectomy in mice is dependent on genetic background susceptibility. The co-expression of LHCGR and GATA-4 in mouse adrenocortical tumors has also been demonstrated (Bielinska *et al.*, 2003, Rahman *et al.*, 2004) (Table 5). However, recent reports have proposed that perhaps LHCGR is not the immediate cause of mice tumorigenesis, but perhaps chronically elevated LH induces activation of stem cells/progenitor cells (Bernichtein *et al.*, 2009), which further promotes tumorigenesis in the presence of other factors. The viral oncogene SV40 Tag used in the present study could be one predisposing factor, the role of which in tumorigenesis has been established. High levels of estradiol or prolactin are also known to promote adrenal hyperplasia (Kero *et al.*, 2000, Li *et al.*, 2001). Perhaps there are also other still unknown factors contributing to mouse adrenal tumorigenesis (Fig. 5).

2.6. Transcription factors GATA-4 and GATA-6 in human and mice adrenals

GATA transcription factors belong to zinc finger proteins. Their role is to regulate cellular development and differentiation (Orkin, 1992). GATA 1-3 are known to be expressed during hematopoiesis, where GATA-1 and -2 are found in multipotential progenitor cells, mast cells and megakaryotic lineages, and GATA-3 is found in T-lymphocytes (Orkin, 1992). GATA 4-6 again are found in organs of endodermal origin. GATA-4 is known to be expressed in the heart, gonads, stomach, intestine and pancreas (Arceci *et al.*, 1993, Heikinheimo *et al.*, 1997, Ketola *et al.*, 1999, Ketola *et al.*, 2004, Haveri *et al.*, 2008), GATA-5 in the heart, stomach, intestine, lung, bladder (Laverriere *et al.*, 1994, Morrisey *et al.*, 1997) and GATA-6 in the heart, gonads, lung, intestine, liver, kidney, pancreas and vascular smooth muscle (Morrisey *et al.*, 1996, Narita *et al.*, 1996, Suzuki *et al.*, 1996, Haveri *et al.*, 2008).

The importance of GATA-4 was shown in GATA-4 deficient mice, who die in utero by day E9.5 because of abnormal cardiac morphogenesis (Kuo *et al.*, 1997, Molkentin *et al.*, 1997). GATA-6 is also needed for embryogenesis, as GATA-6 deficient mice die at day E7.5 due to a defect in visceral endoderm development (Morrisey *et al.*, 1998). In murine adrenals, both GATA-4 and GATA-6 are expressed in the fetal period (Kiiveri *et al.*, 1999, Kiiveri *et al.*, 2002a). Adult WT mice adrenals have been shown to express abundant GATA-6 but no or very little GATA-4, whereas in Inhα/Tag TG mouse adrenal tumors GATA-6 expression is lost but abundant GATA-4 expression is seen (Kiiveri *et al.*, 1999, Kiiveri *et al.*, 2002a, Rahman *et al.*, 2004). The action of GATA-6 thus could be interpreted to support normal adrenal structure in mice, but its absence may predispose to adrenocortical tumor formation (Vuorenoja *et al.*, 2007). GATA-4 and GATA-6 have also been predicted to be responsible for the P450c17 activation at the early stage of mouse embryogenesis (Shi *et al.*, 2009).

Both GATA-4 and GATA-6 are expressed in human fetal adrenal glands (Kiiveri *et al.*, 1999, Kiiveri *et al.*, 2002a). GATA-4 was also found in healthy adult adrenal glands in human (Barbosa *et al.*, 2004), in comparison to murine models, where almost no GATA-4 could be detected in the adult adrenal gland (Kiiveri *et al.*, 1999, Kiiveri *et al.*, 2002b). However, the higher GATA-4 expression seems to be related to advanced human adrenocortical tumors and GATA-4 mRNA and protein expression has been linked to human adrenal adenomas and carcinomas with aggressive behavior (Barbosa *et al.*, 2004, Kiiveri *et al.*, 2004, Kiiveri *et al.*, 2005).

Similar to murine models, GATA-6 expression in human adrenals continues throughout life and it has roles in development and cell differentiation as well as in steroidogenesis (Kiiveri *et al.*, 2005). It is detected in human zona reticularis, the area of androgen production where the co-expression of steroidogenic markers SF-1 and P450c17 are also found (Jimenez *et al.*, 2003, Fluck & Miller, 2004, Kiiveri *et al.*, 2005). Thus

GATA-6 may have a role in androgen synthesis. This is further supported by the fact that even though GATA-6 expression normally diminishes in human ACC, in virilizing carcinomas it is present abundantly (Kiiveri *et al.*, 2005). GATA-6 is also found in human adrenocortical adenomas (Kiiveri *et al.*, 2004). The expression of GATA-6 in human adrenocortical tumors, in contrast to no expression in murine adrenocortical tumors, has suggested that GATA-6 expression is not affected by tumorigenesity (Barbosa *et al.*, 2004).

2.7. Targeted cancer treatment options

2.7.1. Targeted treatments in general

Over 100 years ago, Paul Ehrlich envisioned for the first time the concept of the 'magic bullet', a drug therapy that would only act on the organisms at which they are aimed leaving other cells intact (Stern, 2004). However, this idea still remains one of the major challenges for cancer therapy. At the moment most of the chemotherapeutics in use have considerable side effects due to their action on all dividing cells, including healthy ones. The therapeutic window between curative and toxic effect in these drugs is narrow and the problem with drug resistance is troublesome.

From the time of Paul Erlich a large number of trials in order to reach the above mentioned aim has been carried out. The first main idea was to discover an antibody that could recognize the specific antigen associated to the cancer cells. Antibody-toxin -conjugates (immunotoxins), including ricin, diphtheria and Pseudomonas exotoxin have also been constructed. The idea was to induce an irreversible arrest of protein synthesis in the cells recognized by the conjugate (Pastan & FitzGerald, 1991). Doxorubicin and other antineoplastic agents have also been linked to antibodies or growth factors, such as epidermal growth factor (EGF) or interleukins to be delivered to cancer cells (Hertler & Frankel, 1989, Yeh *et al.*, 1992). Some antibodies carrying radioactive material are already in clinical use e.g. for low grade or follicular B-cell non-Hodgkin's lymphoma (NHL) [ibritumomab with ^{90}Y (Zevalin®) and tositumomab with Iodine I^{131} (Bexxar®)] (Vose *et al.*, 2000, Krasner & Joyce, 2001). The idea in all of these targeted therapies is to deliver the toxin or radioactive material to the antigen carrying cell through antibody recognition. However, there are limitations to antibody-conjugated therapy such as the antibodies' poor penetration to the cancer cells (Halin *et al.*, 2002), the need for tumor-specific antigens and the toxicity of conjugates on other tissues, e.g. the liver (Halpern *et al.*, 1983).

Membrane receptors could be the most suitable candidates for the selective approach of targeted therapy, since by using receptors there would be no need for any intracellular delivery as the receptor is exposed directly to the extracellular environment. The

receptors usually possess high specific affinity to their ligand, which would help in the therapeutic targeting. Some studies on viral vectors on delivering their genetic contents by receptor binding have been reported (Kasahara *et al.*, 1994). There have been some death-inducing receptors (TNF, Fas, TRAIL) in therapeutic use, but due to the side effects on liver cells these trials have been suspended (Kakinuma *et al.*, 1999, Lawrence *et al.*, 2001).

2.7.2. Novel targeted treatment strategies for adrenocortical tumors

Due to the popular use of microarray studies in recent years, knowledge of the genes overexpressed in ACC is increasing (Giordano *et al.*, 2003, de Fraipont *et al.*, 2005, Velazquez-Fernandez *et al.*, 2005, Slater *et al.*, 2006, West *et al.*, 2007, Giordano *et al.*, 2009, Soon *et al.*, 2009). Some trials for targeting drugs for these novel genes have already been started. Epidermal growth factor receptor (EGFR) has been known to be overexpressed in ACC, but unfortunately the two studies carried out thus far have not brought any positive results (Schteingart *et al.*, 2005, Quinkler *et al.*, 2008). Vascular epidermal growth factor (VEGF) has also been shown to be overexpressed in ACC and two multitargeted tyrosine kinase inhibitors that also inhibit VEGF receptor, sunitinib and sorafenib, are in trials at the moment (Berruti *et al.*, 2008). Sunitinib has already been documented to be successful in metastatic ACC (Lee *et al.*, 2009). IGF-II is one of the most commonly found genes overexpressed in ACC (Giordano *et al.*, 2003, de Fraipont *et al.*, 2005, Slater *et al.*, 2006, West *et al.*, 2007, Giordano *et al.*, 2009) and two groups have recently studied the effects of IGF-1R inhibitor in ACC, since IGF-II exerts its mitogenic effects through IGF-1R. One group (Almeida *et al.*, 2008) studied a kinase inhibitor NVP-AEW541 in the H295 cell line and in a new cell line established from pediatric adrenocortical adenoma, where they found IGF-IR overexpression as a biomarker of pediatric adrenocortical carcinomas. IGF-1R kinase inhibitor seemed to have antitumor effects by apoptosis both in adult and pediatric cell lines. The other group also used the H295 cell line as well as xenografts in nude mice to study the same small molecule inhibitor NVP-AEW541, as well as human monoclonal antibody IMC-A12, either alone or combined with mitotane (Barlaskar *et al.*, 2009). IGF-1R kinase inhibitors seemed to be effective in both the cell line and the xenograft and the effect was even more amplified by the combination with mitotane. In addition, it was noted by qRT-PCR that VEGF expression also diminished after the combination treatment perhaps implicating that IGF inhibition may also reduce the tumor angiogenesis (Barlaskar *et al.*, 2009). Since ACCs are also found to express peroxisome proliferator-activated receptor-gamma (PPAR-γ), the antidiabetic agent PPAR-γ -agonists have also been tested with positive results in the treatment of adrenocortical tumors suggesting a role for these agents in the future for ACC treatment (Betz *et al.*, 2005, Ferruzzi *et al.*, 2005).

2.8. Hecate-CGβ conjugate

2.8.1. Lytic peptides

In nature, there are many known membrane disrupting peptides that act as host immune defence mechanisms against pathogens in organisms without an immune system, e.g. in bacteria and invertebrates. These host defence peptides share common features of small size, linearity and multiple Arg and Lys residues causing a net cationic (positive) charge. They have the ability to form amphipathic and α-helical structures especially in a hydrophobic environment and they all destroy rapidly negatively charged membranes (Leuschner & Hansel, 2004). Bacterial membranes are populated with negatively charged phospholipids, whereas most of the normal animal cell membranes are composed of zwitterionic phospholipids with no charge (Matsuzaki, 1999) (Fig. 7).

Membrane disrupting peptides have also been shown to have broad antimicrobial activity in humans and their activity as suggested by their name, is caused by membrane disruption of the lipid bilayer mostly in Gram-negative, but also in Gram-positive bacteria as well as in fungi, enveloped viruses and protozoa (Mader & Hoskin, 2006). This ability gives reason to also call membrane disrupting peptides anti-bacterial peptides. They have been shown to be useful towards many organisms resistant to antibiotics because their action through the cell membranes avoid the resistance problem. On the other hand, they do not usually induce further bacterial resistance (Hancock & O'Reilly, 2005). The most potent members of this group of peptides are cecropins, magainins, defensins, and melittin.

Cecropin was found in the hemolymph of the cecropia moth (*Hyalophora cecropia*), one of the largest moths found in North America (Steiner *et al.*, 1981). It consists of 34-39 amino acids and its actions have been studied both in bacterial and cancer cells (Chen *et al.*, 1997, Chan *et al.*, 1998, Hui *et al.*, 2002, Chen *et al.*, 2003a, Chen *et al.*, 2003b). **Magainin** with 23 amino acid residues was found from the skin of the African clawed frog (*Xenopus laevis*) (Zasloff, 1987). It is an intervertebrate counterpart of cecropin and acts against both Gram-negative and Gram-positive bacteria. Its analogue has been in clinical trials for treatment of infected diabetic foot ulcers (Maloy & Kari, 1995). **Defensins** were found from human neutrofils. They are rich in cysteine and arginine and are known to kill e.g. Staphylococcus aureus, Pseudomonas aeruginosa, and Esherichia coli (Wilde *et al.*, 1989, Ganz *et al.*, 1990, Ganz & Lehrer, 1995). **Melittin**, an active polypeptide from honey bee (*Apis mellifera*) venom, first detected by Habermann in 1958, is composed of 26 amino acids and is known to act through the disruption of lipid bilayers (Sessa *et al.*, 1969). Further studies have also proved its action through aggregating intramembraneous particles especially in erythrocyte membranes (Dufton *et al.*, 1984, Clague & Cherry, 1989, Hui *et al.*, 1990). Melittin in humans after a bee sting causes the membrane disruption of mast cells, thus causing histamine release and inflammatory reaction. Bee venom, where melittin is the major particle has also been

investigated for its antiarthritic effects and the potency of inflammatory inhibition has been shown (Park *et al.*, 2004). A preliminary report shows a role for melittin in the treatment of Lyme disease spirochete *Borrelia burgdorferi* (Lubke & Garon, 1997).

These membrane disrupting peptides and their synthetic analogues (Fink *et al.*, 1989) have also been tried in cancer cells, because they act directly on the cell membranes, thereby bypassing mammalian cell multidrug resistance (Moore *et al.*, 1994). Except for the negative charge of the cell membrane outer leaflet, other features, such as an increase of the surface of cancer cells by membrane fluidity (Sok *et al.*, 1999) and by abundance of microvilli (Chaudhary & Munshi, 1995) in comparison to normal cells, may help the lytic peptides to bind cancer cells and leave the healthy cells unharmed (Mader & Hoskin, 2006). The selectivity towards malignant rather than non-malignant cells is one of the most important characteristics of these membrane disrupting peptides. Lytic peptides have been reported to have almost 50-fold preference in killing cancer cells compared with non-malignant cells (Johnstone *et al.*, 2000). Membrane disrupting peptides, combined with conventional cytotoxic agents, also enhance the toxicity of chemotherapeutics in cancer cells (Johnstone *et al.*, 2000). Since conventional chemotherapeutics are aimed at rapidly dividing cells, slowly proliferating cells can remain outside the treatment efficacy. Membrane disrupting peptides could, by their ability to attack all the negatively charged cells, also reach the dormant tumor cells. Anti-bacterial peptides have also been shown to be less toxic with fewer side effects and less destruction for vital organs. Normally they also have a short systemic half life (Leuschner & Hansel, 2004). Some of these peptides are potent inhibitors of angiogenesis (Mader & Hoskin, 2006).

Recently a melittin-avidin conjugate that recognizes matrix metalloprotease-2 (MMP2) has been developed. MMP2 can be highly expressed by human cancer cells and the idea was to recognize the MMP2 by conjugated avidin (Vihinen & Kahari, 2002, Holle *et al.,* 2003). New ideas such as combining the melittin peptide with prostate cancer specific monoclonal antibody have been introduced (Russell *et al.,* 2004). Melittin has also been combined with a recombinant adenovirus under the control of α-fetoprotein, in order to aim it at human hepatocarcinoma cells producing α-fetoprotein (Ling *et al.,* 2005).

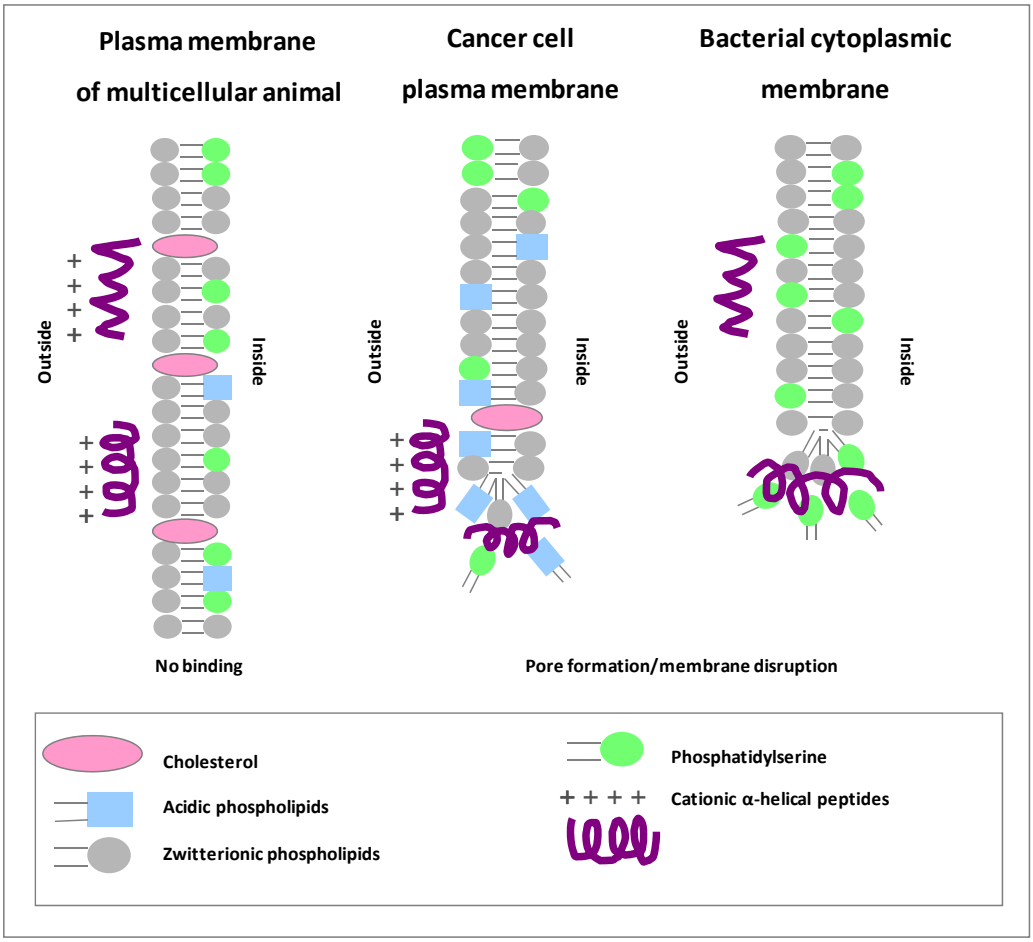


Figure 7. Characteristics of lytic peptides. The positively charged, linear, amphipathic and α-helical lytic peptides destroy the bacterial and cancer cells because of the different membrane structure. Cancer cells are negatively charged due to the increased amount of phosphatidylserines in the outer cell leaflet. Healthy cells remain intact because they have no charge on the cell membrane. Modified from Rivero-Muller *et al*. (2007).

2.8.2. Hecate

Hecate as a name originates from a three headed Greek goddess, who was known to be a goddess of the crossroads, wilderness, childbirth and later on also a goddess of witches. She was considered a mediator of death but was also known for giving back life. Hecate is also a gene e.g. in *Arabidopsis thaliana*, a small flowering plant, where the gene is known to regulate female reproductive tract development (Gremski *et al.*, 2007) and in zebra fish *(Danio rerio)*, where it has a role in the induction of dorsal organizer and in the frequency of intracellular calcium transients (Lyman Gingerich *et al.*, 2005). However, the main interest in Hecate at this time is in a 23 amino acid lytic peptide, a synthetic analogue of melittin, the principal toxic component of natural honeybee venom

(Henk *et al.*, 1995, Baghian *et al.*, 1996). Hecate was designed to change the charge distribution of melittin but to save its α-helical structure (Fig. 8). Hecate is also less hemolytic than its naturally occurring counterpart. As discussed earlier in general, the effectiveness of Hecate was first shown as an antimicrobial agent towards organisms such as *E. Coli, Cryptosporidium parvum and Herpes Simplex Virus-1* (Arrowood *et al.*, 1991, Henk *et al.*, 1995, Baghian *et al.*, 1996). Its effectiveness towards *Cryptosporidium parvum* was especially appreciated because this organism is known to be resistant to multiple antimicrobial agents (Arrowood *et al.*, 1991). Its mechanism of action depends on formation of pores or channels causing osmotic cytolysis and cell death by wedge shaped insertion of monomers, the barrel-stave method (Sui *et al.*, 1994, Leuschner & Hansel, 2004, Mader & Hoskin, 2006), also causing the aggregation of the membrane proteins and expulsion of phospholipids (Tosteson *et al.*, 1990, Leuschner & Hansel, 2004, Mader & Hoskin, 2006) (Fig. 7).

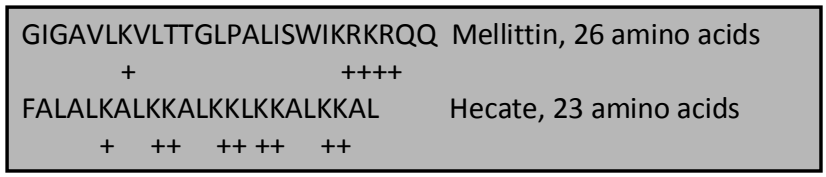


Figure 8. The protein structure of melittin and its analogue Hecate. The + signs indicate the charge of the amino acid. Modified from Baghian *et al*. (1996).

2.8.3. The anticancer agent towards LHCGR

The idea of using LHCGR-aimed targeted therapy was established in the 1990s when LHCGR expression in various tissues, including cancer tissues, was found. One of the main ideas of constructing Hecate-CGβ conjugate was to decrease the potential toxicity of Hecate to other tissues than those expressing LHCGR by fusing it to a fragment of human chorionic gonadotropin β subunit and in this way deliver the peptide selectively to tumor cells expressing LHCGR (Leuschner *et al.*, 2001). Thus, 23 amino acids of lytic peptide Hecate were conjugated to a 15 amino acid segment (81-95) of the β subunit of hCG (Leuschner *et al.*, 2001). This particular β chain fragment possesses high receptor affinity towards luteinizing hormone receptors (Morbeck *et al.*, 1993), which makes the conjugate aim its action selectively at LHCGR expressing tumor cells and improve the cytotoxicity in them, without affecting the healthy cells (Leuschner *et al.*, 2001, Bodek *et al.*, 2003) (Figs. 7 and 9). The efficacy of Hecate-CGβ conjugate has been investigated in prostate cancer cells and xenografts (Leuschner *et al.*, 2001, Bodek *et al.*, 2005), in ovarian (Gawronska *et al.*, 2002, Zaleska *et al.*, 2003) and testicular Leydig tumor cells (Zaleska *et al.*, 2003, Bodek *et al.*, 2003) as well as in mammary gland tumor cells, xenografts and in induced mammary gland tumors (Bodek *et al.*, 2003, Leuschner *et*

al., 2003b, Zaleska *et al.*, 2004, Hansel *et al.*, 2007a). All of these tumor cells expressed abundant LHCGRs.

The effect of Hecate-CGβ conjugate was studied intensively *in vitro*, and it was shown that the toxicity of the peptide is highly dependent on the amount of LHCGR in tumor cells. The CHO cells transfected with LHCGR responded more efficiently to Hecate-CGβ conjugate and the $ZnCl_2$ pretreatment augmented the cytotoxic effect due to increased amount of LHCGR (Leuschner *et al.*, 2001). Pretreatment of cells or xenograft-bearing nude mice with estradiol (E2) or FSH increased LHCGR expression and, consequently, the efficacy of the treatment (Hansel *et al.*, 2001, Leuschner *et al.*, 2001). In contrast, the removal of steroids from the media made the treatment effect decrease (Leuschner *et al.*, 2001). Cells without LHCGR are not affected by the lytic peptide in normal concentrations (Zaleska *et al*., 2003). Hecate-CGβ conjugate competes with LH/hCG for the binding sites to LHCGR (Leuscher *et al.*, 2001, Gawronska *et al*., 2002, Zaleska *et al.*, 2003, Bodek *et al.*, 2003) and the pretreatment of tumor cells with CG inhibits dose dependently the lytic effect of Hecate-CGβ conjugate (Leuschner *et al.*, 2003b, Bodek *et al.*, 2005).

The ability of Hecate-CGβ conjugate to selectively kill cancer cells expressing LHCGR is dose dependent, as has been reported by cytotoxicity measurements (Bodek *et al.*, 2003, Zaleska *et al.*, 2003, Bodek *et al.*, 2005). In very high doses Hecate-CGβ conjugate causes cell destruction even in the cells without LHCGR (Zaleska *et al.*, 2003). Further, the *in vivo* experiments done with diethylstilbestrol/dimethylbenz[a]anthracene (DES/DMBA) - induced rat mammary gland carcinoma showed that even in the presence of very low LHCGR Hecate-CGβ conjugate can also be effective (Zaleska *et al.*, 2004). The treatment effect in this case could have additionally been mediated through a systemic effect, as Hecate-CGβ conjugate treatment significantly decreased prolactin (PRL) and growth hormone (GH) levels in comparison to Hecate treated groups (personal communication with N. Rahman and A. Ziecik).

Hecate-CGβ conjugate has been shown to induce a rapid and cell-specific membrane permeabilization of LHCGR expressing cells *in vitro* (Bodek *et al.*, 2005). It induces plasma membrane disruption within a short period of time (Bodek *et al.*, 2005, Bogacki *et al.*, 2008). It is metabolized fast, as its serum levels begins to decrease already after 60 minutes and the lytic peptide is unmeasurable four hours after the injection (Bogacki *et al.*, 2008).

In addition to Hecate-CGβ conjugate, other membrane disrupting lytic peptides combined to 15 AA fragment of human CGβ have also been constructed and studied. Another synthetic lytic peptide, Phor(14)-βCG, which contains two 7 amino acid (KFAKFAK) segments, proved to be less effective in tumor burden reduction in comparison to Hecate-CGβ conjugate in prostate cancer xenografts (Hansel *et al.*, 2001). Phor(14)βCG(ala)

as well as Phor(21)βCG(ala), where three cysteines of CGβ have been substituted with alanine, seems to be highly effective in endocrine tumor treatment (Leuschner & Hansel, 2004, Leuschner & Hansel, 2005). These latter two lytic peptides have also been studied in metastatic cancers with promising results (Leuschner & Hansel, 2005, Hansel *et al.*, 2007a). Thus conjugated peptides could be useful especially in post-operative treatment before metastatic tumors tend to appear. LHRH-Hecate, where Hecate is combined with a fragment of gonadotropin/luteinizing hormone releasing hormone, is effective in prostate and estrogen-dependent mammary cancer treatment in the cells that possess gonadotropin/luteinizing hormone releasing hormone (GnRH/LHRH) receptors (Leuschner *et al.*, 2003a, Leuschner *et al.*, 2003b). Hecate has also been conjugated with magnetic nanoparticles and this has been shown to be therapeutically active in breast cancer cell lines (Kumar *et al.*, 2004).

Hecate-CGβ conjugate provides a very effective model for studying the treatment possibilities in LHCGR-possessing tumors as it is similar to other membrane disrupting peptides with the ability to be rapidly metabolized (Bogacki *et al.*, 2008). The non-immunogenicity is explained by the relatively short size of the peptide (less than 40 amino acids). Several studies have shown Hecate-CGβ conjugate to have no side effects on any other non-gonadal tissues (Hansel *et al.*, 2001, Leuschner *et al.*, 2001, Leuschner *et al.*, 2003b, Zaleska *et al.*, 2004). It is highly likely that phosphatidylserine distribution in the outer leaflet of tumor cells attracts the Hecate-CGβ conjugate to attack the negatively charged tumor cells alone, sparing the healthy positively charged ones (Papo & Shai, 2003). Because of the negative membrane charge and LHCGR-spesific action of Hecate-CGβ conjugate, the treatment-induced side effects were minimal or none.

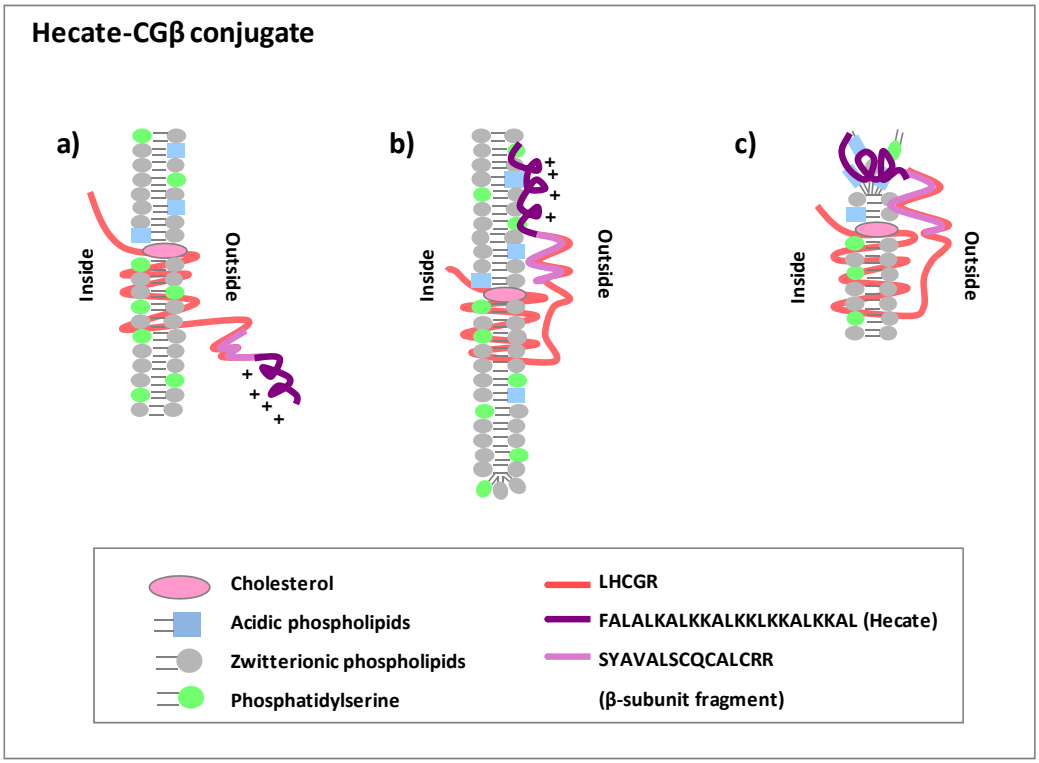


Figure 9. The structure of Hecate-CGβ conjugate and its mode of action by the cell membrane. Hecate-CGβ conjugate binds to LHCGR and becomes more stable (a). It exposes the α-helix of the Hecate molecule in close proximity to the cell membrane (b) and disrupts the membrane of high membrane potential (c). Modified from Rivero-Muller *et al.* (2007).

2.9. Gonadotropin suppression

2.9.1. Gonadotropin-releasing hormone

Gonadotropin-releasing hormone (GnRH), also called gonadorelin, is a decapeptide secreted from the hypothalamus. It acts through GPCR-family gonadotropin-releasing hormone receptors (GnRHR) in the pituitary gland and stimulates the synthesis and secretion of LH and FSH. The signaling pathway of GnRHR, similar to LHCGR, is through PKC and cAMP with a consequent Ca^{2+} release (Stojilkovic *et al.*, 1994). The pulsatile secretion of GnRH is the requirement for normal action of gonadotropin release. When GnRH secretion is sustained, gonadotropin release becomes arrested due to receptor desensitization (Belchetz *et al.*, 1978). According to some reports, this desensitization mechanism is not yet fully understood (Conn & Crowley, 1994). Interestingly, GnRH receptors are found in many cancers (mammary gland, ovary, endometrium, prostate) and also in adrenocortical adenomas (Ye *et al.*, 2007, Zwermann *et al.*, 2009). GnRHRs are also present in multiple and diverse extra-pituitary tissues like placenta, gonads,

pancreas, liver, kidney, heart, and they are known to have many roles in reproduction functions (Hapgood *et al.*, 2005). A recent study has also shown vast expression of GnRHR in the fetal adrenal gland, suggesting the role of GnRH in the function of this gland (Xing *et al.*, 2009).

2.9.2. GnRH agonists

GnRH agonists mimick the action of GnRH. They take advantage of the phenomenon of receptor desensitization, when constantly high doses of the agonists are used for a longer period to reduce gonadotropin secretion. However, GnRH agonists first cause an initial stimulation of gonadotropins, causing hypersecretion or 'flare' but later, upon prolonged use, block gonadotropin secretion. GnRH agonists have long been in use both in infertility treatment (Neveu *et al.*, 1987) and in cancer treatment, especially in prostate cancer (Borgmann *et al.*, 1982). GnRH agonist leuprolide acetate has also been used for adrenocortical diseases, such as for gonadotropin dependent, LHCGR bearing adrenal adenoma/Cushing's syndrome (Lacroix *et al.*, 1999).

2.9.3. GnRH antagonists

GnRH antagonist treatment in men causes a very fast and strong decrease of circulating testosterone, similar to what can be achieved by surgical castration. GnRH antagonists contain changes in the amino acid sequence in comparison to natural GnRH (Huirne & Lambalk, 2001, Huhtaniemi *et al.*, 2009). The action of binding occurs by competitive receptor occupancy without the receptor activation (Huirne & Lambalk, 2001, Huhtaniemi *et al.*, 2009). Earlier, the older generation of GnRH antagonists were known to cause allergic reactions through histamine release, which made them less popular in use in comparison to GnRH agonists. With the new generation of GnRH antagonists this allergy problem is faced very rarely.

In clinics, GnRH antagonists are generally and successfully used in *in vitro* fertilization 'short-protocols', when a fast blockage of endogenous gonadotropins is required to prevent too early ovulation during ovarian stimulation, before oocyte collection (Detti *et al.*, 2008, Lainas *et al.*, 2008). Cetrorelix was the first antagonist to get approval for in vitro fertilization (IVF) -treatments (Reissmann *et al.*, 2000). The idea of using GnRH antagonist in comparison to GnRH agonist in infertility treatment was to shorten the period of use with less given hormones, but it seems that the pregnancy rates remain lower after GnRH antagonist than after GnRH agonist treated cycles. There are many reviews to compare these two hormonal treatments showing equivocal results (Al-Inany *et al.*, 2007, Huirne *et al.*, 2007, Li *et al.*, 2008). GnRH antagonists are also used for the treatment of benign prostate hyperplasia (Lepor, 2006), leiomyomas (Gonzalez-Barcena *et al.*, 1997) and endometriosis (Kupker *et al.*, 2002). The effectiveness of GnRH-antagonist in cancer treatment, namely in prostate cancer and mammary gland tumors

was first established in animal models in the 1980s (Redding *et al.*, 1982, Redding & Schally, 1983, Schally *et al.*, 1983), but cetrorelix came in use 10 years later (Srkalovic *et al.*, 1990, Szende *et al.*, 1990, Korkut *et al.*, 1991). At present, there are many on-going clinical trials with several GnRH antagonists (abarelix, teverelix, cetrorelix, ganirelix, iturelix, acyline, degarelix, ornirelix) in order to get further approval for their use in the treatment of prostate cancer. Abarelix was the first to be clinically tried. Due to the apparent allergic reactions it did not get an approval in the USA but is currently in use in Germany (Huhtaniemi *et al.*, 2009). Degarelix has recently been clinically tested in the treatment of human prostate cancer up to phase III studies (Gittelman *et al.*, 2008, Klotz *et al.*, 2008, Huhtaniemi *et al.*, 2009) and cetrorelix has gone through phase I studies in patients with advanced prostate cancer (Gonzalez-Barcena *et al.*, 1994, Gonzalez-Barcena *et al.*, 1995). A single injection of cetrorelix can reduce testosterone levels to subnormal values from a few hours to couple of days and it also reduces prostate specific antigen (PSA) values to close to normal. Furthermore, no flare symptoms have been reported after cetrorelix. Several studies to further characterize the actions of GnRH antagonists have been carried out on prostate cancer xenografts (Jungwirth *et al.*, 1997a, Jungwirth *et al.*, 1997b, Stangelberger *et al.*, 2007). The tumor inhibion by GnRH antagonist was suggested to take place either by decreasing the production of IGF-II mRNA (Lamharzi *et al.*, 1998b), by decreasing the tumor LHRH levels (Lamharzi *et al.*, 1998a) or through apoptosis (Jungwirth *et al.*, 1997b, Pareek *et al.*, 2007). There are also reports on GnRH antagonists' effects on endometrial (Kleinman *et al.*, 1993) and mammary gland tumors (Hershkovitz *et al.*, 1993), where their actions are believed to be mediated by IGF-II decline and by estrogen deprivation. Ovarian epithelial cancer xenografts have been successfully treated by a GnRH antagonist (Yano *et al.*, 1994). Human trials with GnRH antagonist for gynecological and breast cancer patients seem promising in patients who no longer respond to other treatments (Emons *et al.*, 2003).

3. AIMS OF THE PRESENT STUDY

Endocrine tumors such as ovarian granulosa, testicular Leydig cell and many adrenocortical tumors have one thing in common: they all abundantly express LHCGR. In the present study, Hecate-CGβ conjugate, a lytic peptide constructed in order to kill tumor cells possessing LHCGR, was used in a transgenic Inhα/Tag TG mouse model. The general aim was to establish the principle that lytic peptides can be successfully used in the treatment of endocrine tumors targeting their action at their ectopic hormone receptors.

The specific aims of the study were:

1. to investigate the antitumoral efficacy of fusion protein Hecate-CGβ conjugate for gonadal somatic cell and adrenocortical tumor cells with ectopic LHCGR in Inhα/Tag TG mice

2. to study the molecular mechanism underlying the mode of cell death caused by Hecate-CGβ conjugate

3. to study the effects of gonadotropin blockage on tumor treatment in parallel to Hecate-CGβ conjugate, as well as the gonadotropin dependency of adrenocortical tumor progression

4. to find tumor specific molecular markers to monitor treatment efficacy for adrenocortical tumorigenesis

4. MATERIALS AND METHODS

4.1. Animal model used (I, II, III)

For the mice experiments, Inhα/Tag TG mice were used. The PCR-positive mice were selected according to earlier protocols (Kananen *et al.*, 1995) using DNA samples either from tail (I) or ear biopsies (II-III). For gonadal experiments these TG mice were chosen at the age of 5.5 months (seven to ten per group). The tumor size was verified by making longitudinal laparotomies under Avertin anesthesia (Hogan *et al.*, 1994) and measuring the gonadal volume. In order to study the adrenocortical tumors, the mice were gonadectomized before puberty (six to ten per group). Operations were carried out under Avertin anesthesia and postoperative analgesia (buprenorphine) was administered. According to extensive previous characterization (Kananen *et al.*, 1996b, Rilianawati *et al.*, 1998, Kero *et al.*, 2000, Rilianawati *et al.*, 2000, Rahman *et al.*, 2004) tumors are known to develop by the age of 6 mo. Treatment to the adrenal tumor bearing mice was started at the age of 6.5 months in order to ensure that the tumors had appeared and thus the treatment would have the anti-tumoral and not tumor-preventing effect. Wild type control littermate mice (C57Bl/6N) were used as controls, and for the adrenal studies these mice were also gonadectomized. The mice were weaned at the age of 21 days and placed two to four per cage, females and males separately, in a room of controlled light (12 h light, 12 h darkness) and temperature (21 ± 1 ˚C). They were fed with mouse chow SDS RM-3 (Whitham, Essex, UK) and tap water *ad libitum*. The mice were kept in specific pathogen-free surroundings and were routinely screened for common mouse pathogens. The Ethics Committees for Animal Experimentation of the University of Turku and the State Provincial Office of Southern Finland approved all the animal experiments.

4.2. Cell lines and cultures

There were two murine LHCGR-positive Leydig tumor cell lines, mLTC-1 (Rebois, 1982) and BLT-1 (Kananen *et al.*, 1996a) and two other LHCGR-positive cell lines, murine granulosa KK-1 (Kananen *et al.*, 1995) and prostate cancer PC-3 (Kaighn *et al.*, 1979) in use. As a control, LHCGR-negative colon carcinoma cell line HT-29 (Thomas *et al.*, 1974) was used. The mLTC-1 cell line was cultured in Waymouth medium (Sigma Chemical Co., St. Louis, MO) with supplementation of 9% heat-inactivated horse serum (Life Technologies, Paisley, Scotland, UK) and 4.5% heat-inactivated fetal calf serum (iFCS, Bioclear, Wokingham, Berks, UK) with 0.1 g/L gentamicin (Gibco BRL, Gaithersburg, MD, USA). Other cell lines were maintained in DMEM/Ham's F-12 1:1 medium (Sigma), supplemented with 10% iFCS, 50 IU/I penicillin and 0.5 g/L streptomycin (Sigma). The cells were allowed to grow on Petri dishes with a 9.6 cm diameter (Greiner Labortechnik,

Frickenhausen, Germany) or on 6- or 24-well plates (Greiner) to 70-80% confluency under a humified atmosphere of 95% air and 5% CO2 at 37 ˚C.

4.3. Hecate and Hecate-CGβ conjugate treatments (I, II, III)

For gonadal tumors, treatment was started at the age of 5.5 mo and before treatment longitudinal laparotomy was performed as described above. For adrenal tumors, treatment was commenced at the age of 6.5 mo. Animals were treated with Hecate only (12 mg/kg), Hecate-CGβ conjugate (12 mg/kg) or saline by intraperitoneal injections once per week for three consecutive weeks according to the earlier established protocols (Leuschner *et al.*, 2001, Gawronska *et al.*, 2002, Leuschner *et al.*, 2003b). As no significant weight changes could be observed between Hecate and Hecate-CGβ conjugate by adding hCG to Hecate, both Hecate and Hecate-CGβ conjugate were given in the same dose (Leuschner *et al.*, 2001, Gawronska *et al.*, 2002). As Hecate only was shown to have an effect similar to control treatment in publications I and II, only Hecate-CGβ conjugate was used in publication III. One week after the last treatment the mice were sacrificed by cervical dislocation, preceded by blood collection by cardiac puncture performed under Avertin anesthesia. Body, tumor and different organ weights were recorded. Tissues were either fixed for histological analysis or snap-frozen in liquid nitrogen for later analyses. Hecate and Hecate-CGβ conjugate were synthesized and purified in the Peptide and Protein Laboratory, Department of Virology, Haartman Institute, University of Helsinki (Bodek *et al.*, 2003).

4.4. Hormonal treatments (III)

In order to prove the hypothesis that blocking the circulating LH would increase the possibilities for Hecate-CGβ conjugate to bind to LH receptors, the mice were also treated with a GnRH antagonist, cetrorelix acetate (10mg/kg) (Merck Serono, Geneva, Switzerland), subcutaneously every 84 hours starting at the age of 6.5 months. The injections were given either alone or in combination with Hecate-CGβ conjugate.

Since estradiol was already known to improve the treatment efficacy from the first lytic peptide conjugate studies (Leuschner *et al.*, 2001), the 8 mm silastic implant tubes, filled with estradiol powder (Sigma) and sealed at both ends with silastic adhesive (Elastosil RTV-1 Silicone Rubber, Wacker-Chemie, GmbH, Munich, Germany) were implanted under the back skin of female mice (Pakarainen *et al.*, 2005b) at the age of 6.5 months. The mice were either given the estradiol implant only or treated simultaneously with Hecate-CGβ conjugate according to the protocol.

Both hormonal treatments were given for a four week period after which the mice were sacrificed and the tissues were collected as mentioned above. The efficacy of estradiol treatment was monitored by the uterine weight of treated female mice.

4.5. Histological and immunohistochemical analyses (I, II, III)

For histological analysis, tissues were fixed either in Bouin's solution or in 4% paraformaldehyde and embedded in paraffin. The paraffin sections were cut in 5 µm thickness. For histological analysis the sections were stained with hematoxylin-eosin and for immunohistochemical analysis the sections were deparaffinized and rehydrated. After peroxide blocking (3% H_2O_2) and antigen retrieval (15 mins microwave in 10 mM citric acid, pH 6.0) the sections were incubated at +4˚ C overnight with the following primary antibodies:

1. rabbit polyclonal anti-LHCGR antibody (directed against human peptide sequence, KKLPSRETFVNLLEA, dilution 1:1000, a gift of Dr Asgi Fazleabbas, Department of Obstetrics and Gynecology, University of Illinois, Chicago, IL, USA)
2. rabbit polyclonal anti-GATA-4 (dilution 1:800, Santa Cruz Biotechnology, Santa Cruz, CA, USA)
3. goat polyclonal anti-SF-1 (dilution 1:400, Santa Cruz Biotechnology)
4. goat polyclonal anti-GATA-6 (dilution 1:250, Santa Cruz Biotechnology)
5. goat polyclonal anti-p53 (dilution 1:100, Santa Cruz Biotechnology)
6. rabbit monoclonal anti-Ki-67 (SP3) (dilution 1:2000, NeoMarkers, Fremont, CA, USA)

After the application of primary antibody, the slides were incubated with anti-rabbit (dilution 1:400, Vector Laboratories, Inc., Burlingame, CA, USA) or anti-goat (dilution 1:400, Santa Cruz Biotechnology) secondary antibodies. The avidin-biotin immunoperoxidase system was used to visualize bound antigen-antibody complexes (Vectastain Elite ABC Kit, Vector Laboratories) with 3.3' – diaminobenzidine (Sigma) as a substrate following the manufacturer's instructions. The washes between the incubations were done with 0.05M Tris and 150 mM NaCl (TBS) with 0.1% Tween (TBS-T). The Novolink™ Polymer Detection System Kit (Novocastra™, Benton Lane, UK) was used with anti-GATA-4 antibody, EnVision+ System-HRP labelled Polymer (DakoCytomation Inc., Carpinteria, CA, USA) was used with anti-Ki-67 antibody and PowerVision+™ Poly-HRP IHC Kit for goat (ImmunoVision Technologies, Hague, the Netherlands) for the detection of p53. For more detailed protocols, please see the original publications.

4.6. Morphometric analyses (III)

To quantify the histological differences between the study groups, serial sections (n = 6/ group) of HE-stained slides from each treatment group were analyzed morphometrically by a point counting technique as described earlier (Haapasalo *et al.*, 1990, Howard &

Reed, 1998). The volume fraction estimation (in %) was done by counting the number of crossing points of an orthogonal grid placed on the top of the section. Specific types of tissue of interest (i.e. tumor, healthy, fibrotic/necrotic, cyst-type formation) were counted separately and also the crossing points of the whole section were counted. The number of the points of each tissue type of interest was then divided by the total number of points.

4.7. LH, FSH, progesterone and corticosterone assays (I, II, III)

Serum samples were used for the analyses of LH, FSH, progesterone and corticosterone. LH and FSH were measured by immunofluorometric assays for rat (Delfia; Wallac, Turku, Finland) as described previously (Haavisto *et al.*, 1993, van Casteren *et al.*, 2000). Corticosterone was measured from diethyl extracts of the sera by RIA using a kit for rats and mice (MP Biomedicals, Orangeburg, NY, USA). Progesterone was either measured by RIA (I) as described previously (Vuorento *et al.*, 1989) or by the Delfia Progesterone Kit (II-III) (Wallac). The approximate assay sensitivity for LH was 0.0075 µg/L (0.75 pg/tube), for FSH 0.1 µg/L, for progesterone 0.5 nmol/L (50 fmol/tube) and for corticosterone 30 fmol/tube, respectively. The intra- and interassay coefficients of variation for these assays were below 10-15%.

4.8. Laser capture microdissection (II)

Laser pressure catapulting (LPC) / laser capture microdissection (LCM) were performed using a protocol described previously (Westphal *et al.*, 2002). For microdissection, cryosections of 5 µm were prepared from three independent specimens on special slides (PALM PEN MembraneSlides, PALM Microlaser Technologies, GmbH, Bernried, Germany) and stored in 70% EtOH until staining. The slides were first dehydrated according to the protocol and then stained with hematoxylin. The slides were stored under laminar flow for 2-10 minutes and kept in dry ice. LCM was performed by using a Zeiss PALM laser capture microdissection instrument (PALM Microlaser Technologies). The areas of 100 µm in diameter from the wanted area (tumor/non-tumor areas in adrenal cortex) were catapulted into the special eppendorf tubes (PALM AdhesiveCaps, PALM Microlaser Technologies) and immediately placed in dry ice. The RNA was extracted immediately using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

4.9. Fluorescence microscopy (I)

To prove the preferential destruction of LHCGR-expressing cells and to determine the mechanisms involved in cell death caused by the Hecate-CGβ conjugate, two different appearing cells lines, LHCGR positive mLTC-1 or KK-1 cells and LHCGR negative

HT-29 cells were co-cultured on the same glass well slides. After 24 hour incubation in complete Waymouth medium and a phosphate buffered saline (PBS) wash, the cells were incubated with 0.5 μM Hecate or Hecate-CGβ conjugate for 15 minutes, 0.5, 1 and 5 μM Hecate for 30 minutes or 0.5 μM Hecate-CGβ conjugate for 30 minutes. The medium was then replaced with fresh medium containing propidium iodide (PI) and the lysed and intact cells were differentiated by fluorescence microscopy, where the lysed cells reflected the fluorescence light.

4.10. FACS analysis (I)

In order to count the percentage of apoptotic cells, mLTC-1 and BLT-1 as well as the prostate cancer cell line PC-3 were used. After incubation at 37 ˚C in 5% CO_2 for 24 hours, followed by one PBS wash, the cells were incubated for four hours at increasing concentrations (0.1, 0.5, 1 μM) of Hecate or Hecate-CGβ conjugate. As an apoptotic positive control, 0.1% of hydrogen peroxide was added to the culture media. Both adherent and floating cells were collected and stained with PI and cellular DNA content was analyzed by FACSCalibur multicolor flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) as previously described (Fried *et al.*, 1978, Nicoletti *et al.*, 1991). The apoptotic cells were detected by percentages of cells stained with PI, indicating the nuclear fragmentation.

4.11. MTT analysis (I)

Cells (BLT-1, mLTC-1) were seeded into six-well plates for twenty-four hour incubation with phenol-red/DMEM medium. The incubation was continued with new medium containing 20 μM pan-caspase inhibitor Z-VAD.fmk (Calciobiochem, Nottingham, UK) for 1 hour. Hecate and Hecate-CGβ conjugate were then added at concentrations of 0.1, 0.5 and 1 μM and incubated either overnight (Hecate) or for 16 hours (Hecate-CGβ conjugate). A colorimetric MTT assay was then performed to measure cell survival (Mosmann, 1983). Briefly, MTT (3-(4,5-dimethylthiatzol-2-yl)-2-5-diphenyl tetrazolium bromide) was added to wells of an assay and the plates were incubated at 37 ˚C for 4 hours. Acid-isopropanol was added and after some minutes the plates were read on the ELISA reader. 0.1% H_2O_2 was used as the positive control for apoptosis. Viability in the treated cells was expressed as a percentage of controls, the untreated controls were assigned a value of 100%. The color in this experiment was produced only by the living cells.

4.12. RT-PCR and quantitative RT-PCR analysis (II, III)

Snap-frozen adrenal samples were used for RNA analyses. For both analyses, RNA was extracted with the RNeasy Mini Kit (Qiagen) according to the manufacturer's

protocol. For reverse-transcription polymerase chain reaction (RT-PCR), one µg of DNase-treated RNA was reverse transcribed using avian myeloma virus reverse transcriptase (Promega Corp., Madison, WI, USA) and amplified using thermostable DNA polymerase (Dynazyme, Finnzymes, Espoo, Finland) in the same reaction tube in a thermal cycler.

For real-time quantitative (q) RT-PCR the extracted RNA was treated with amplification grade DNaseI (Invitrogen, Carlsbad, CA, USA). For cDNA synthesis and subsequent qRT-PCR, the SYBR Green DyNAmo HS qRT-PCR kit (Finnzymes, Espoo, Finland) was used with 1:50 diluted aliquots. qRT-PCR analysis was performed using a DNA Engine Thermal Cycler (BioRad, Hercules, CA, USA) with continuous fluorescence detection. The endogenous control gene *L19* (L19 ribosomal protein), was run in parallel for each cDNA template. Amplification products were separated on 1% agarose gel to stain with ethidium bromide. The primer pairs used for RT-PCR reactions are shown in Table 7. The primer pairs for qRT-PCR are shown in Table 8.

Table 7. Oligonucleotides used in RT-PCR analysis.

Gene	*Primers*	*Sequence*
L19	Forward	5'-GAAATCGCCAATGCCAACTC-3'
	Reverse	5'-TCTTAGACCTGCGAGCCTCA -3'
GATA-4	Forward	5'-AAACGGAAGCCCAAGAACCTGAAT-3'
	Reverse	5'-GGCCCCCACGTCCCAAGTC-3'
LHCGR	Forward	5'-CTCTCACCTATCTCCCTGTC-3'
	Reverse	5'-TCTTTCTTCGGCAAATTCCTG-3'

Table 8. Oligonucleotides used in qRT-PCR analysis.

Gene	*Primers*	*Sequence*	*T (annealing) (°C)*
L19	Forward	5'-GGACAGAGTCTTGATGATCTC-3'	56
	Reverse	5'-CTGAAGGTCAAAGGGAATGTG-3'	
P450c17	Forward	5'-GGCCCCAGATGGTGACTCT-3'	54
	Reverse	5'-GGACTCCCCGTCGTATGTAA-3'	
ERα	Forward	5'-CCGTGTGCAATGACTATGCC-3'	56
	Reverse	5'-GTGCTTCAACATTCTCCCTCCTC-3'	
GATA-4	Forward	5'-TCTCACTATGGGCACAGCAG-3'	61
	Reverse	5'-CGAGCAGGAATTTGAAGAGG-3'	
GATA-6	Forward	5'-GAGCTGGTGCTACCAAGAGG-3'	61
	Reverse	5'-TGCAAAAGCCCATCTCTTCT-3'	
LHCGR	Forward	5'-CAATGGGACGACGCTAATCT-3'	56
	Reverse	5'-CTGGAGGGCAGAGTTTTCAG-3'	

4.13. Western, Northern and Southern hybridization analysis (I, II)

Southern hybridization was used to confirm the RT-PCR results in publication II. The oligonucleotide corresponding to LHCGR cDNA was

5'-TGGAGAAGATGCACAGTGGA-3' and corresponding to GATA-4 it was 5´-AGTGGCACGTAGACGGGCGAGGAC- 3´. The membranes were washed according to the manufacturer's instructions and exposed to Kodak X-ray film (XAR 5; Eastman Kodak, Rochester, NY, USA).

Western blot hybridizations were done to monitor caspase-3 activation in mLTC-1 cells or GATA-4 and LHCGR expression in adrenal tissue samples after Hecate or Hecate-CGβ conjugate treatment. Cells were incubated for 60 minutes at 0.5, 1 and 5μM Hecate or Hecate-CGβ conjugate. Total cell lysates were prepared as described previously (Yang & Sladek, 1995). The tissue samples were homogenized and the protein concentration was measured using the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Electrophoresis was carried out through a 12.5% SDS-PAGE gel and transfered to a Hybond-P PVDF nitrocellulose membrane (Amersham Biosciences/GE Healthcare, Buckinghamshire, UK). After blocking the membranes incubation with the following primary antibodies was performed at +4 ˚C overnight:

1. rabbit monoclonal antibody anti-caspase-3 (dilution 1:500, Cell Signaling, Beverly, MA, USA)

2. goat polyclonal antibody anti-GATA-4 (dilution 1:100, Santa Cruz Biotechnology, C-20, sc-1237)

3. rabbit polyclonal anti-LHCGR (dilution 1:4000, Acris Antibodies, Hiddenhausen, Germany)

As secondary antibodies, bovine anti-goat IgG-HRP (dilution 1:10000, Santa Cruz Biotechnology) or ECL anti-rabbit IgG, HRP-linked whole AB (dilution 1:10000, Amersham Biosciences) were used. Signals were visualized using ECL Plus Western blotting detection reagents (Amersham Pharmacia Biotech/GE Healthcare, Buckinghamshire, UK) and finally exposed to Fuji X-ray film (Super RX, Fuji Photo Film Ltd., Bedford, UK).

For Northern blot analysis total RNA was isolated from the whole testis, ovary or adrenal of the wild type and TG mice using the single-step method as described previously (Chomczynski & Sacchi, 1987). Twenty μg of RNA per lane was resolved on 1.2% denaturing agarose gel and transferred onto Hybond-XL nylon membranes (Amersham International). For LHCGR, membranes were prehybridized overnight at 65 ˚C. A complementary RNA probe for the rat LHCGR subcloned into the pGEM-4Z plasmid was used for hybridization (LaPolt *et al.*, 1990). The [32P]dUTP-labeled (800Ci/mmol; Amersham International) probe was generated using a Riboprobe system II kit (Promega, Madison, WI, USA). For GATA-4, the prehybridization was done at 42 ˚C. The cRNA probes were cut out from pMT2-GATA4 (Arceci *et al.*, 1993) using EcoRI/PstI and SmaI restriction enzymes respectively, and labeled with a Prime-a-Gene kit (Pharmacia,

Stockholm, Sweden) using [α-P^{32}]-dCTP. The probes were purified with NickColumns (Pharmacia) and added to the prehybridization solution for 20 hours. After washes the membranes were exposed to Kodak X-ray films (Kodak XAR-5; Eastman Kodak) at -70 ˚C for 4 to 7 days or to a phosphor imager (Fujifilm BAS-5000; Fujifilm IaI, Tokyo, Japan) for 4 to 24 hours. The intensities of specific bands for both Western and Northern blotting analysis were quantified using the Tina Software (Raytest, Staubenhardt, Germany). The molecular sizes in Northern blots were estimated by comparison with mobilities of the 18S and 28S ribosomal RNA.

4.14. Statistical analysis (I, II, III)

Statistical ANOVA paired *t* tests or ANOVA with Bonferroni tests or non-parametric Mann-Whitney Rank test were carried out using the StatView program for Windows (version 5.0.1) or the SAS Enterprise Guide 3.0 program (SAS Institute Inc., Cary, NC, USA). Logarithmic changes were used to normalize the distribution, and in the case of unequal distribution non-parametric tests were carried out. All data are presented as mean ± SEM.

5. RESULTS

5.1. Mode of antitumor effect with Hecate-CGβ conjugate (I)

The molecular mechanisms underlying the mode of antitumoral effect by the lytic peptide was studied by caspase-3 to activate the pathways or to block the caspase pathways with a pan-caspase inhibitor. PI staining for the cell cultures was also carried out. It was shown by light and fluorescence microscopy, Western blot analysis, flow cytometry and MTT-studies that the mode of antitumor effect of Hecate-CGβ conjugate is by necrosis. A 30 minute incubation with 0.5 μM of Hecate-CGβ conjugate caused a swelling of LHCGR-bearing mLTC-1 cells, which is strong evidence towards necrosis (Papo *et al.*, 2003). In flow cytometry, the apoptotic controls showed nuclear fragmentation by an average of 68%, whereas after Hecate-CGβ conjugate the apoptotic rate was only around 4%. In Western blot analysis of mLTC-1 cells no activated form of caspase-3 could be seen after 90 minutes incubation with 0.5 μM Hecate-CGβ conjugate, suggesting that cell death did not occur with the proteolysis by procaspase. In order to check whether any other caspases in addition to caspase-3 activation of the apoptotic pathways could have taken place by the Hecate-CGβ conjugate mode of killing, the pan-caspase inhibitor Z-VAD.fmk was added to the media. Results seen by MTT analysis showed that cell viability was the same with the inhibitor or without it in Hecate or Hecate-CGβ conjugate treatments. However, in H_20_2-treated cells as apoptotic control, the apoptosis was blocked significantly and 50% more living cells could be seen. All these results confirmed that the mode of cell death caused by Hecate or Hecate-CGβ conjugate was not apoptosis, but necrosis. This was also confirmed by the light microscopy findings.

5.2. The treatment efficacy of Hecate-CGβ conjugate (I, II, III)

5.2.1. Tumor volume reduction

Gonadal or adrenal tumors of both sexes in Inhα/Tag TG mice were treated with either Hecate or Hecate-CGβ conjugate for one month. With Hecate-CGβ conjugate treatment testicular tumor size (measured by weight and volume) diminished by 58% ($p<0.05$). The ovarian tumor size diminished by 36% but did not reach the level of significance compared to the volume before treatment. The adrenal tumor size (measured by weight) diminished by an average of 65% (59% in II and 72% in III) ($p<0.01$) in adrenal tumors in males, whereas in female adrenal tumors the average reduction of tumor volume was only 22% (18%-25%) in comparison to Hecate or control treatment.

As the age related tumor progression rate is known to vary among individuals in Inhα/Tag TG and Tag TG mice (Hanahan, 1989, Kananen *et al.*, 1995, Kananen *et al.*, 1996a, Rahman *et al.*, 1998), the tumor burden (tumor weight or volume/body weight) was also measured. The gonadal tumor burden in both sexes decreased significantly. However, in adrenal tumors, tumor burden values reached significance only in male mice. The total body weights did not differ significantly in any of the treatment groups, indicating that the drug was not generally toxic. Hecate-CGβ conjugate or Hecate did not show any significant effects on the gonadal volumes of WT mice or on the adrenal volumes of gonadectomized WT mice.

The treatment efficacy of Hecate-CGβ conjugate proved to be better in males both in gonadal and adrenal tumors. In gonadal tumors this difference might be explained by the slightly higher circulating LH in females, which could have competed in the binding to LHCGR with Hecate-CGβ conjugate (Bodek *et al.*, 2003). In gonadectomized mice with adrenal tumors, however, no sex differences in the gonadotropin levels could be detected but the mRNA expression of LHCGR was found to be higher in male adrenal tumors than those in the female.

5.2.2. Histopathology

Histopathologic examination of gonads after Hecate or control treatment showed multiple mitoses and cellular atypia. The histology of adrenals after control or Hecate treatment in males and after Hecate-CGβ conjugate treatment in females showed loose endothelium and lack of stromal tissue with blood-filled cyst-like formations. Cell layers were unorganized and most of the tumor cells seemed to be of the zona fasciculata type.

However, Hecate-CGβ conjugate treatment caused a degradation of tumor mass both in the gonads and in male adrenals. The antitumoral effect was directly shown by necrotic cells that were present especially in testicular sections. In male adrenal tumors the residual tumor tissue/hyperplasia could only be observed in a reduced area of zona glomerulosa and the sinusoidal structure of zona fasciculata was preserved. In general, Hecate-CGβ conjugate treatment seemed to cure the tissue, sparing the normal adrenal structure in the males. Morphometrical analysis showed that TG female tumors treated with Hecate-CGβ conjugate did not respond to the treatment and the structure did not differ from the control-treated group; over 90% of the tissue was either tumorous or blood filled (Fig. 10). However, in males Hecate-CGβ conjugate was proven to be effective. There was only 10% tumorous tissue left after the Hecate-CGβ conjugate treatment, the remainder of the tissue seemed to be healthy (Fig. 10).

Other non-gonadal tissues (lung, liver, pancreas, spleen, uterus) examined after the treatment appeared unaffected. However, occasionally metastases could be found in the lungs in the gonadal tumor bearing mice and liver metastases in adrenal tumor bearing

mice were detected. Overall, all metastases were very rare and found in Hecate only treated groups. No metastases were ever found after Hecate-CGβ conjugate treatment.

5.2.3. Endocrine consequences

Both gonadal and adrenal tumors in Inhα/Tag TG mice are known to secrete progesterone (Kananen *et al.*, 1995, Kananen *et al.*, 1996a, Kananen *et al.*, 1996b) and as a consequence, the levels of gonadotropins are known to decline along with tumorigenesis. Hecate-CGβ conjugate treatment in gonadal tumors in both sexes decreased the progesterone levels and increased LH levels, thus showing the positive treatment effects of the lytic peptide.

In adrenal tumors there was a clear elevation in basal LH levels induced by the gonadectomy and in the WT mice the elevation of LH was more obvious, partly due to the lack of negative feedback from the gonads and partly because of the lower progesterone values than in TG mice. In males, similar as in gonadal tumors, the progesterone values were decreased after the Hecate-CGβ conjugate treatment. In female adrenal tumors no clear differences in progesterone could be seen after the treatments. These endocrine hormone data correlated with the treatment efficacy results and tumor volume reduction data. No changes in corticosteroid levels could be seen after the treatments in comparison to the wild type mice.

5.3. LHCGR specific action of Hecate-CGβ conjugate (I, II, III)

In order to discover the LHCGR specific action of Hecate-CGβ conjugate, *in vitro* experiments were carried out and protein and mRNA levels were measured from the treated cells. First, in a co-culture of LHCGR-positive (Leydig and granulosa cell lines) and LHCGR-negative (colon carcinoma cell line) cells, Hecate-CGβ conjugate killed only LHCGR-bearing cells and spared the LHCGR-negative cells. Northern blot analysis of the treated gonadal tissues showed a significant decline of LHCGR mRNA levels after Hecate-CGβ conjugate treatment, in comparison to Hecate or control treatment. In the adrenals, a similar phenomenon was observed by Western blot, where the protein expression of LHCGR was significantly downregulated after the Hecate-CGβ conjugate treatment but not in control or Hecate treated groups. LHCGR could not be detected in the Hecate-CGβ conjugate treated adrenal sections by immunohistochemistry. The qRT-PCR results confirmed the protein expression findings. No LHCGR mRNA expression could by detected in adrenals after Hecate-CGβ conjugate in comparison to Hecate or control treatments. These results confirmed that Hecate-CGβ conjugate acts on endocrine tumors by destroying the LHCGR bearing cells only.

5.4. Co-localization of LHCGR and GATA-4 in the tumors (II, III)

GATA-4 is known to be expressed in adrenal tumors (Kiiveri *et al.*, 1999) and the co-initiation of GATA-4 and LHCGR expression in adrenal tumors has also been earlier demonstrated (Rahman *et al.*, 2004). Thus the idea was to see whether GATA-4 would also be diminished along with LHCGR after Hecate-CGβ conjugate treatment, to further establish the use of these two markers for monitoring the treatment outcome. The expression of LHCGR and GATA-4 in adrenals was studied by immunohistochemistry, Western blot, RT-PCR/Southern blot and qRT-PCR. The data from LPC/LCM-extracted tumorous and non-tumorous adrenal tissues from the same slides, further evaluated by RT-PCR, showed that both GATA-4 and LHCGR expression can be found in adrenal tumor tissue but not in WT or non-tumorous adrenal tissue sections. This verified the co-localization of LHCGR and GATA-4 in the same adrenocortical tumor region. The same could be shown by immunohistochemistry, where the co-localization of LHCGR and GATA-4 antibodies was strictly restricted to the tumor area verified in the hematoxylin-eosin stained histological sections. In Western blot analysis it was shown that both LHCGR and GATA-4 protein expressions were significantly downregulated after the Hecate-CGβ conjugate, although downregulation of GATA-4 was not as clear as that of LHCGR. The results in qRT-PCR showed a similar pattern in mRNA expression of GATA-4 and LHCGR: along with LHCGR mRNA depletion after Hecate-CGβ conjugate treatment also GATA-4 mRNA expression was undetectable. After Hecate and control treatment, adrenocortical tumors showed strong expressions of both LHCGR and GATA-4 mRNAs, whereas in WT control mouse adrenals neither of them was expressed. These results thus showed the co-localization of GATA-4 and LHCGR in adrenal tumors and the co-disappearance of them by Hecate-CGβ conjugate treatment.

5.5. Gonadotropin suppression as a treatment strategy (III)

As Hecate-CGβ conjugate treatment for adrenocortical tumors was not effective in females, but only males, the treatment needed improvement. GnRH antagonist or estradiol was thus added to the treatment. The hypothesis was that the decrease in circulating LH would diminish the potential competition between Hecate-CGβ conjugate and LH for binding to LHCGR. This could possibly increase the efficacy of the Hecate-CGβ conjugate treatment particularly in the female TG mice.

5.5.1. Tumor volume reduction

In males, GnRH antagonist alone reduced the tumor volume by 94% in comparison to the control and was thus even more efficient than Hecate-CGβ conjugate. In females, the reduction of tumor volume by GnRH antagonist was 96% in comparison to the control and 95% in comparison to Hecate-CGβ conjugate, which was found not to be an

efficient mode of treatment in females. Estradiol treatment reduced the tumor volume in females by 77%. The treatment efficacy of estradiol was monitored by uterine weight measurements. The uterine weights were significantly increased (up to 60%) after estradiol treatment. The combination of Hecate-CGβ conjugate to the treatments had no additive effect in either of the sexes.

5.5.2. Histopathology

GnRH antagonist alone or in combination with Hecate-CGβ conjugate drastically reduced the tumor volume in males. However, histopathologically GnRH antagonist seemed to destroy the normal adrenal structure, leaving only a thick outer capsule with matured fibrosis/necrotic tissue with hemosiderin-like remnants. Hecate-CGβ conjugate alone did not affect the normal adrenal tissue structure. In females after GnRH antagonist treatments there were areas of hyperplasia left. Estradiol alone or combined with Hecate-CGβ conjugate also decreased the tumor size, but some tumor nodules could still be found in histological evaluation.

The morphometrical analysis showed that in males GnRH antagonist combined with Hecate-CGβ conjugate were able to reduce the tumoral tissue to only 10%. However, after the combined treatment 40% of the fibrosis remained. GnRH antagonist alone treatment also left more tumorous tissue (35%) in comparison to the combination treatment (Fig. 10). In females, even though GnRH antagonist alone or in combination with Hecate-CGβ conjugate left more tumorous tissue than in males (30% *vs*. 10%, females *vs.* males), only a minor fraction of fibrotic tissue could be found in post-treatment samples. From the two hormonal treatment, GnRH antagonist seemed to be the better treatment choice in females, since after estradiol more tumorous tissue, up to 55%, could be observed compared to GnRH antagonist (Fig. 10).

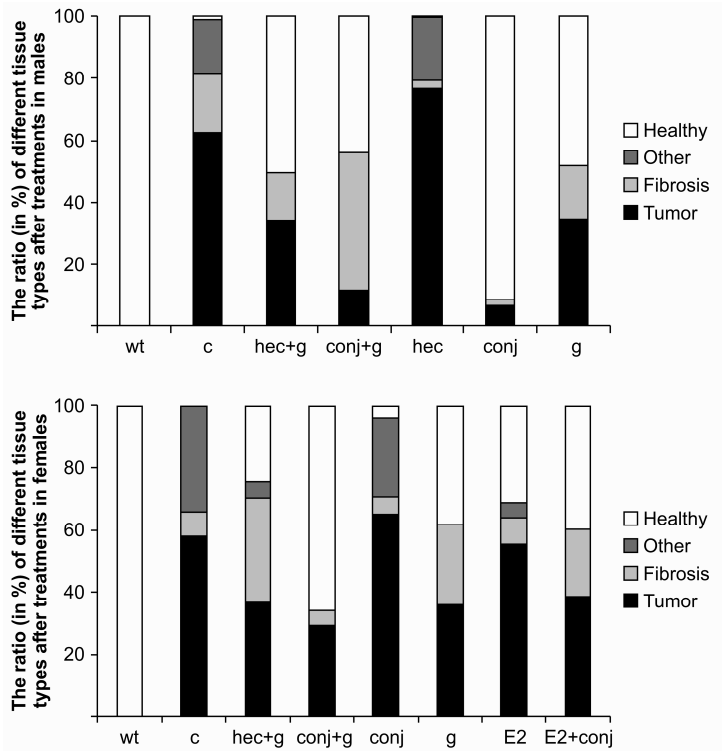


Figure 10. The post-treatment tissue structures by morphometric analysis in both male and female adrenocortical tumors. The results showed that in males Hecate-CGβ conjugate was the best treatment whereas the combination of Hecate-CGβ conjugate and GnRH antagonist caused considerable fibrosis. However, in female tumors after Hecate-CGβ conjugate there were tumor and blood filled cysts (named as 'other' in the figure) left but the combination with GnRH antagonist and Hecate-CGβ conjugate or GnRH antagonist alone worked significantly better. wt, wild type; c, control; hec + g, Hecate + GnRH antagonist; conj + g, Hecate-CGβ conjugate + GnRH antagonist, g, GnRH antagonist; E2, estradiol; E2 + conj, estradiol + Hecate-CGβ conjugate.

5.5.3. Endocrine consequences

Both in females and males, the progesterone levels decreased after GnRH antagonist, or after estradiol in females, in comparison to the control tumor group. This supported the effectiveness of gonadotropin suppression in tumor reduction. GnRH antagonist and E2 inhibited progesterone production apparently by blocking gonadotropin secretion. GnRH antagonist treatment blocked gonadotropin secretion more efficiently in males than in females, and the levels of LH in all the GnRH antagonist treated groups were almost unmeasurable.

5.5.4. Two distinct modes of antitumor effect

GnRH antagonist or estradiol alone were able to induce tumor mass reduction. Adding Hecate-CGβ conjugate to GnRH antagonist or to estradiol did not improve the positive outcomes, which further emphasized the role of GnRH antagonist or estradiol as a

possible treatment for female adrenocortical tumors. Tumor reduction with estradiol was significantly poorer than with GnRH antagonist. The same phenomenon was seen by qRT-PCR, where more LHCGR mRNA could be detected after estradiol than GnRH antagonist treatment in females. In males, in comparison to Hecate-CGβ conjugate treatment where LHCGR mRNA was undetectable, some LHCGR mRNA could be detected in male adrenals after GnRH antagonist treatment. The experiments with hormonal treatment also showed two distinct mechanisms of action in adrenocortical tumor treatment. Hecate-CGβ conjugate caused tumor destruction by killing LHCGR-bearing tumor cells, whereas GnRH antagonist inhibited tumor growth by blocking the gonadotropin dependent tumor progression through systemic effects.

5.6. Expression of GATA-6 in adrenocortical tumors (III)

GATA-6 expression has been shown in normal murine adrenocortical tissue but in adrenocortical tumors it regresses (Kiiveri *et al.*, 1999). GATA-6 mRNA measured by qRT-PCR showed, according to earlier findings, downregulation in control and Hecate-treated tumors in comparison to WT, but the expression was significantly increased in the Hecate-CGβ conjugate -treated group. In immunohistochemistry, in contrast to GATA-4, GATA-6 expression was shown in WT and Hecate-CGβ conjugate -treated male tumors and in GnRH antagonist -treated male and female tumors, whereas in control adrenal tumors no expression of GATA-6 could be detected. These observations emphasized the novel finding that GATA-6 can re-appear in adrenals by the successful treatment of adrenocortical tumors.

5.7. Expression of CYP17 (P450c17) and ERα in adrenocortical tumors (unpublished results)

Although CYP17 (P450c17) is not expressed in normal mouse adrenals, several murine adrenocortical tumors are known to secrete steroids needing this enzyme for their synthesis (Bielinska *et al.*, 2003, Bielinska *et al.*, 2005, Looyenga & Hammer, 2006). As the expression of CYP17 in Inhα/Tag TG murine tumors had not been studied earlier, it was now checked after different treatments. Adrenocortical tumor tissue showed abundant CYP17 (P450c17) mRNA expression, which became downregulated due to the treatments in comparison to control mice. This observation suggests the potential usefulness of CYP17 as a marker for tumor treatment efficacy, as downregulation of CYP17 expression would additionally show the ability of the treatment to reduce the steroidogenic tumorous tissue.

As human adrenals and adrenocortical tumors have been reported to express ERs (Barzon *et al.*, 2008), was expression of ERα was studied in the Inhα/Tag TG mice after different treatments. The finding of elevated ERα expression in the adrenal tumors, albeit not

significantly affected by the treatments, is in line with a study carried out on humans, where ERα was upregulated in ACCs (Barzon *et al.*, 2008) (Fig. 11).

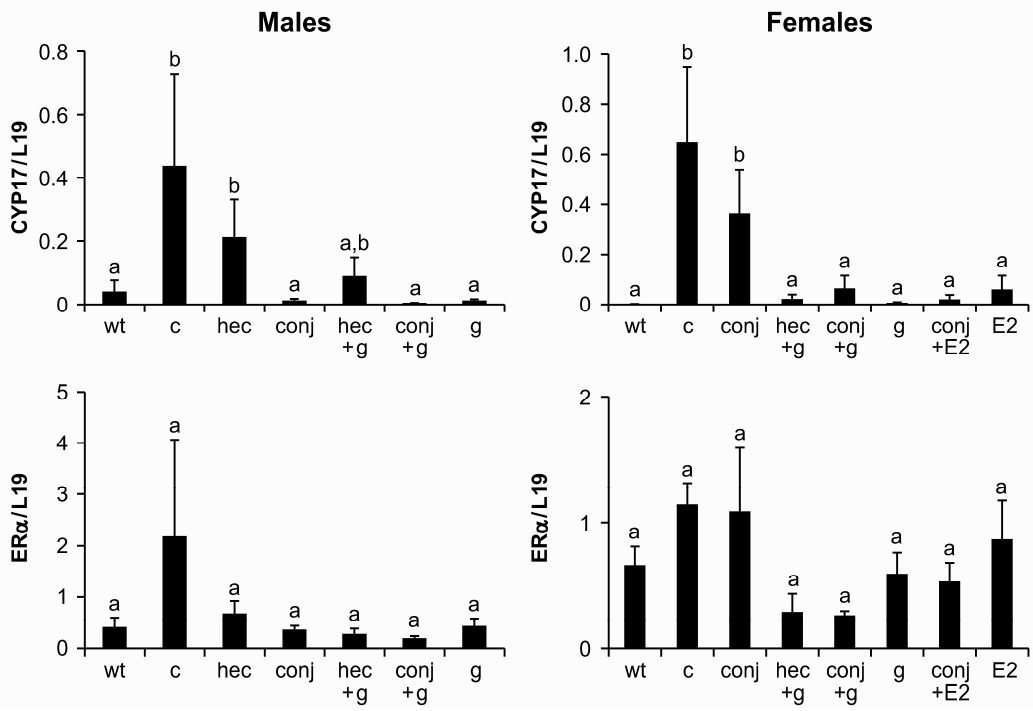


Figure 11. The qRT-PCR analysis of CYP17 and ERα mRNA in adrenocortical tumors. There is significant downregulation of the expression of CYP17 mRNA after Hecate-CGβ conjugate (conj) or GnRH-antagonist (g) or their combination in males (the left panel) in comparison to control (c) or to Hecate (hec) treatment. In females (right panel) GnRH antagonist (g) alone or combined with Hecate (hec + g) or with Hecate-CGβ conjugate (conj + g) as well as estradiol (E2) alone or combined with Hecate-CGβ conjugate (conj + E2) caused downregulation of CYP17 in comparison to control (c) or Hecate-CGβ conjugate (conj). Wt; wild type. (n = 5 in each group). ERα mRNA in males had a trend to decrease by the treatments but the changes were not significant due to high variation in the control group. In females, the differences between the groups were also not significant. The house-keeping gene L19 was run in parallel for each cDNA template. Different letters above the bars indicate that the difference between them is statistically significant ($P < 0.05$).

5.8. Tumor proliferation markers as indicators for treatment efficacy (III)

The proliferation markers Ki-67 and p53 were used in order to monitor adrenal tumor residues after the treatments. Proliferating cells could be detected throughout the tissue after control treatment in both sexes, as well as after Hecate treatment in males and Hecate-CGβ conjugate treatment in females. Hecate-CGβ conjugate -treated adrenals in males showed both Ki-67 and p53 expression only in patchy areas whereas after GnRH antagonist the proliferating areas in both sexes could be seen as tumor islets. After combined treatment with Hecate-CGβ conjugate and GnRH antagonist very few marker-positive cells were present.

6. DISCUSSION

6.1. The role of suitable murine models in cancer studies

The lack of suitable animal models for adrenocortical tumors has been a major obstacle for unraveling the molecular mechanisms of their pathogenesis, as well as for improving their diagnostic and therapeutic strategies. In the present work, advantage was taken of the Inhα/Tag TG mouse model that expresses gonadal somatic cell tumors with 100% penetrance and also adrenocortical tumors after prepubertal gonadectomy by the age of 5-8 months (Kananen *et al.*, 1996b). Due to the extensive characterization of this murine model (Kananen *et al.*, 1995, Kananen *et al.*, 1996a, Kananen *et al.*, 1996b, Kananen *et al.*, 1997, Rilianawati *et al.*, 1998, Rilianawati *et al.*, 1999, Kero *et al.*, 2000, Rilianawati *et al.*, 2000, Rahman *et al.*, 2001, Rahman *et al.*, 2004) both the pathophysiological and endocrine consequences of tumorigenesis were well known, which helped to monitor any tumor treatment outcome. Similar to human LHCGR-expressing adrenocortical tumors Inhα/Tag TG mice have chronically elevated LH, ectopic/upregulated LHCGR expression, hyperplasia-adenoma-carcinoma sequence in tumor formation, slow tumor growth, late discernible tumor incidence (analogous to human postmenopausal/andropausal age) and low metastasis frequency (Rahman *et al.*, 2004). These characteristics of adrenocortical tumorigenesis make Inhα/Tag TG mice a good tumor model for human ACC.

6.2. The role of Hecate-CGβ conjugate in targeted therapy of endocrine tumors

Intensive trials to find novel cancer treatment strategies are currently important research areas in medical science. There has been rapid advancement of chemotherapy with an increasing number of available cytotoxic agents. However, the treatment outcome has remained non-satisfactory, as these cytotoxic agents possess equal toxicity against all the rapidly dividing cells, including healthy cells. During recent years the concept of membrane disrupting peptides in cancer treatment has become important and relevant, as these lytic peptides, in contrast to standard chemotherapeutic treatments, are less toxic and have the ability to escape multi-drug resistance (and apoptosis resistance) due to their direct action on the cell membrane. Most importantly, they only affect the negatively charged cancer cells, and spare the healthy, positively charged cells (Utsugi *et al.*, 1991, Mader & Hoskin, 2006). Due to this selective method based on membrane charge instead of cell division, dormant cancer cells were also attacked by the lytic peptide with probably less possibilities for early recurrence. When the lytic peptide is further conjugated to a ligand that enables the targeting of the drug directly to cells having the ligand receptor, the effect becomes even more precise.

Hecate, a synthetic form of lytic peptide melittin, has all the characteristics of action of membrane disrupting peptides (Leuschner & Hansel, 2004, Mader & Hoskin, 2006). However, in the studies carried out on endocrine tumors *in vitro*, Hecate alone was not as effective as Hecate-CGβ conjugate (Zaleska *et al.*, 2004, Bodek *et al.*, 2005a), although in high concentrations Hecate alone killed the tumor cells with or without LHCGR (Bodek *et al.*, 2003). In the present *in vivo* studies, it was also shown that Hecate alone did not show any differential curative effects compared to control treatment.

One of the major goals in constructing Hecate-CGβ conjugate was to increase the efficacy of Hecate to disrupt cancer cell membranes by delivering peptide selectively to LHCGR expressing tumor cells. Hecate-CGβ conjugate, as earlier shown in *in vitro* and xenograft studies (Gawronska *et al.*, 2002, Bodek *et al.*, 2003, Leuschner *et al.*, 2003b, Zaleska *et al.*, 2003) as well as in induced mammary gland tumors *in vivo* (Zaleska *et al.*, 2004), showed effective tumor destruction through LHCGR. In the present studies, Hecate-CGβ conjugate was also highly effective both in gonadal somatic cell and adrenal tumors in males, due to its high selective specificity for abundant LHCGR bearing tumor cells. However, the results were not as promising in female granulosa cell and adrenocortical tumors. The male preference in the gonads could be explained by the established phenomenon that LHCGR expression is higher in the Leydig than in the granulosa cells (Kananen *et al.*, 1995, Kananen *et al.*, 1996a). The higher LHCGR could thus attract more Hecate-CGβ conjugate to bind and improve the treatment effect (Zaleska *et al*., 2003). Males also had lower circulating serum LH. This might have helped Hecate-CGβ conjugate to bind with higher affinity to existing LHCGR because of the lower competition between Hecate-CGβ conjugate and LH to the binding site. In the adrenals, the preference of Hecate-CGβ conjugate towards male adrenal tissue could partly be explained by the higher LHCGR expression in male adrenals in comparison to females. However, there could be many other predisposing factors to this phenomenon. Firstly, the relatively more aggressive adrenocortical tumor formation in females than in males is a known fact also in other murine strains (Bielinska *et al.*, 2003, Looyenga & Hammer, 2006). This could be due to the higher sensitivity to elevated serum LH like in double-positive TG mice, where bLHβ-CTP mice with constitutively elevated LH levels (Risma *et al.*, 1995) were crossbred with Inhα/Tag TG mice. In these bLHβ-CTP/Inhα/Tag mice the abundant ectopic LHCGR were present and adrenocortical tumors were formed even in the non-gonadectomized females, but this never occurred in double-positive male mice (Mikola *et al.*, 2003). Further, immortalization of a tumor cell line (Cα1) from female founder Inhα/Tag TG tumor mice was possible in contrast to the male founder mice (Kananen *et al.*, 1996b). Women are also more prone to develop ACC than men and this usually occurs after menopause (Schulick & Brennan, 1999a). These above mentioned findings could suggest that female adrenocortical tumors are more prone

to respond to elevated LH and LH suppression in females should, therefore, be more pronounced than in males in order to get similar treatment effects in both sexes.

The endocrine consequences of Hecate-CGβ conjugate treatment showed that progesterone secretion was clearly diminished after treatment and, consequently, the LH that was downregulated due to negative feedback from the progesterone, was increased. This showed the endocrinological action of the tumors that is well in line with previous findings of gonadal somatic cell and adrenal tumors (Kananen *et al.*, 1995, Kananen *et al.*, 1996a, Kananen *et al.*, 1996b, Kananen *et al.*, 1997). Progesterone could also serve as a good tumor marker. The levels of corticosteroids did not change after the treatments, which indicates that the treatment did not affect either normal adrenal steroidogenesis or destroy adrenal tissue.

No remarkable side effects after Hecate-CGβ conjugate could be detected in earlier or in the present studies in any of the non-gonadal organs with the given doses. The conjugated lytic peptides could affect the steroidogenic tissue of testis and ovaries during the treatment of xenografts in nude mice models (Hansel *et al.*, 2001, Leuschner *et al.*, 2001, Gawronska *et al.*, 2002, Leuschner *et al.*, 2003b, Hansel *et al.*, 2007). However, no such side effects have been reported after Hecate-CGβ conjugate treatments in earlier *in vivo* experiment (Zaleska *et al.*, 2004). In our experiments, there was no change in the gonadal volume of the WT mice treated with either Hecate or Hecate-CGβ conjugate in comparison to the non-treated gonads. The tissue structure after the treatments of Leydig cell tumors was not notably affected although the tumor mass was abolished. In the mice with adrenocortical tumors the gonads were removed prepubertally, thus no data on side effects to healthy gonads in the case of adrenal tumors could be obtained in the present studies. None of the other organs with ectopic LHCGR were affected by the treatment. This phenomenon might be explained by the comparatively lower LHCGR expression levels in non-gonadal organs or by the inability of the ectopic LHCGR to bind the CGβ-fragment (Hansel *et al.*, 2007b). Moreover, these lytic peptides are known to attack only the cancer cells with negatively charged membranes and to leave the positively charged healthy cells intact (Utsugi *et al.*, 1991, Leuschner & Hansel, 2004, Mader & Hoskin, 2006). Thus, Hecate-CGβ conjugate killed only the tumor cells possessing LHCGR, but spared the healthy LHCGR bearing cells, as they were not negatively charged.

No signs of metastases could be found after Hecate-CGβ conjugate treatment of gonadal or adrenal tumors. In fact, the studies carried out by another group even showed that this lytic peptide may prevent prostate or breast cancer cell xenograft induced metastases in lymph nodes and bones in nude mice (Leuschner & Hansel, 2005, Hansel *et al.*, 2007a). These results might suggest a role for Hecate-CGβ conjugate e.g. in the prevention of metastases after primary cancer surgery (Hansel *et al.*, 2007a).

6.2.1. Mode of cell death induced by Hecate-CGβ conjugate treatment – apoptosis vs. necrosis

Death of cells or tissues in a living organism, with a failure of membrane integrity, followed by cell swelling and lysis, is referred to as necrosis. It is a pathological process following cellular injury which usually involves a solid mass of tissue and cell groups. Often an inflammatory reaction is evoked by the tissue. In contrast to necrosis, in apoptosis the cell membrane remains intact and there is no inflammatory reaction. Apoptosis is an energy-dependent process for deletion of unwanted cells which occurs either physiologically or due to pathologic stimuli. Normally in apoptosis the cell shrinks and becomes fragmentated. Apoptosis results from the activation of caspases either via the action of bcl-2 family proteins or via binding of Fas ligand to its receptor. P53, the tumor suppressor protein, is also involved in apoptosis by checking the integrity of mitosis. If the dividing cells are defective, apoptosis is induced. In many neoplasias the loss of p53 function or activation of bcl-2 expression may result in failure of initiation of apoptosis causing cell accumulation.

In this study, the mode of cell death of Hecate-CGβ conjugate was studied more precisely. The lytic peptide conjugate caused a rapid permeabilization of the cell membrane by disturbing it with consequent cell death. Fluorescence microscopy and cell viability tests showed that cell death occurred through injury, swelling and bursting, suggesting death by necrosis rather than apoptosis. No signs of apoptosis could be seen. The necrotic mode of cell death after Hecate-CGβ conjugate treatment was further shown by DNA fragmentation tests (Bodek *et al.*, 2005).

Most of the antitumor effects of cytotoxic agents are induced through apoptosis. However, there are often anti-apoptotic genes evolved in the cancer cells and resistance to apoptosis is an often faced problem (Martinez-Lorenzo *et al.*, 1998). The present results indicating the necrotic mode of cell death of Hecate-CGβ conjugate show that in the future, this lytic peptide might have a role in the treatment of tumor cells with apoptosis resistance.

6.2.2. Hecate-CGβ conjugate vs. other cytotoxic agents

Leydig and granulosa cell tumors in older patients can be aggressive with a higher rate of recurrence (Kim *et al.*, 1985, Jamieson & Fuller, 2008). In these cases, chemotherapeutics are often needed, but for Leydig cell tumors no efficient chemotherapeutics exist (Bertram *et al.*, 1991) and myelotoxicity is an obstacle with chemotherapeutics used for granulosa cell tumors (Zambetti *et al.*, 1990, Homesley *et al.*, 1999). Thus, Hecate-CGβ conjugate could serve as one of the agents to be considered for the treatment of these gonadal somatic cell tumors.

In ACCs, surgery with total primary resection is the only curable treatment with 5-year survival up to 60% (Schulick & Brennan, 1999a). The mitotane treatment used in the advanced, non-resectable tumors has not been shown to be very effective, with the survival rates only up to 30% (Ng & Libertino, 2003). Mitotane as adjuvant treatment has shown some improvement to survival rates (Terzolo *et al.*, 2007). However, the side effects (neurological, gastrointestinal, adrenolysis) and the need for a strict follow-up of the mitotane concentration makes this agent problematic. Nevertheless, mitotane has proved its place in the combination treatment with conventional chemotherapeutics, such as etoposide-doxorubicin-cisplatin (Berruti *et al.*, 2005) or streptozocin (Khan *et al.*, 2000), since it is known to prevent multidrug resistance, which often hampers the use of these conventional cytotoxic agents in ACCs. The mode of cell death caused by mitotane, similar to Hecate-CGβ conjugate, is necrosis (Schulick & Brennan, 1999a), also preventing apoptosis resistance. However, combination treatments with mitotane still cause the usual cytotoxic problems to healthy cells. It is tempting to consider the idea of combination of Hecate-CGβ conjugate and conventional chemotherapeutics in the treatment of ACC, which could improve the curative effects. Hecate-CGβ conjugate would prevent the multidrug resistance and apoptosis resistance by the direct action of membrane effects on the LHCGR bearing cells and thus would help conventional chemotherapeutics to enter the tumor cells only, putatively saving the healthy cells.

6.3. LHCGR and ectopic hormone receptors

The relevance and significance of extragonadal LHCGR expression is quite well established, although sometimes debated (Rao, 2001, Pakarainen *et al.*, 2007). These receptors have been suggested to be functional and of use e.g. in early pregnancy and embryo implantation (Reshef *et al.*, 1990). LHCGRs in the brain have been suggested to play a role in the regulation of sexual behavior and e.g. in nausea connected in pregnancy (Lei *et al.*, 1993a, Apaja *et al.*, 2004). Some studies were done in our laboratory where the functionality of ectopic non-gonadal LHCGRs was not found in the female reproductive tract (Pakarainen *et al.*, 2005a, Pakarainen *et al.*, 2005c) or in tumorigenesis of hCG overexpressing mice (Rulli *et al.*, 2003, Ahtiainen *et al.*, 2005). However, according to recent findings, LHCGRs in the mammary gland may have a function in LH-induced gene expression (Rönnblad, 2008).

LHCGRs are found in normal adrenals (Pabon *et al.*, 1996b), but also in many hormone secreting adenomas (Leinonen *et al.*, 1991, Saner-Amigh *et al.*, 2006) and carcinomas (Wy *et al.*, 2002, Barbosa *et al.*, 2004). They are also present, together with many other ectopic receptors such as those for gastric inhibitory peptide (GIP) and 5-hydroxytryptamine 4 (5-HT4) in ACTH-independent Cushing's syndrome (Lacroix *et al.*, 2000). Circulating LH binds to these ectopic receptors and causes the secretion of adrenal hormones. Often,

the affected adrenals secrete glucocorticoids, but aldosterone production is possible e.g. through LH-dependent increased CYP11B2 (P450aldo) activity (Saner-Amigh *et al.*, 2006). Hence, there are LHCGR dependent mechanisms in adrenal pathogenesis and they may be clinically important e.g. in polycystic ovary syndrome and in postmenopausal women.

The earlier reported fact from *in vitro* and xenograft studies that Hecate-CGβ conjugate targets its action directly to cells with LHCGR (Leuschner *et al.*, 2001, Bodek *et al.*, 2003, Zaleska *et al.*, 2003), was confirmed in all the present experiments of thesis. Western blot, cell culture studies, immunohistochemistry and qRT-PCR all proved that Hecate-CGβ conjugate was able to specifically kill the cancer cells with LHCGR. Decrease of tumor size was also seen macroscopically. It can therefore be concluded that LHCGRs do have a distinct functional relevance as ectopic receptors in the adrenals.

The co-expression of LHCGR and GATA-4 in Inhα/Tag TG mice, as shown previously (Rahman *et al.*, 2004) was further confirmed by many methods, such as Western and Southern blot analysis, immunohistochemistry, RT-PCR and qRT-PCR. The idea was to establish a role for GATA-4 and GATA-6 as treatment outcome monitoring markers and with the results obtained in the present study, it can be postulated that these transcription factors can be useful in monitoring the treatment outcome. It is still not clear which one of LHCGR or GATA-4 regulates the other and which one would appear first during adrenocortical tumorigenesis. Ki-67 and p53 are common markers for many cancers and are also used in clinical practice for differentiating the adrenocortical carcinomas from adenomas (Arola *et al.*, 2000). In this study we showed that the control mouse adrenal tumors had high expression of both of these proliferation markers, which then got reduced by the efficient treatment. This finding established the role of p53 and Ki-67 in mouse adrenocortical tumor studies. The results also gave information about the type of adrenocortical tumors in these mice: In humans it has been shown that p53 is abundantly present only in carcinomas, not in adenomas (Arola *et al.*, 2000). The strong expression of both p53 and Ki-67 in control-treated adrenocortical tumors hints at the possibility that adrenocortical tumors in Inhα/Tag TG mice may be more carcinoma-like tumors than adenomas.

6.4. Hecate-CGβ conjugate *vs.* gonadotropin suppression

Since Hecate-CGβ conjugate was not effective in treating female adrenal tumors, the treatment strategy needed improvement. The aim was to improve the binding of Hecate-CGβ conjugate to LHCGR by decreasing the circulating LH by GnRH antagonist or estradiol-mediated gonadotropin suppression. Estradiol was also known to improve the cytotoxicity of Hecate-CGβ conjugate in cell culture studies *in vitro*, perhaps by increasing the number of LHCGRs on the cells (Leuschner *et al.*, 2001, Gawronska

et al., 2002). In our *in vivo* experiments estradiol was thought to act mainly through negative feedback to gonadotropins as suggested by endocrine evaluation.

GnRH antagonist in males seemed to cause a significant tumor regression but it also destroyed the adrenal structure. The reason for this tissue structure impairment of GnRH antagonist -treated adrenal tumors remain unknown. However, due to the findings, GnRH antagonist may not be recommended for use in males. In females, GnRH antagonist treatment also diminished tumor size and in contrast to male adrenals, only some fibrosis without impairment in tissue structure was detected. Estradiol was also effective in tumor shrinkage, but it was not as good as GnRH antagonist: the tumor size remained bigger, more proliferation marker-positive cells were left, and LHCGR and GATA-4 were found more abundantly after the treatment. Since estradiol is also known to increase the risk of e.g., thromboembolism, uterine cancer and breast cancer, GnRH antagonist should be considered as better hormonal treatment option in females. In fact, gonadotropin suppression in a form of GnRH agonist is already in use for adrenocortical adenomas (Lacroix *et al.*, 1999), and the findings of this study would further support the idea of the use of gonadotropin suppression in female LH-responsive adrenocortical tumors.

GnRH antagonists seem to work through a different mechanism than Hecate-CGβ conjugate, which was shown to cause tumor destruction of LHCGR-bearing cells. GnRH antagonist inhibited the tumor growth. The tumor ontogeny of Inhα/Tag TG mice has been shown to be gonadotropin dependent (Kananen *et al*., 1997) and in the present study it was possible to show that the adrenocortical tumor progression was also dependent on gonadotropins. SV40-Tag oncogene -induced tumorigenesis has been considered irreversible. However, the results showed that the SV40-Tag influence in Inhα/Tag TG tumorigenesis could also be reversible. Several studies have reported the downregulative effect of GnRH antagonist also on IGF-II expression, e.g. in mammary and prostate gland tumors (Hershkovitz *et al.*, 1993, Lamharzi *et al.*, 1998b) and the mechanism of action of GnRH antagonist has been proposed to be through induction of apoptosis (Pareek *et al.*, 2007). IGF-II has also been shown to be upregulated in human adrenocortical carcinomas (Ilvesmaki *et al.*, 1993, Giordano *et al.*, 2003, de Fraipont *et al.*, 2005, Slater *et al.*, 2006, Giordano *et al.*, 2009, Soon *et al.*, 2009). There is not yet evidence on high IGF-II levels in Inhα/Tag TG mice adrenocortical carcinomas, but in the light of the recent findings as discussed above, it would be intriguing to speculate about the role of IGF-II downregulation as a reason for tumor regression in GnRH antagonist treated mice. If the action of GnRH antagonist would also be mediated through apoptosis, perhaps even directly through the GnRH receptors in the adrenals and not by pituitary effects, this would give yet another aspect to the different mechanisms of action of Hecate-CGβ conjugate and the GnRH antagonist. These hypotheses require further studies.

6.5. Clinical relevance of the study

Many microarray studies have been carried out to find out the genes, pathways and molecular mechanisms underlying adrenocortical tumor development. Novel treatments are being developed towards the genes found and at present, some, such as IGF-1R antagonists, are been tested in *in vitro* and xenografts (Almeida *et al.*, 2008, Barlaskar *et al.*, 2009) as IGF-II has been shown to be strongly affected in adrenocortical tumorigenesis. In the microarray studies of human ACCs, LHCGR has not appeared in the top priority lists for gene alterations in adrenocortical carcinomas and LHCGR bearing adrenocortical tumors are also quite rare. However, Hecate-CGβ conjugate has proven its efficacy in the *in vivo* studies of gonadal and adrenocortical tumors carried out in the present thesis. As there are LHCGR bearing tumors in the human gonads and adrenal glands, this lytic peptide would deserve consideration for further use. At present, lytic peptide conjugate, Phor21(ala)-CGβ has approached clinical trials for prostate cancer (Leuschner & Hansel, 2005, Jia *et al.*, 2008), showing the importance and usefulness of the concept of targeted lytic peptides in clinical use.

None of the microarray studies of human ACC have been carried out on the post-treatment adrenocortical tissues, which could have shown the genes that are functionally affected by the treatment. In the future, it would be interesting to carry out microarray studies on the tumors of the Inhα/Tag TG mice, before and after the Hecate-CGβ conjugate and GnRH antagonist treatments, to evaluate the molecular mechanisms and the genes involved in pathogenesis and in the recovery process. The Inhα/Tag TG adrenocortical model could be ideal for such a treatment-induced gene array studies. These results, if proven to be representative, could further establish a place for LHCGR-targeted membrane disrupting peptides in adrenocortical tumor treatment. In the future, a feasible method of imaging mouse adrenocortical tumors, such as PET/CT or MRI, will also be a challenge.

Taken together, Hecate-CGβ conjugate could be used either alone or in connection with conventional cytotoxic agents to selectively treat LHCGR bearing tumors. Its mechanism of action prevents both multidrug and apoptosis resistance which may improve the current treatments in human. It might also serve as an adjuvant agent to prevent LHCGR dependent metastases after primary surgery.

7. SUMMARY AND CONCLUSION

At present, a great need exists for new methods for targeted cancer therapies. Taking advantage of an earlier established transgenic mouse line Inhα/Tag suitable for studying both gonadal and adrenal tumors, the lytic targeted peptide Hecate-CGβ conjugate, known to direct its action to LHCGR, was examined *in vivo*. The present thesis showed the following:

1. Hecate-CGβ conjugate works efficiently both in gonadal somatic cell and in adrenocortical tumors. It causes tumor destruction by killing the LHCGR bearing cells without side effects to any other organs. The action is more efficient in male tumors, where LHCGR expression was found to be higher in the target organs in comparison to females. The positive results in adrenocortical tumors further proves the principle of the ability of Hecate-CGβ conjugate to destroy any tumor cells that ectopically express LHCGR. This proves the functionality of LHCGR in extragonadal tissues such as the adrenals. These studies also proved the principle that cytotoxic agents can be delivered to malignant cells expressing ectopic receptors by targeted action without affecting the normal healthy cells.

2. The mode of cell death by Hecate-CGβ conjugate was shown to be necrosis without activation of the apoptotic machinery. The mechanism of cell death by necrosis is quite rare with cytotoxic agents, since most are known to induce apoptosis. This can be a positive finding for the future use of these lytic peptides, since in cancer apoptosis-related genes are often mutated, making them apoptosis-resistant.

3. Gonadotropin suppression by GnRH antagonist was found to be effective in the regression of female adrenal tumors. It works through a systemic effect by inhibiting tumor progression. It shows the gonadotropin dependency of the tumors in transgenic mice, not only in ontogeny but also during tumor progression. The positive treatment effects also proved that the tumorigenesis induced by SV40/Tag oncogene can be reversed.

4. The transcription factor GATA-4 and LHCGR hereby became established as tumor markers monitoring the treatment outcomes in murine adrenocortical tumors, as their expression diminished precisely along with the adrenocortical tumor mass. They could therefore also be considered as potential and useful tumor and treatment monitoring markers in clinical use. The novel finding of the re-appearance of GATA-6 in the adrenal cortex after the efficient treatment of adrenocortical tumors, could also suggest its role as a potential ‘curing effect’ marker for adrenocortical tumors. The proliferation markers Ki-67 and p53, which are rather commonly and routinely used in the diagnosis of human adrenal tumors, were also highly specific in determining the treatment efficacy of lytic peptide Hecate-CGβ conjugate and gonadotropin suppression in Inhα/Tag TG mice. Consequently, they further emphasized the relevancy of the Inhα/Tag TG murine adrenocortical model for studying human adrenocortical tumorigenesis.

8. ACKNOWLEDGEMENTS

This work was carried out at the Department of Physiology, University of Turku.

My deepest gratitude goes to my supervisors Docent Nafis Rahman, MD, PhD and Professor Jorma Toppari, MD, PhD, the Head of Department. It has been a pleasure to be your student. Nafis, I admire your enthusiasm towards science. Thank you for introducing me to the world of research and always finding time for my numerous questions and problems. I appreciate our team-work and the trust you had in me from the first moment. Jorma, I am thankful to you for the opportunity to work at the department and the possibility to be involved in teaching. I admire your knowledge and your way of making things work and setting up guidelines. I warmly appreciate your contribution, patience, fatherly guidance and the words of encouragement during this project. I want to thank Professor Ilpo Huhtaniemi MD, PhD, the former head of the department for letting a young medical student join the research group and for being a member of my thesis supervisory committee. You have always shown interest towards my research project. I admire your strong scientific experience and dedication, which also influenced this work. I also want to thank Professor Markku Heikinheimo, MD, PhD, another member of my thesis supervisory committee for valuable ideas and discussions.

I am very thankful to reviewers of my thesis, Docent Päivi Heikkilä, MD, PhD and Professor Raimo Voutilainen, MD, PhD for a careful review and lively discussions. Your comments and criticism improved the quality of the thesis. I wish to acknowledge my co-authors Johanna Arola MD, PhD, Gabriel Bodek PhD, Bidut Prava Mohanty, PhD, Adolfo Rivero-Müller, PhD and Professor Adam Ziecik, PhD for important contributions to the studies. Adolfo Rivero-Müller is especially acknowledged for his kind help in teaching the secrets of molecular biology. I wish to thank Harry Kujari, MD, for his help with morphometric analysis and Tero Vahlberg, MSc with statistical matters. Michael Nelson, PhD is acknowledged for revising the language of the thesis and Mr Timo Kattelus for help with the figures.

Professors Matti Poutanen, PhD, Markku Ahotupa, PhD, Antti Pertovaara, MD, PhD, Lauri Pelliniemi, MD, PhD and Emeritus Professor Martti Parvinen, MD, PhD, as well as senior scientists of the department Sonia Bourguiba, PhD, Noora Kotaja, PhD, Tarja Lamminen, PhD, Antti Perheentupa, MD, PhD, Saija Savolainen, PhD, Petra Sipilä PhD, Leena Strauss, PhD and Matti Vierula, PhD, are thanked for many discussions, advice and creating a open atmosphere for learning. Ms Anneli Vesa, Pirjo Pakarinen, PhD, Tuula Hämäläinen, PhD, Ms Leena Karlsson and Ms Marjatta Keistinen are gratefully thanked for all possible help, friendly smiles and support during these years. Ms Katri Hovirinta, Ms Nina Messner, Ms Erja Mäntysalo, Ms Heli Niittymäki are thanked for all their help with the mice experiments and the personnel of Turku University Animal

Facility for taking good care of the mice. Ms Tarja Laiho, Ms Aila Metsävuori, Ms Taina Kirjonen, Ms Hannele Rekola, Ms Eila Virransuo, Ms Johanna Vesa, Ms Taija Leinonen, Ms Erica Nyman, Ms Jonna Palmu, Ms Johanna Järvi, Ms Liubov Hännikäinen, Ms Merja Leppiaho-Vallinhovi, Ms Katja Suomi, Ms Mona Laaksonen, Ms Maire Tohv, Kalle Vaahtoranta, MSc and Marko Tirri, MSc Engineering, are acknowledged for their valuable help in the laboratory. Aila Metsävuori is also acknowledged for help with teaching matters. Ms Sirpa Saarinen is thanked for her friendly help with the computers.

All the friends and colleagues in teaching: Petteri Ahtiainen, MD, PhD, Jenni Jalkanen, MD, PhD, Jaakko Kytölä, MD, Juho-Antti Mäkelä, MSc, Tomi Pakarainen, MD, PhD, Aino Rönnblad, MD, PhD, Janne Suominen, MD, PhD, Helena Virtanen, MD, PhD as well as Kirsti Ellonen, MSc Nursing and Päivi Erkko, MSSc are thanked for all the valuable moments together. Juho-Antti Mäkelä and Sonia Bourguiba are also thanked for many help in the laboratory and Kaisa Huhtinen, MSc for friendship. My former and present room-mates, Anastasios Damdimopoulos, PhD, Päivi Järvensivu, MSc, Raimo Lindqvist, PhD, Taija Saloniemi, MSc and Shen Zhongyi, MSc are thanked for a good working atmosphere. Annika Adamsson, MSc, Maarit Ahtiainen, PhD, Ritva Berg, MSc, Ida Björgren, MSc, Leon Brokken, PhD, Marcin Chruciel, MSc, Matteo Da Ros, MSc, Nimisha Desai, MSc, Kame El-Darwish, PhD, Antti Ellonen, Eve Etuaro, MB, Veera Hämäläinen MSc, Min Jiang, MD, PhD, Marko Kaleva, MD, PhD, Jenny Kallio MSc, Tero Karhi, MSc, Heli Koivuniemi, MSc, Heidi Lagerbohm MB, Heidi Liljenbäck, MSc, Veronika Mamaeva, MD, PhD, Mari Mansikkamäki, MB, Ilkka Martikainen, MD, Oliver Meikar, MSc, Katja Mäkelä MSc, Mirja Nurmio MSc, Sakari Pakarinen, Jenni Paviala MSc, Dwi Ari Pujianto, PhD, Pia Rantakari, MSc, Pia Roering, MSc, Emmi Rotgers, MB, Vuokko Saario, Anu Salminen MSc, Anu Sironen, PhD, Heidi Sulonen, MSc, Ms Leena Söderholm, Emma Tobjörk, MD, Jussi Toivanen, Maria Tuominen, MB, Heikki Turunen, MSc, YangYang Wang, MD, PhD, as well as all other former and present work-mates in the Department of Physiology are thanked for creating such a friendly working atmosphere. I'll miss the time with you!

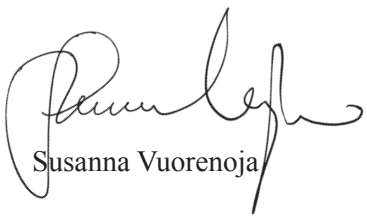
The Head of the Department of Pediatrics in the Turku University Hospital, Marja-Riitta Ståhlberg MD, PhD and Professor Olli Simell MD, PhD are thanked for the opportunity for clinical work during the research years. Professor Toivo T. Salmi MD, PhD, Päivi Lähteenmäki MD, PhD, Kirsi Jahnukainen MD, PhD and Marika Grönroos, MD, PhD are warmly thanked for introducing and guiding me in the fascinating world of hematology and oncology. The heads of the Hematological Laboratory, TYKSLAB, Tarja-Terttu Pelliniemi, MD, PhD and Irma Matinlauri, MD, PhD as well as Tarja-Leena Penttilä, MD, PhD and Anri Tienhaara, MD, PhD are thanked for a warm welcome and educative teaching. I also want to thank the colleagues and the personnel in both departments for pleasant co-work.

My dear parents, Liisa and Pekka—you have always sacrificed your life for me and helped and supported with all possible matters. I am very thankful for the trust you have always had in my abilities – that's the greatest thing that parents can give. I wish I could be able to share all the good will and love you have given me. My parents-in-law Hilkka and Timo: it's been wonderful to get to become a part of your family. Thank you for all the care and understanding during these years. My sisters (-in-law) and their husbands, Anna-Kaisa and Kimmo, Annemari and Juha—thank you for the friendship and all the good moments and cheerful travels outside work. Anna-Kaisa is especially acknowledged for all the practical help during the thesis writing. Eki and Marja, your encouragement to research was crucial for this project. Thank you. My friends Anu-Riikka, Liina and Riitta; Jenni, Jaana, Maarit, Miia, Pieta, Salla and Satu with their spouses, friends in Akademiska Orkestern and all the friends throughout life are thanked for their friendship and joy! Sara ja Jussi, thank you for always supporting and living alongside our lives. Jaana and Kari, Jonna and Tuomas and Riikka and Lassi, we have shared so many important days and happenings together. Thank you for always being there for me. I especially want to thank my precious godsons Eemil and Lauri for bringing joy to my life numerous times during these years. You are true gifts of God!

Finally, my beloved husband Ari-Matti. There exist no words to express my gratitude for all the support you have given and the sacrifices you have made during these years. The joyful days together and your love have been the strength for this work. I admire your attitude of life, your bright ideas and the endless trust that things will work out if one just stays positive. Thank you for everything.

This study was financially supported by grants from the Academy of Finland, Centre for Reproductive and Developmental Medicine, Finnish Cancer Organisations, Finnish Cultural Foundation at Southwestern Finland, the Finnish Endocrine Society, the Finnish Medical Foundation, the Ida Montin Foundation, Moikoinen Cancer Research Foundation, Sigrid Juselius Foundation, Turku University, Turku University Hospital and the Turku University Foundation.

Turku, June 2009



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