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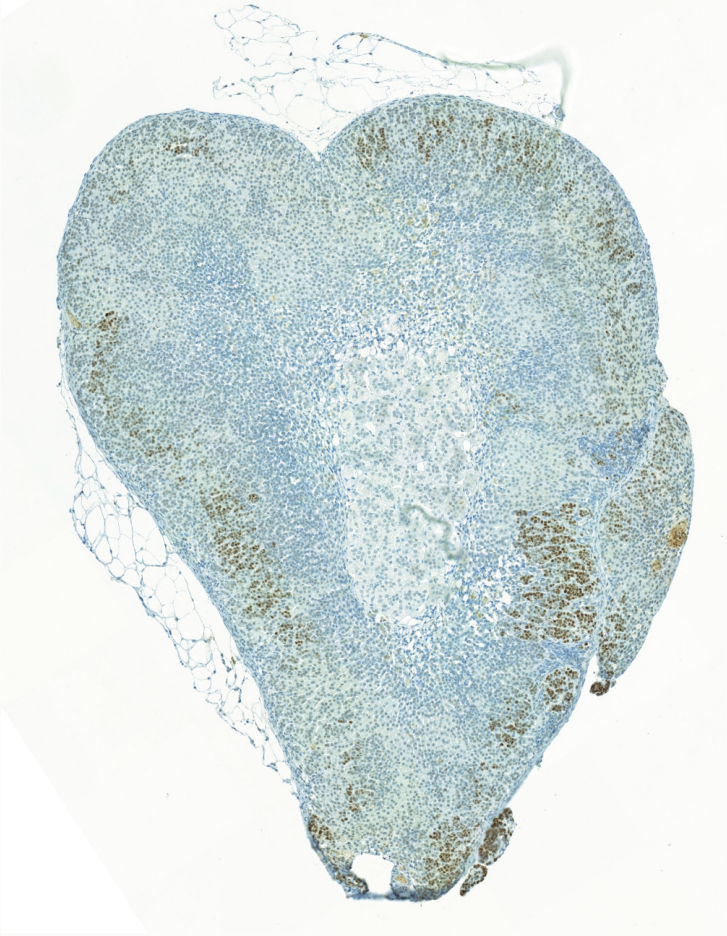

IDENTIFICATION OF NOVEL GENES INVOLVED IN THE PATHOPHYSIOLOGY OF ADRENOCORTICAL TUMORIGENESIS

Milena Doroszko

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ABSTRACT

Milena Doroszko

Identification of novel genes involved in the pathophysiology of adrenocortical tumorigenesis

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Turku, 2017

Adrenocortical tumors (ACT) are relatively common, with a prevalence of 6% in the aging population. They can be divided into benign adenomas (ACA) and malignant carcinomas (ACC). Tumors responding to luteinizing hormone (LH)/choriogonadotropin (CG) belong to a subgroup of hormonally active ACTs. Such tumors may develop during the chronic elevation of LH (menopause) or hCG (pregnancy), and result in cortisol, aldosterone, or/and androgen overproduction.

In the present thesis, transgenic mice expressing SV40Tag oncogene under the inhibin α promoter (inhα/Tag), were utilized to identify novel biomarker genes and unravel the molecular mechanisms in the gonadotropin-dependent adrenocortical tumorigenesis. The effects of GnRH antagonist treatment were investigated on human and mouse adrenocortical cell lines *in vitro*, and on mouse adrenocortical tumors *in vivo.* Finally, the role of LHCGR in the pregnancy-induced Cushing syndrome was characterized in a case study.

Estrogen receptor alpha (ESR1) expression was identified to be associated with the adrenocortical tumors in inhα/Tag mice, whereas several other molecules, such as GRB10, RERG, GNAS, and NFATC2, were linked to healthy adrenal tissue. Gonadotropin-dependent adrenocortical tumors of inhα/Tag mice were found to originate from zona fasciculata and LHCGR to be a prerequisite for their onset, presumably through the cell fate reprogramming by GATA6 to GATA4 transcription factor switch. After induction, GATA4 took over the transcriptional control in the adrenal gland, prompting the tumor progression and gonadal-like phenotype, and sequentially adrenocortical tumors became independent of LHCGR signaling. The GnRH antagonist cetrorelix acetate acted directly on human and mouse adrenocortical tumor cells, inducing apoptosis. Moreover, it appeared that chronically high LH/hCG concentrations promoted the transformation of *LHCGR*-positive cells into LH/hCG-responsive adrenocortical cells, which gave rise to the cortisol and androgen-producing hyperplastic cells in human, and estrogen-producing neoplasms in mice.

In conclusion, LH/LHCGR signaling plays a crucial role in the induction of some adrenocortical tumors. Regulatory role of GATA4 was shown to be required for tumor progression in mice. Cetrorelix acetate could be considered for improving the ACT therapy, either through its systemic or direct action, or their combined effects on adrenocortical tumor cells.

Keywords: LHCGR, LH, adrenal, adrenal tumor, pathophysiology, gonadotropin, GATA4, biomarker, molecular mechanisms, transgenic mice, gonadectomy-induced, GnRH antagonist, Cushing syndrome, gonadectomy

TIIVISTELMÄ

Milena Doroszko

Lisämunuaiskuoren kasvainten kehittymisen patofysiologiaan liittyvien uusien geenien tunnistaminen

Turun yliopisto, Lääketieteellinen tiedekunta, Biolääketieten laitos, Fysiologia, Molekyylilääketieteen tohtoriohjelma (TuDMM), Turku, Suomi.

Turku, 2017

Lisämunuaiskuoren kasvaimet ovat suhteellisen yleisiä, niitä esiintyy kuudella prosentilla ikääntyvästä väestöstä. Ne jaetaan hyvänlaatuisiin adenoomiin (ACA) ja pahanlaatuisiin karsinoomiin (ACC). Kasvaimet, jotka vastaavat luteinisoivaan hormooniin (LH)/istukkagonadotropiiniin (CG), kuuluvat hormonaalisesti aktiivisten kasvainten alaryhmään. Tälläiset kasvaimet voivat kehittyä LH:n (menopaussi) tai hCG:n (raskaus) kroonisen lisääntymisen aikana ja ne johtavat kortisolin, aldosteronin ja androgeenien ylituotantoon.

Tässä väitöskirjassa hyödynnettiin inhibiini α-promoottorin alaisuudessa SV40Tag-onkogeeniä ilmentäviä siirtogeenisiä hiiriä gonadotropiiniriippuvaisen lisämunuaiskuoren tuumorigeneesin uusien biomarkkerigeenien tunnistamisessa ja molekulaaristen mekanismien selvittämisessä. GnRH-antagonistihoidon vaikutuksia tutkittiin ihmisen ja hiiren lisämunuaisperäisissä solulinjoissa *in vitro*, sekä hiirten lisämunuaiskuoren kasvaimissa *in vivo*. Lopuksi analysoitiin LHCGR:n roolia raskauden aiheuttamassa Cushingin oireyhtymässä potilastapauksen valossa.

Estrogeenireseptori alfan (ESR1) ilmentymän tunnistettiin liittyvän lisämunuaiskuoren kasvaimiin inhα/Tag –hiirissä, kun taas useat muut molekyylit kuten GRB10, RERG, GNAS ja NFATC2 liittyivät terveen lisämunuaiskudoksen toimintaan. Inhα/Tag hiirten gonadotropiiniriippuvaisten kasvaimien huomattiin syntyvän zona fasciculatassa (juostevyohyke) ja LHCGR:n olevan edellytys niiden ilmaantumiselle. Oletettavasti syynä LHCGR:n ilmentymälle on solukohtalon uudelleenohjelmoituminen GATA6 – GATA4- transkriptiotekijäkytkennän kautta. Induktion jälkeen GATA4 otti hallintaansa transkriptiokontrollin lisämunuaisessa, täten kiihottaen kasvaimen sekä gonadin kaltaisen ilmiasun kehittymistä. Vähä vähältä, kasvaimet itsenäistyivät LHCGR:n soluviestinnästä. GnRH-antagonisti, setroreliksiasetaatti, vaikutti suoraan ihmisen ja hiiren kasvainsoluihin aiheuttamalla apoptoosia eli solukuolemaa. Lisäksi kroonisesti suuret LH/hCG-pitoisuudet edistivät LHCGR-positiivisten solujen muuttumista LH/hCG:lle reagoiviksi lisämunuaiskuoren soluiksi. Tämä aiheutti kortisolia ja androgeenejä tuottavien hyperplastisten solujen lisääntymisen esimerkkipotilaan lisämunuaiskuoressa ja estrogeeneja tuottavien kasvainten kasvun hiiren lisämunuaisissa.

Yhteenvetona, LH/LHCGR soluviestinnällä on keskeinen osa joidenkin lisämunuaiskuoren kasvainten kehityksessä. GATA4:n säätelytehtävä osoitettiin edellytykseksi hiirten kasvainkehitykselle. Setroreliksiasetaattia voidaan harkita lisämunuaiskuoren kasvainten hoitojen parantamiseksi, joko sen systeemisen tai suoran vaikutuksen välityksellä, tai näiden yhteisvaikutuksella lisämunuaiskuoren kasvainsoluihin.

Avainsanat: LHCGR, LH, tuumorigeneesi, patofysiologia, gonadotropiiniriippuvaisuus

TABLE OF CONTENTS

ABBREVIATIONS

ACA	adrenocortical adenoma
ACC	adrenocortical carcinoma
ACT	adrenocortical tumor
ACTH	adrenocorticotropin
AGP	adrenogonadal primordium
Amh	anti-Mullerian hormone
Amhr2	anti-Mullerian hormone receptor
ARMC5	armadillo repeat containing 5
BIMAH	bilateral macronodular adrenal hyperplasia
cAMP	3',5'-cyclic adenosine monophosphate
Ccna1	cyclin A1
cDNA	complementary DNA
CRH	corticotropin releasing hormone
CS	Cushing syndrome
CTNNB1	catenin beta 1
CTX	cetrorelix acetate
CV	coefficient of variation
CYP11A1	cytochrome p450 family 11 subfamily A member 1
CYP11B1	cytochrome p450 family 11 subfamily B member 1
CYP11B2	cytochrome p450 family 11 subfamily B member 2
CYP17A1	cytochrome p450 family 17 subfamily A member 1
CYP19A1	cytochrome p450 family 19 subfamily A member 1
CYP21A2	cytochrome p450 family 21 subfamily A member 2
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DNA	deoxyribonucleic acid
E	embryonic day
ESR1	estrogen receptor alpha
ESR2	estrogen receptor beta
FBS	fetal bovine serum

FSH	follicle stimulating hormone
FSHR	follicle-stimulating hormone receptor
G0s2	G0/G1 switch 2
GATA4	GATA binding protein 4
GATA6	GATA binding protein 6
gDNA	genomic DNA
GDX/OVX	gonadectomy/ovariectomy
GIACT	gonadectomy-induced adrenocortical tumorigenesis
Gnas	guanine nucleotide binding protein (G protein) alpha subunit
GnRH	gonadotropin releasing hormone (as a hormone)
GNRH	gonadotropin releasing hormone (gene/protein)
GNRHR	gonadotropin releasing hormone receptor (gene/protein)
GPCR	G-protein coupled receptor
Grb10	growth factor receptor bound protein 10
hCG	human chorionic gonadotropin
HIFs	hypoxia-inducible transcription factors
HPA	hypothalamus-pituitary-adrenal axis
HSD3B2/ 3βHSD	hydroxy-delta-5-steroid dehydrogenase
IGF2	insulin-like growth factor 2
INHA	inhibin alpha subunit
Inha$^{-/-}$ mice	inhibin alpha subunit knockout mice
inhα/Tag mice	transgenic mice expressing SV40Tag under inhibin alpha promoter
KCNJ5	potassium voltage-gated channel subfamily J member 5
KREMEN1	kringle containing transmembrane protein 1
LH	luteinizing hormone
Lhb	luteinizing hormone beta subunit
LHCGR	luteinizing hormone/chorionic gonadotropin receptor
LuRKO mice	luteinizing hormone receptor knockout mice
MC2R	adrenocorticotropic hormone receptor
Mmp24	matrix metallopeptidase 24
mRNA	messenger RNA
Nedd4	E3 ubiquitin-protein ligase

Nfatc2	nuclear factor of activated T-cells 2
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline with Tween20
PFA	paraformaldehyde
PRKACA	protein kinase C alpha subunit
PRKAR1A	cAMP-dependent protein kinase type I-alpha regulatory subunit
Prl-rs1	Prolactin receptor isoform 1
qPCR	quantitative polymerase chain reaction
Rasgrf2	Ras protein specific guanine nucleotide releasing factor 2
Rerg	RAS-like estrogen-regulated growth inhibitor
RNA	ribonucleic acid
SEM	standard error of the mean
SF1/AD4BP/NR5A1	steroidogenic factor 1/ adrenal 4 binding protein/ nuclear receptor subfamily 5 group a member 1
Sgcd	sarcoglycan delta
Srd5a1	steroid 5 alpha-reductase 1
Star	steroidogenic acute regulatory protein
SV40Tag	simian virus 40 large T antigen
TART	testicular adrenal rest tumors
TBS	tris-buffered saline
TBS-T	tris-buffered saline with Tween20
TGFβ	transforming growth factor beta
Tusc5	tumor suppressor candidate 5
VEGF	vascular endothelial growth factor
WT	wild type
xZ	x zone
zF	zona fasciculata
ZFPM2	zinc finger protein multitype 2
zG	zona glomerulosa
ZNRF3	zinc and ring finger 3
zR	zona reticularis

LIST OF ORIGINAL PUBLICATIONS

This doctoral thesis is based on the following publications that are referred in the text by Roman numerals (I-IV). The original publications have been reproduced with the permission of the copyright holder.

I. **Doroszko M.**, Chrusciel M., Belling K., Vuorenoja S., Dalgaard M., Leffers H., Nielsen H.B., Huhtaniemi I., Toppari J., and Rahman N.A., Novel genes involved in pathophysiology of gonadotropin-dependent adrenal tumors in mice. *Mol Cell Endocrinol* 2017;444:9-18.

II. **Doroszko M.**, Chrusciel M., Stelmaszewska J., Slezak T., Rivero-Muller A., Padzik A., Anisimowicz S., Wolczynski S., Huhtaniemi I., Toppari J., and Rahman N.A., Luteinizing hormone and GATA4 action in the adrenocortical tumorigenesis of gonadectomized female mice. *Cell Physiol Biochem* 2017;43:1064–1076

III. **Doroszko M.**, Chrusciel M., Stelmaszewska J., Slezak T., Anisimowicz S., Plöckinger U., Quinkler M., Wolczynski S., Huhtaniemi I., Toppari J., and Rahman N.A. Gonadotropin releasing hormone (GnRH) antagonist treatment of adrenocortical cell tumors. *Manuscript*

IV. Plöckinger U., Chrusciel M., **Doroszko M.**, Saeger W., Blankenstein O., Weizsäcker K. Kroiss M., Hauptmann K., Radke C., Pöllinger A., Tiling N., Steinmüller T., Huhtaniemi I., Quinkler M., Bertherat J., Lacroix A., and Rahman N.A., Functional implications of LH/hCG receptors in pregnancy-induced Cushing syndrome. *J Endocr Soc* 2017,1;57-71

1 INTRODUCTION

Adrenocortical tumors (ACT) can be divided into benign adrenocortical adenomas (ACA) observed in ~5% of population over the age of 50 (Thompson and Young Jr 2003), and malignant adrenocortical carcinomas (ACC) diagnosed yearly in ~2 patients per million (Allolio and Fassnacht 2006, Kirschner 2006, Fassnacht and Allolio 2009). Among hormonally active adrenocortical tumors, a group responding to luteinizing hormone (LH)/choriogonadotropin (CG) can be specified (Alevizaki et al. 2006, Carlson 2007, Huhtaniemi 2010). Such ACT may arise during the chronic elevation of LH (menopause) or hCG (pregnancy) and result in Cushing syndrome (CS) (Zubair et al. 2006, Carlson 2007). Surgical removal remains the most common treatment strategy for adrenocortical tumors, and on the basis of pathological and molecular features, further radiation or adjuvant therapies are applied (Creemers et al. 2016). Difficulties to distinguish between ACAs and ACCs, as well as the lack of efficient post-operative therapies, result in a 5-year survival rate below 30% in patients with ACC (Bernichtein et al. 2008b).

Transcriptome studies revealed that one of the most common alterations in ACTs include germline and somatic mutations in tumor protein p53 (*TP53*) (Barlaskar and Hammer 2007). The inactivation of p53 enables cell cycle progression despite DNA damage, which promotes the neoplastic cell differentiation (Levine et al. 1991). The p53 inhibition has been successfully tailored in animal models by transgenic expression of Simian Virus 40 T-antigen (SV40Tag), which results in the development of tumors in various organs (Hudson and Colvin 2016). These animal models provided a useful insight into the mechanisms leading to tumor formation and progression due to the inactivation of p53 mutations.

The inhα/Tag transgenic mouse model combines a gonadotropin-dependent tumor onset along with the p53 inactivation process. Inhα/Tag mice express SV40Tag oncogene regulated by inhibin-α 6kb promoter, and have been extensively characterized as models to investigate gonadal and adrenocortical tumorigenesis (Kananen et al. 1995, Kananen et al. 1996b, Rahman et al. 1998, Rilianawati et al. 1998, Rilianawati et al. 1999, Rilianawati et al. 2000, Bodek et al. 2005, Vuorenoja et al. 2007, Chrusciel et al. 2014). Adrenocortical tumorigenesis in inhα/Tag mice is induced by prepubertal gonadectomy (GDX) (Kananen et al. 1996b). The hallmarks of adrenocortical tumors in inhα/Tag mice are the abundant expression of LHCGR, inhibin alpha (INHA), and GATA binding protein 4 (GATA4) (Kananen et al. 1996b, Rilianawati et al. 1998, Rahman et al. 2001, Rahman et al. 2004, Vuorenoja et al. 2007). However, the interaction of these genes as well as the cascade of events leading to tumorigenesis are still

poorly understood. Additionally, inhα/Tag mice have been shown to be a good model for testing novel treatment strategies for adrenocortical tumors (Vuorenoja et al. 2009). It is noteworthy that the inhα/Tag model is superior to the xenograft models for therapy testing, as inhα/Tag mice develop tumors endogenously and possess an intact immune system. Moreover, the Cα1 cell line derived from inhα/Tag mouse adrenocortical tumor (Kananen et al. 1996b), provides an *in vitro* drug screening tool.

The present study describes findings on LHCGR-dependent adrenocortical tumorigenesis in inhα/Tag mice, and in a pregnancy-induced adrenal hyperplasia, as well as on the molecular mechanisms of the cetrorelix acetate direct action on the adrenocortical tumor cells. The data provide an interesting insight into the adrenocortical tumor biology and highlights the possibilities for a novel therapy of adrenocortical tumors.

2 REVIEW OF THE LITERATURE

2.1 The adrenal gland

2.1.1 Development of the adrenal gland and its functional zonation

The embryonic adrenogonadal primordium (AGP) is the origin of progenitors for the major steroidogenic organs: ovaries, testes, and adrenal cortex (Pihlajoki et al. 2015). In mice, the separation of AGP cells is induced around embryonic day (E) 9.5-11, which results in the development of a bipotential gonad and an adrenal anlage (Adams and McLaren 2002). The adrenal anlage is colonized with sympathoblasts that later form the medulla, and it is surrounded by capsule cells (Wood et al. 2013). Functional development of the cortical zones starts at E16 and ends when the mouse turns 3 weeks old, shortly before reaching puberty (Zubair et al. 2006). The subcapsular region is the lifetime progenitor reservoir for adrenal cortex replenishment (Pihlajoki et al. 2015). Recent reports show that among the adrenal progenitors in an adult mouse adrenal gland, long-lived AGP-like cells can be localized. These AGP-like cells differentiate into normal adrenal steroidogenic cells. Any distortion of hormonal homeostasis, like gonadectomy (GDX), may promote their differentiation into gonadal-like tissue (Bandiera et al. 2013, Dörner et al. 2017).

The steroid producing adrenal cortex in human and mice consists of 3 layers: outer zona glomerulosa (zG), middle zona fasciculata (zF), and inner zona reticularis (zR) in human, or x zone (xZ) in mice (Pihlajoki et al. 2015). Cells of each layer express steroidogenic enzymes, which results in a zone-specific production of mineralocorticoids (zG), glucocorticoids (zF), or androgens (zR) (Pihlajoki et al. 2015). The xZ in mice is steroidogenically inactive (Miller and Auchus 2011) and regresses in males after reaching puberty, whereas remains in females until the first pregnancy (Howard-Miller 1927, Holmes and Dickson 1971). The highly differentiated cells of the adrenocortical zones do not proliferate, but are replaced by new cells migrating from the subcapsular niche of stem/progenitor cells towards the medulla (Pihlajoki et al. 2015).

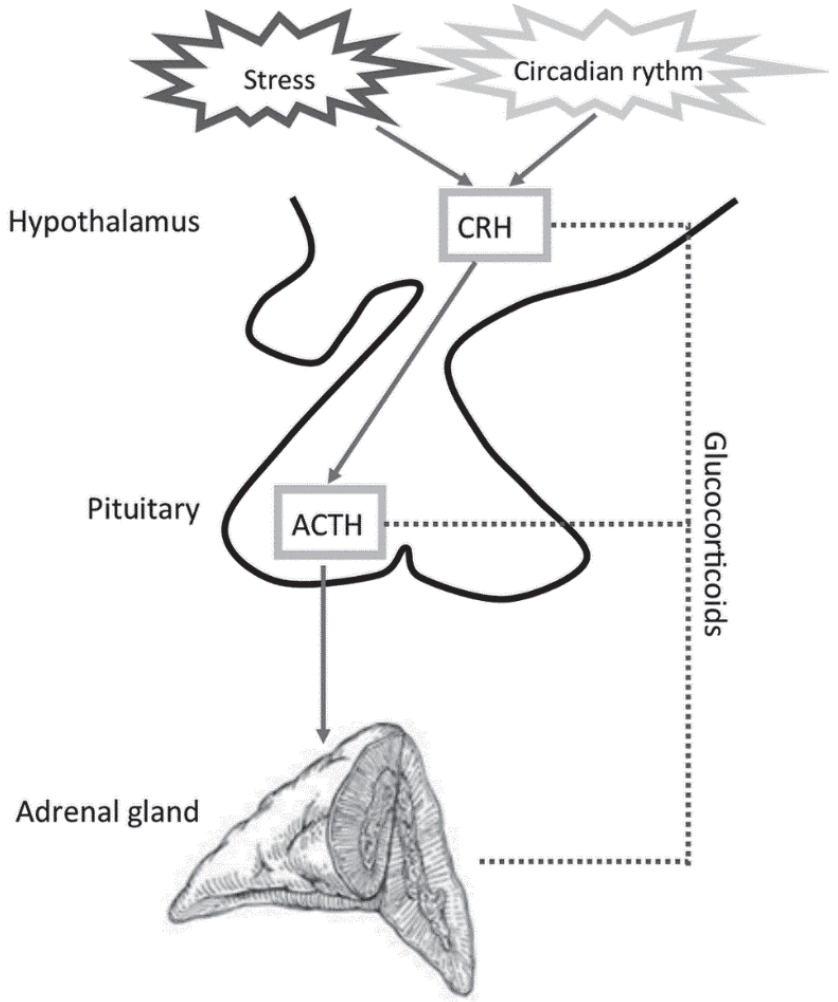

Figure 1. Hypothalamus-pituitary-adrenal axis
Regulation of hypothalamus-pituitary-adrenal axis is illustrated. Solid lines represent stimulatory, whereas dashed lines inhibitory effects. Abbreviations: CRH, corticotropin-releasing hormone; ACTH, adrenocorticotropin.

2.1.2 Regulation and steroidogenic activity of the adrenal cortex

The adrenal gland is a major source of steroid hormone production. The activity of the cortical zones is controlled by specific hormones that regulate the transcription and/or activity of the steroidogenic enzymes, causing the production of zone-specific steroids. In mouse and human regulation of zG and zF function is

similar. The activity of zG is controlled by the renin-angiotensin system, which leads to the production of aldosterone, whereas zF is regulated by the hypothalamus-pituitary-adrenal (HPA) axis (Figure 1) (Goodman 2003, Miller and Auchus 2011). Corticotropin-releasing hormone (CRH) from the hypothalamus stimulates the pituitary gland to release adrenocorticotropin (ACTH), which by acting on ACTH receptors (MC2R) in zF induces cortisol or corticosterone production. Human zR is less active steroidogenically than zG and zF, but upon ACTH stimulation may produce dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S) (Goodman 2003, Miller and Auchus 2011). Mouse xZ is steroidogenically inactive (Howard-Miller 1927, Holmes and Dickson 1971, Zubair et al. 2006), however it is responsive to LH stimulation (Jones 1949). A simplified steroidogenic pathway in human and mouse adrenals is illustrated in Figure 2. In contrast to humans, the mouse adrenal gland does not produce androgens under physiological conditions (Jones 1949, Dunn 1970, Zubair et al. 2006) due to the methylation of cytochrome p450 family 17 subfamily A member 1 (*Cyp17a1*) (Missaghian et al. 2009). As a consequence, adrenal steroid biosynthesis may only proceed in a cholesterol-pregnenolone-progesterone-deoxycorticosterone sequence, making corticosterone the main glucocorticoid in mice (Figure 2) (Dunn 1970, Miller and Auchus 2011).

A major factor regulating the development and function of the adrenal and gonadal steroidogenic cells is the steroidogenic factor 1 [SF1; also called adrenal 4-binding protein (*Ad4bp*) or nuclear receptor subfamily 5 group A member 1 (*Nr5a1*)]. SF1 regulates the expression of steroidogenic genes (Lala et al. 1992, Morohashi et al. 1992), as well as defines the definitive (differentiated) adrenal cortex (Wood et al. 2013). Its dominant function in mouse steroidogenic cell development is manifested by the fact that *Sf1* null mice do not develop either gonads or adrenal glands (Luo et al. 1994, Sadovsky et al. 1995). In contrast, heterozygous *Sf1* mutants, presented with normal gonads but smaller adrenals and impaired corticosterone release in response to stress (Bland et al. 2000). Furthermore, the over-expression of *Sf1* in mice results in the development of adrenocortical tumors that express gonadal-type markers (Doghman et al. 2007). In human, haploinsufficiency of *Sf1* may result in different levels of adrenal and gonadal insufficiency (Jameson 2004). On the other hand, *Sf1* overexpression has been found in childhood ACTs (Pianovski et al. 2006).

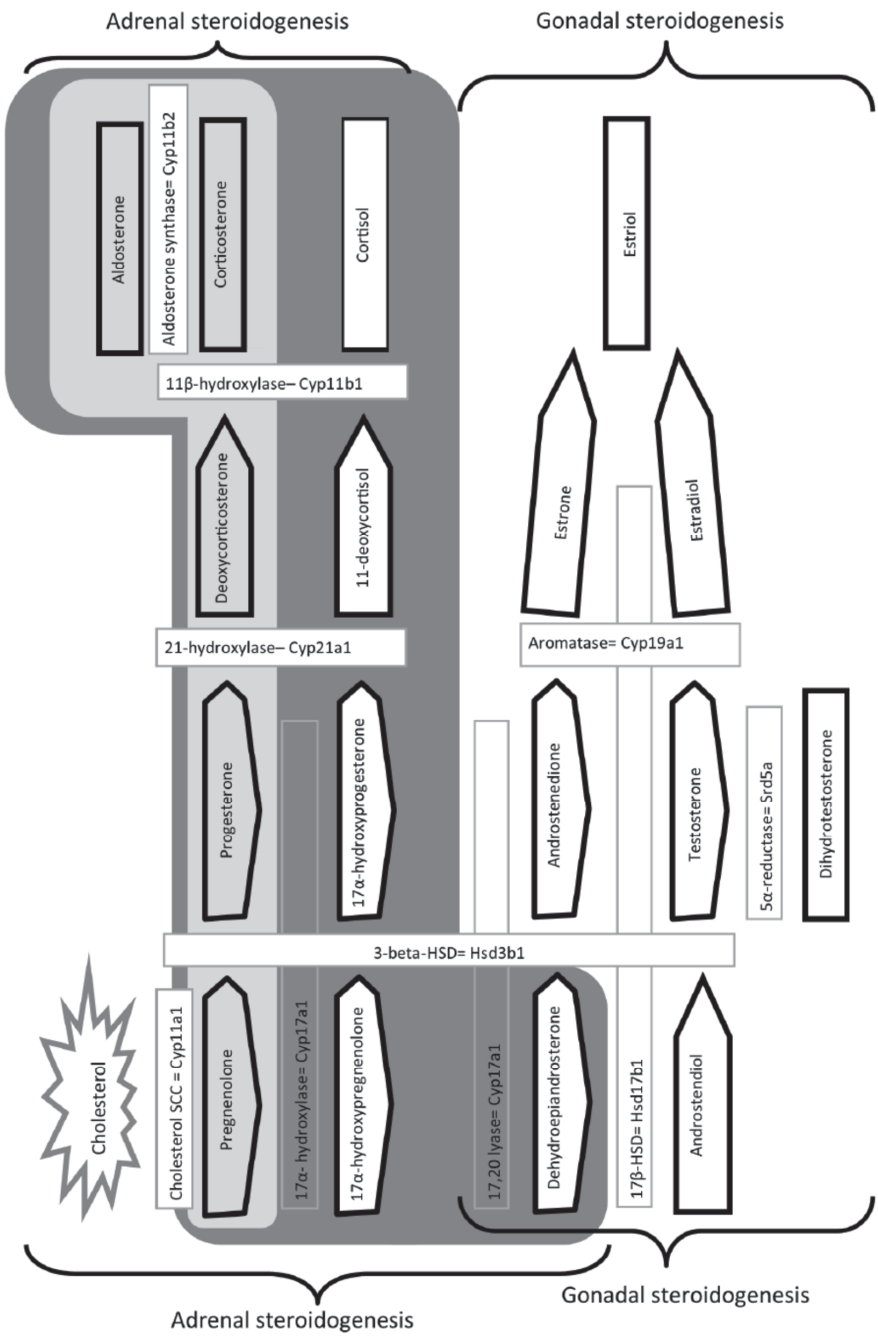

Figure 2. Steroidogenic pathways in human and mouse adrenal gland. The expression of distinct steroidogenic enzymes in human and mouse adrenals determines their ability to produce specific steroid hormones. Lack of CYP17A1 expression in mouse adrenal gland allows only the production of corticosterone aldosterone (light grey field). Human adrenal gland, beyond the production of glucocorticoids and mineralocorticoids, can also produce androgens, mainly dehydroepiandrosterone (dark grey field). Modified from (Miller and Auchus 2011)

2.1.3 GATA binding proteins in the adrenal gland development

The GATA transcription factors family consists of six members (GATA1-6) that recognize the (A/T)GATA(A/G) motif of the promoter regions of the target genes. Their essential role is expressed by the lethality of most GATA gene defects (Lentjes et al. 2016). Among this family, GATA4 and GATA6 are the key regulators of adrenal development and functions (Tevosian et al. 2015). Mice with germline null mutations in GATA4 or GATA6 die during gastrulation as they are required for the extra-embryonic endoderm development (Molkentin et al. 1997, Koutsourakis et al. 1999).

In the adult mouse adrenal gland, conflicting reports exist on GATA6 localization. Some authors show GATA6 protein localization in the capsular and subcapsular regions (Pihlajoki et al. 2013), although *in situ* RNA detection showed uniform distribution among all adrenal layers (Kiiveri et al. 1999). In contrast, GATA4 expression in the adult mouse adrenal gland is associated with the presence of neoplastic/tumor cells (Bielinska et al. 2003, Chrusciel et al. 2013, Tevosian et al. 2015). Simultaneous conditional loss-of-function mutations of GATA4 and GATA6 in the adrenal cortex under a strong adrenal *Sf1* promoter (*Sf1*-Cre), blocks the adrenal gland development. Therefore, females die shortly after birth, whereas males survive due to adrenal steroid production by the adrenal-like tissue formed in the testis (Tevosian et al. 2015). Mice lacking GATA6 in the *Sf1*-Cre model display adrenal aplasia, cytomegalic cells, lack of the xZ, and promotion of the neoplastic gonadal-like cells that express GATA4 (Pihlajoki et al. 2013). Mice lacking GATA4 in *Sf1*-Cre (Manuylov et al. 2011), or in cells expressing anti-Mullerian hormone receptor 2 (*Amhr2*-Cre model), presented with normal adrenal glands (Krachulec et al. 2012). Taken together, GATA6 is clearly a transcription factor controlling the homeostasis and development of a healthy adrenal gland, whereas GATA4 is important in adrenal development but in adult adrenal it is associated with neoplastic cells.

2.2 Tumorigenesis and cancer

2.2.1 Current theories regarding the origin of tumor cells

Spontaneous mutations in human cells occur with a frequency of 2.3×10^{-8}/ nucleotide/cell generation during DNA replication (Nachman and Crowell 2000). Oncoviruses and environmental factors, such as skin exposure to sunlight or smoking cigarettes increase the prevalence of spontaneous mutations (Fouad and Aanei 2017). Under physiological conditions, cells with DNA damage either

undergo DNA repair, or are designated to a programmed cell death, apoptosis (Houtgraaf et al. 2006). Occasionally, cells with DNA damage survive and acquire abnormal morphology, growth, ability to proliferate, and novel functions that lead to tumor development, tumorigenesis (Hanahan and Weinberg 2011, Balani et al. 2017). Tumors are divided into benign masses and carcinomas, the latter having the ability to invade and metastasize (Fouad and Aanei 2017). Two theories exist on cancer development. The first views the malignant transformation of neoplastic cells as an evolutionary process (Balani et al. 2017). The genome of a mutated tumor cell becomes unstable and accumulates novel mutations in the progeny. The most desirable changes (genetic and epigenetic) that predispose mutants to malignant growth would include genes responsible for DNA damage control, cell cycle progression, or tumor suppression. Genome instability favors diversification of mutant colonies that in turn becomes the source of heterogeneity of the cancer cells within its foci (Balani et al. 2017). Generally, it takes years for a benign mass to evolve into cancer (Balani et al. 2017, Fouad and Aanei 2017), but as recently showed in pancreatic cancer, a rapid eruption of genomic changes is also possible (Notta et al. 2016).

The second theory suggests a presence of so-called cancer stem cells. They are a sub-population of stem cells of an organ that have undergone mutation and give rise to the primary malignant cells (Balani et al. 2017). Supporting this theory is the fact that cancer stem cells and stem cells of an organ share similar phenotype and transcriptional patterns. The presence of cancer stem cells has been reported in breast, brain, colon and blood cancers (Balani et al. 2017). The attribute of dormant growth that cancer stem cells share with normal cells, makes cancer cells resistant to common chemotherapies, which disables efficient tumor treatment (Balani et al. 2017).

2.2.2 Enabling characteristics and hallmarks of tumor cells

Benign tumor and cancer cells have certain features distinguishing them from normal cells. It is considered that these hallmarks of tumor cells lay downstream to so called enabling characteristics, namely, ‘genome instability and mutation’ and ‘tumor-promoting inflammation’ (Hanahan and Weinberg 2011). Genome instability and mutation trait relates to the evolutionary theory of tumorigenesis, viewing acquired mutations as attributes for e.g. unlimited growth, escape from apoptosis, and immune system recognition. On the other hand, the tumor-promoting role of inflammation is associated with the progression of existing tumor cells. Previously, the inflammatory process was considered to be a response of the immune system to eliminate tumor cells (Hanahan and Weinberg 2011). However, in the last 20 years it has become clear that inflammation provides

valuable supplies in a form of bioactive compounds to the tumor-microenvironment (Hanahan and Weinberg 2011). Among them are growth, survival, and pro-angiogenic factors that result in sustained proliferation signals and minimized cell death, and provide nourishing vascular architecture. Moreover, extracellular matrix modifying proteins, beyond promoting the angiogenesis, prompt the invasiveness of the tumor cells and allow metastatic cell formation (Hanahan and Weinberg 2011).

The common hallmarks for benign and malignant tumor cells are the ability for continuous proliferation, bypassing the growth suppression, unlimited cell division, resistance to programmed cell death, as well as the ability to develop the vascular architecture, escaping immune destruction, and dysregulation of energy metabolism (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011, Fouad and Aanei 2017). However, the abilities of invasion and metastasis are mainly hallmarks of cancer cells (Fouad and Aanei 2017).

The most crucial property of a tumor cell is the ability of continuous proliferative signaling. The proliferation of healthy cells is strictly regulated to ensure tissue homeostasis. Tumor cells sustain proliferative signaling by, for example, producing growth factors themselves or stimulating neighboring cells to produce them, as well as by increasing the number of receptors for growth factors (Hanahan and Weinberg 2000). Tumor cells can bypass growth suppression also by inhibiting the function of tumor suppressor genes (Hanahan and Weinberg 2011, Fouad and Aanei 2017). The major growth suppressor gene, which is found mutated in over 50% of sequenced tumor samples, is the tumor suppressor p53 (*TP53*) (Fouad and Aanei 2017). The p53 protein is a fundamental factor that initiates the repair, or if the damage is impossible to fix, directs the cell towards senescence/apoptosis (Levine et al. 1991). The lifetime likelihood of developing tumor when harboring p53 inactivating mutations is 75% in men and nearly 100% in women (Guha and Malkin 2017).

Normal cells have a limited number of cell divisions before senescence or apoptosis is induced. Tumor cells have unlimited division capacity and are reluctant to cell senescence, which effectively makes them immortal (Hanahan and Weinberg 2000, Fouad and Aanei 2017). Tumor cells also develop mechanisms that allow them to avoid programmed cell death by means of genetic and epigenetic changes, such as loss of pro-apoptotic genes, and up-regulation of anti-apoptotic genes (Hanahan and Weinberg 2011). A good example is the loss of p53 that makes the cell insensitive to numerous apoptotic stimuli (Fouad and Aanei 2017). However, once the apoptotic signals are induced, tumor cells are more prone to undergo apoptosis than normal cells. This phenomenon could be related to the evolution of the tumor cells, namely, eliminating not-well-suited lineages and clearing the area for the well-fitted mutants that promote tumor progression (Labi and Erlacher 2015).

Vascularization of the tumor is crucial for its growth beyond 3 mm (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011, Fouad and Aanei 2017). This process is mainly maintained through sprouting, division, and migration of the endothelial cells from already existing vessels, in a process called angiogenesis (Carmeliet 2000). The major factor for angiogenesis is hypoxia, which induces hypoxia-inducible transcription factors (HIFs) that sequentially activate pro-angiogenic factors like vascular endothelial growth factor (VEGF). Tumor vasculature has quite different properties, such as irregular endothelium that causes an increased permeability of the vessels. In addition, the blood flow is disorganized, leaving some parts of the tumor hypoxic, which in turn may stimulate angiogenesis or mutagenesis (Fouad and Aanei 2017).

According to the cancer immune surveillance theory, immune cells recognize and eliminate potentially dangerous (e.g. mutated) cells (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011, Fouad and Aanei 2017). Therefore, tumor cells that escape the immune system recognition undergo so-called "immune-editing", either by the absence of tumor-antigen recognition or resistance to apoptosis, or by production and release of immuno-suppressing molecules (Fouad and Aanei 2017). This 'escape' plays a crucial role during the initial tumor formation and progression (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011, Fouad and Aanei 2017).

The difference between normal and tumor cells in energy metabolism has been reported nearly a century ago (Warburg 1930). As their main source of energy, cancer cells utilize glycolysis instead of the 18-fold more efficient mitochondrial oxidative phosphorylation (Hanahan and Weinberg 2011). The rationale to this inefficient glycolytic switch is that increased glycolysis increases the levels of glycolytic intermediates that can be used to various biosynthetic processes, facilitating the production of organelles and macromolecules that are necessary for the proliferating cell (Vander Heiden et al. 2009).

The major properties differentiating benign tumor cells from malignant cells are invasion and metastasis (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011, Fouad and Aanei 2017). Patients rarely die of primary tumors, whereas over 90% of cancer-related deaths are caused by a metastatic disease (Steeg 2006). The cell has to develop a series of adaptations to leave the native organ and to be able to grow in a distant organ. This sequence is referred to as 'invasion-metastasis cascade'. It is a multi-step process that includes local invasion, intravasation into lymphatic and blood vessels, passage through the lymphatic and blood circulation, and extravasation into the destination tissue (Hanahan and Weinberg 2000, Hanahan and Weinberg 2011, Fouad and Aanei 2017).

2.3 Adrenocortical tumors

Adrenocortical tumors (ACT) are relatively common neoplasms observed in ~6% of the aging population. Majority of them are adrenocortical adenomas (ACA) with a prevalence of around 5% in a cohort over the age of 50 (Thompson and Young Jr 2003), whereas prevalence of fatal adrenocortical carcinoma (ACC) is 0.7-2 cases/million/year (Allolio and Fassnacht 2006, Kirschner 2006, Fassnacht and Allolio 2009). ACCs have a bimodal distribution, either clustering in adults aged 40-50, more commonly in women than in men, or in children under the age of 10 (Kirschner 2006, Marques-Pereira et al. 2006). ACTs can be divided into silent, and hormonally active masses. The silent ones, referred to as incidentalomas, are symptom-free and found 'by incident' either during examinations irrelevant to ACT diagnosis, or during autopsy (Arnaldi and Boscaro 2012). Hormone producing masses represent 20% of ACAs and are associated with hypercortisolism (Cushing syndrome), mineralocorticoid excess (Conn's syndrome), or/and hyperandrogenism (virilizing syndrome) (Lacroix et al. 2010, Arnaldi and Boscaro 2012). The most common clinical treatment is the tumor resection surgery (Creemers et al. 2016). Possible further adjuvant or post-operative radiation treatments are applied after the type of the tumor is established, on the basis of its pathological and molecular features (Creemers et al. 2016).

2.3.1 Differential diagnosis

Distinguishing between malignant ACCs and benign ACAs is a challenging task, but crucial for successful treatment (Fassnacht and Allolio 2009). Difficulties in the identification of ACCs, combined with the lack of efficient therapies, result in a 5-year survival rate of only 10-25% (Bernichtein et al. 2008b). Because the prevalence of ACAs is over 25'000-fold higher than that of ACCs, only very few ACAs undergo malignant transformation. The hypothesis is that ACTs are mostly an effect of multiple chromosomal alterations that additively lead towards cancer development (Hanahan and Weinberg 2011). This makes ACCs a highly heterogeneous group of tumors (Allolio and Fassnacht 2006, Fassnacht and Allolio 2009).

The most commonly used histopathological parameter to differentiate ACCs from ACAs is the Weiss score (Fassnacht and Allolio 2009). It combines tumor structure (cytoplasm, architecture and necrosis description), cytology (cellular atypia, count of mitotic figures and the presence of abnormal mitoses), and invasion (vasculature, sinusoids, tumor encapsulation). Moreover, the MKI67 score can be helpful in differentiating between benign and malignant tumors as well as give the outcome prognosis for the latter (Fassnacht and Allolio 2009).

In recent years, a great effort has been made to identify dysregulated molecular pathways in ACTs, which has resulted in the discovery of novel candidate genes for ACT distinction and malignant transformation (Assie et al. 2014, Zheng et al. 2016). The factors resulting in hormone excess symptoms have been broadly examined to aid patients with functional ACAs (Lacroix et al. 2010).

2.3.2 The molecular genetics of adrenocortical tumors

High-throughput data experiments on large ACT cohorts reveal many novel mutations linked to the pathogenesis of adrenal tumors. Dysregulation of Wnt/β-catenin signaling is very frequent. It involves β-catenin (*CTNNB1*) activating mutations, or loss-of-function mutations in Wnt pathway inhibitors, which include zinc and ring finger 3 (*ZNRF3*), and kringle containing transmembrane protein 1 (*KREMEN1*) (Tissier et al. 2005, Espiard and Bertherat 2015, Zheng et al. 2016). Other common alterations that could be named are tumor suppressor p53 gene (*TP53*) germline (6% of adult and 80% of pediatric ACT), and somatic (25–70% of adult ACC) mutations (Lerario et al., 2014). Inactivation of p53 in both alleles enables cells to progress through the cell cycle despite their DNA damage (Levine et al., 1991), which leads to tumor formation (Lerario et al. 2014).

The most common hormone producing ACTs are those with an inactivating mutation of armadillo repeat containing 5 (*ARMC5*), and those with an activating mutation in protein kinase A catalytic subunit (*PRKACA*) – both of which cause cortisol over-production (Lerario et al. 2014, Espiard and Bertherat 2015). Aldosterone over-production can be associated with an increased cytosolic calcium levels caused by abnormal membrane depolarization e.g. due to mutation in potassium voltage-gated channel subfamily J member 5 (*KCNJ5*) (Lerario et al. 2014, Espiard and Bertherat 2015). Moreover, dysregulated expression of the following genes have been documented: insulin-like growth factor 2 (*IGF2*) (Espiard and Bertherat 2015), inhibin-α (*INHA*) that belongs to the transforming growth factor beta family (TGF-β) (Hofland et al. 2006), and *GATA* family transcription factors (Kiiveri et al. 2005). Relevant adrenocortical tumor mouse models and their underlying mechanisms of tumorigenesis are discussed below in detail.

2.3.3 Impaired adrenal function due to LH/hCG action

Normal human fetal and adult adrenal glands express low levels of luteinizing hormone receptor (LHCGR) in zR that produces DHEA-S upon hCG stimulation. However, the physiological role of LHCGR in adult adrenal remains

unknown (Pabon et al. 1996, Carlson 2007, Lacroix et al. 2010). During adrenocortical tumor (ACT) development, chronically elevated LH or human chorionic gonadotropin (hCG) concentrations may up-regulate the expression of LHCGR, and functionally result in cell proliferation and/or hormone production (Carlson 2007, Bernichtein et al. 2008b, El Ghorayeb et al. 2015). This phenomenon occurs occasionally in pregnant or post-menopausal women, leading to an over-production of cortisol and subsequent Cushing syndrome (CS) development. Moreover, LH/hCG stimulation can cause an over-production of androgens or aldosterone in ACTs (Carlson 2007), as well as DHEA-S in H295R adrenocortical tumor cells (Rao et al. 2004).

In the murine adrenal gland, the expression of LHCGR as well as gonadotropin action is only known within the context of pathological changes. Transgenic expression of luteinizing hormone beta subunit (*Lhb*) fused with the human chorionic gonadotropin β-subunit C-terminal peptide in *bLHβ*-CTP mice (Risma et al. 1995), resulted in up to 15-fold higher circulating LH levels, 80% larger adrenal gland and 14-fold corticosterone levels as compared with the wild-type littermates (Kero et al. 2000). Also, LHCGR knockout (LuRKO) mice have been produced by inactivation of the exon 11 of *Lhcgr* gene (Zhang et al. 2001). However, its adrenocortical phenotype was never investigated.

Moreover, aging animals of all mouse strains develop basophilic, non-steroidogenic, spindle-shaped cells that descend in a wedge shape from the sub-capsular area towards the peri-medullary region. These are called type A cells and they do not form tumors (Bielinska et al. 2003, Johnsen et al. 2006, Krachulec et al. 2012, Chrusciel et al. 2013). The abundancy of A cells notably increases after GDX (Bandiera et al. 2013), which may be stimulated by high levels of LH. The A cells resemble the human ovarian theca-like cells that are seen in human in a metaplasia of the adrenal gland (Fidler 1977). Recently, it has been shown that the A cells originate from the capsular cells expressing GLI1 (GLI-Kruppel family member GLI1) (Dörner et al. 2017) (Figure 3).

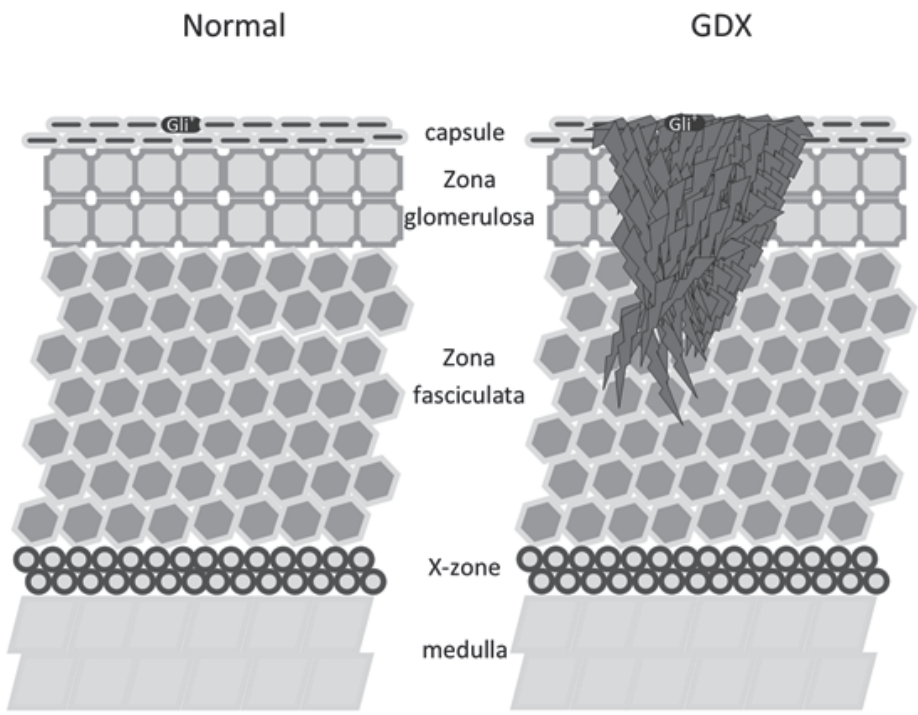

Figure 3. Current view on the ontogeny and appearance of sub-capsular A cells

Sub-capsular A cells originate form GLI1-positive pool of stem/progenitor cells in the capsule of aging or GDX mice and migrate from the sub-capsular region towards the adrenal peri-medullary region (Dörner et al. 2017).

In consistence with the human cases, increased LH levels trigger adrenocortical tumor formation in certain inbred and transgenic mouse strains (Matzuk et al. 1994, Kananen et al. 1996b, Kumar et al. 1996, Rilianawati et al. 1998, Kumar et al. 1999, Bielinska et al. 2003, Bielinska et al. 2005, Krachulec et al. 2012, Chrusciel et al. 2013, Basham et al. 2016). In contrast to humans, these tumors mostly present with the gonadal-like gene expression profile and produce sex steroids. Tumorigenesis in these mice depends on GDX, hence they are referred to as models of genetic susceptibility to gonadectomy-induced adrenocortical tumorigenesis (GIACT). The mouse strains considered susceptible to GIACT are DBA/2J, B6D2F1, NU/J, BALB/c, CE/J, and C3H (Basham et al. 2016). Conversely, C57Bl/6 and FVB/N mice are considered genetically non-susceptible to GIACT (Basham et al. 2016).

2.3.4 Gonadotropin releasing hormone analogue treatments for hormone symptom suppression

Adrenal over-function due to LH/hCG action can efficiently be blocked by the ablation of circulating gonadotropins (Lacroix et al. 1999, Carlson 2007, Hammer et al. 2015). For this purpose, gonadotropin-releasing hormone (GnRH) analogues have been used, namely GnRH agonists and GnRH antagonists (Lacroix et al. 1999, Lacroix et al. 2010, Manuylov et al. 2011). GnRH agonists bind to the GNRHR, initially causing the release of high levels of gonadotropins, both LH and follicle stimulating hormone (FSH), with a subsequent long-term desensitization of the receptor resulting in gonadotropin blockage. Among possible common side effects of the GnRH agonist treatment are hot flashes and flare phenomenon, which may cause spinal cord compression that may lead to paralysis (Limonta et al. 2012). On the other hand, GnRH antagonist binds to the GNRHR in the pituitary, and by blocking the receptor immediately ablates gonadotropin release. As of current, various types of antagonists have been developed, providing a blockage lasting from few days (e.g., cetrorelix) up to 60 days (degarelix) without the flare effect (Limonta et al. 2012). In recent years, GNRHR has also been localized in peripheral tissues (splenocytes, thymocytes, lymphocytes, and ovarian granulosa cells), as well as in tumor tissues (prostate, ovary, breast, endometrium, lung, pancreas, melanoma, glioblastoma, and adrenal gland), which makes them a direct target of GnRH analogue action (Ziegler et al. 2009, Limonta et al. 2012, Seitz et al. 2014, Sakai et al. 2015). Interestingly, as displayed on tumor cells, GnRH antagonists can evoke a signal transduction, therefore acting in non-pituatary cells as receptor agonists (Limonta et al. 2012). In tumor cells, direct effects of GnRH analogues are manifested in decreased cell proliferation, metastatic ability, and angiogenesis (Limonta et al. 2012).

In human, the GNRHR has been localized in healthy adrenal glands (medulla and cortex), SW13 adrenocortical carcinoma cell lines (Ziegler et al. 2009), and adrenocortical adenomas (Ziegler et al. 2009, Lacroix et al. 2010, Albiger et al. 2011, Nakamura et al. 2014). Recently, it has been shown that GnRH and its analogues may stimulate the expression of cytochrome p450 family 11 subfamily B member 2 (CYP11B2), resulting in aldosterone production (Albiger et al. 2011, Nakamura et al. 2014), which ultimately proves that the adrenal gland can be a direct target of GnRH analogue action.

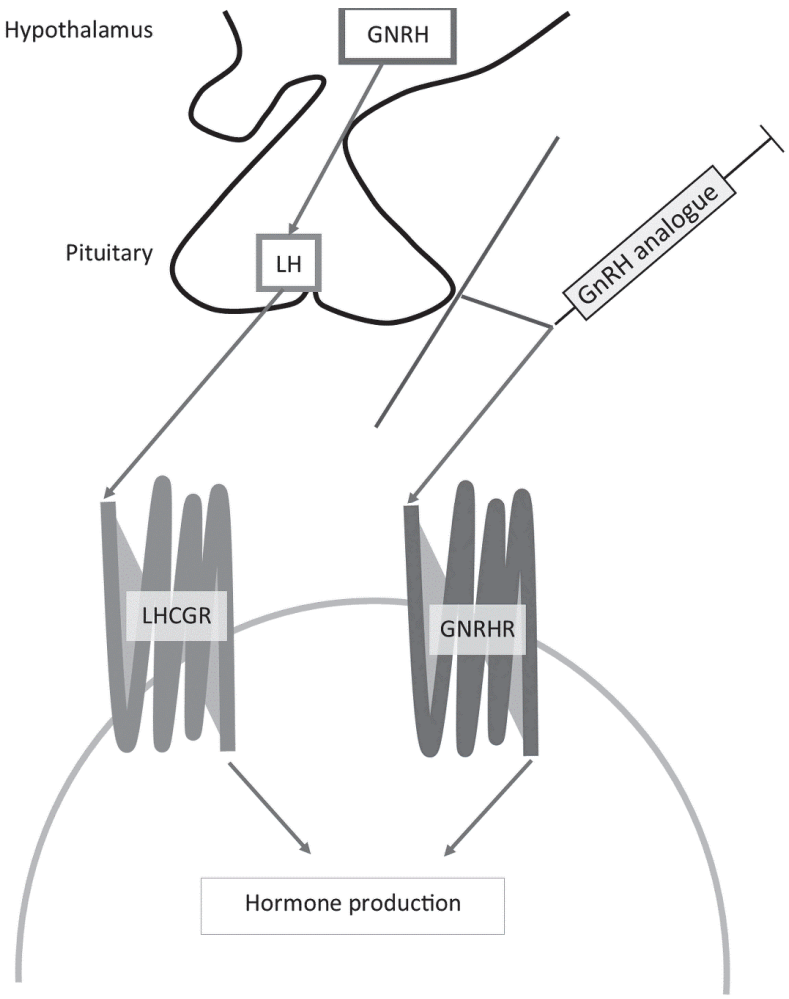

Figure 4. Human adrenal gland as a target for gonadotropin action- a schematic view.

Human ACTs expressing LHCGR are responding to LH/hCG stimulation by hormone production. To abolish this effect, GnRH analogues can be used. Systemically, they block LH release but in certain cases, they may stimulate Cyp11b2 expression resulting in aldosterone release.

2.4 Mouse models to study gonadotropin dependent tumorigenesis

GIACT models develop adrenal tumors of gonadal-like phenotype (Matzuk et al. 1994, Kananen et al. 1996b, Kumar et al. 1996, Rilianawati et al. 1998, Kumar et al. 1999, Bielinska et al. 2003, Bielinska et al. 2005, Krachulec et al. 2012, Chrusciel et al. 2013, Basham et al. 2016). This phenomenon is attributed to the common origin of gonads and adrenals (see 2.1.1 Development of the adrenal gland and its functional zonation), and to the fact that they express similar sets of transcription factors. However, gonadal-like phenotype of adrenocortical tumors

in GIACT is most probably associated with the expression of GATA4 in tumor cells. An opposite phenomenon could be observed, when high circulating ACTH concentration induces human testicular adrenal rest tumors (TART) (Claahsen-Van der Grinten et al. 2009). These TART tumors express adrenal-type markers such as cytochrome p450 family 11 subfamily B member 1 (CYP11B1), cytochrome p450 family 21 subfamily A member 2 (CYP21A2), MC2R, and GATA6 (Claahsen-Van der Grinten et al. 2009).

2.4.1 Adrenocortical B cells

Gonadectomy-induced adrenocortical tumors of inbred (DBA/2J) or transgenic (21-OH-GATA-4) mouse strains develop tumors consisting of type A and type B cells. B cells are large, lipid-laden, steroidogenic cells that form in between the patches of A cells (Bielinska et al. 2005, Krachulec et al. 2012, Chrusciel et al. 2013). Previous studies have shown that the susceptibility to adrenal tumor formation is an effect of intrinsic genetic traits (Bernichtein et al. 2008a). Experiments with conditional GATA4 knockout (Krachulec et al. 2012), and transgenic over-expression of GATA4 under adrenal-specific 21-hydroxylase (*Cyp21a1*) promoter (21-OH-GATA-4) (Chrusciel et al. 2013), have shown that GATA4 plays a crucial role in triggering the appearance of B cells. However, there is no tumor development in intact 21-OH-GATA-4 mice, suggesting that in addition to GATA4, additional hormonal/genetic factors may be needed for efficient tumorigenesis (Chrusciel et al. 2013).

The B cells express an array of gonadal factors such as GATA4, *Lhcgr*, *Cyp17a1*, cytochrome p450 family 19 subfamily A member 1 (*Cyp19a1*), and anti-Mullerian hormone (*Amh*) and its receptor (*Amhr2*) (Bielinska et al. 2003, Krachulec et al. 2012, Chrusciel et al. 2013). These genes are involved in steroidogenic activity that leads to estrogen production (Bielinska et al. 2003, Bielinska et al. 2005, Johnsen et al. 2006).

2.4.2 Adrenocortical tumors in Inha knockout mice ($Inha^{-/-}$)

Genetically modified inhibin alpha knockout mice ($Inha^{-/-}$) develop fatal gonadal tumors by the age of 4 weeks, but when they are GDX, they develop malignant adrenocortical tumors (Matzuk et al. 1994). When crossbred with mice lacking gonadotropin-releasing hormone (*Gnrh*; *hpg* mice), $Inha^{-/-}$ mice do not develop tumors, which ultimately proves that gonadotropins are upstream factors in tumorigenesis (Kumar et al. 1996). Moreover, $Inha^{-/-}$ mice, intercrossed with *bLHβ*-CTP mice (Risma et al. 1995), show 6.7-fold higher circulating LH levels,

resulting in an earlier onset, faster progression, and higher lethality of mice bearing gonadal and adrenocortical tumors, when compared with *Inha*$^{-/-}$ mice (Beuschlein et al. 2003). Additionally, the intercross of *Inha*$^{-/-}$ with *FSHβ* knock out mice (Kumar et al. 1997) does not prevent gonadal tumor formation, indicating that LH is the key gonadotropin leading to tumorigenesis in that model (Kumar et al. 1999). Consecutively, a study revisiting the adrenocortical tumor ontogeny in *Inha*$^{-/-}$ model showed that these tumors originate from the pool of stem/progenitor cells, but not from the xZ as initially suggested. Moreover, it was suggested that the key factor for malignant transformation of adrenocortical cells is gonadotropin-mediated transcription factor switch from GATA6 to GATA4 (Looyenga and Hammer 2006).

2.4.3 Inhα/Tag mouse model

Inhα/Tag mice develop gonadal and adrenocortical tumors under inhibin alpha (*Inha*) promoter-controlled expression of Simian Virus T-antigen (SV40Tag) oncogene (Kananen et al. 1995, Kananen et al. 1996b). Functionally, SV40Tag binds to the tumor suppressor p53 and retinoblastoma (RB) proteins. This blocks the function of p53 as an apoptotic gatekeeper, and RB's downstream inhibition of cell cycle-promoting genes (Hudson and Colvin 2016). These two mechanisms result in the development of highly proliferative tumors (Hudson and Colvin 2016). Intact inhα/Tag mice develop gonadal tumors by the age of 5-6 months, but when prepubertally GDX, they develop adrenocortical tumors by the age of 5-8 months (Kananen et al. 1995, Kananen et al. 1996a, Kananen et al. 1996b, Chrusciel et al. 2014). Similar to the *Inha*$^{-/-}$ model (Kumar et al. 1996), crossbreeding inhα/Tag with *hpg* mice as well as chemical ablation of gonadotropins prevent adrenocortical tumor formation (Rilianawati et al. 1998). Also, inhα/Tag intercrossed with *bLHβ*-CTP mice, develop gonadal and adrenocortical tumors simultaneously (Mikola et al. 2003). Based on the above-mentioned data, adrenocortical tumorigenesis in inhα/Tag mice appears to be gonadotropin-dependent. The expression of SV40Tag allowed the isolation and establishment of an adrenocortical tumor Cα1cell line (Kananen et al. 1996b), and its utilization as an *in vitro* model (Rilianawati et al. 1998, Rilianawati et al. 2000, Rahman et al. 2004). Both adrenocortical tumors and Cα1 cells express abundantly an array of gonadal factors, including *Lhcgr* and *Gata4* (Kananen et al. 1996b, Bielinska et al. 2003, Rahman et al. 2004). However, the inter-relationship between *Gata4*, *Lhcgr,* and hyperplasia–adenoma–adenocarcinoma transition is yet to be elucidated.

3 AIMS OF THE STUDY

1. To identify and validate novel genes for adrenocortical tumorigenesis in inhα/Tag mice.
2. To establish the ontogeny and characterize LH/LHCGR signaling and GATA4 impact on tumorigenesis in inhα/Tag mice.
3. To analyze the molecular mechanisms underlying the GnRH-antagonist cetrorelix action on adrenocortical tumors in vitro and in vivo.
4. To characterize the role of LH/LHCGR signaling in pregnancy-induced Cushing syndrome

4 MATERIALS AND METHODS

4.1 Model animals (I, II, III)

Inhα/Tag transgenic mice were used as a model for adrenocortical tumorigenesis (I, II, III) (Kananen et al. 1996b). For tumor onset studies we crossed inhα/Tag with LuRKO mice (II) (Zhang et al. 2001). C57Bl/6N wild-type (WT) littermates were used as control animals (I, II). Mice were genotyped using DNA isolated from the ear biopsies by PCR as described earlier (Kananen et al. 1995, Zhang et al. 2001). Mice were gonadectomized (GDX) prepubertaly (I, II, III) at 21-24 days of age under isoflurane anesthesia (2-4%) (Isoflo, Orion Pharma, Turku, Finland). Before the surgery mice were injected subcutaneously with analgetic Temgesic (buprenorphine, 0.1 mg/kg/8 h) (Schering-Plough, Brussels, Belgium) and after the surgery with Comforion® (ketoprofen, 5 mg/kg/24h) (Orion, Turku, Finland). All experimental mice (n=7-14/group) were sacrificed by terminal cardiac puncture under isoflurane anesthesia. Blood was collected into a tube containing 0.5 M sterile EDTA solution, centrifuged at 3000 RPM for 10 minutes in 4 °C and separated plasma was stored in -80 °C. Tissues weights were recorded, adrenals snap-frozen in liquid nitrogen and/or fixed with 4% paraformaldehyde (PFA). Mice were housed in a room with controlled light (12 h light and 12 h darkness) and temperature (21±1 ^{0}C) in a specific pathogen-free surrounding. Mice were fed with commercial mouse chow SDS RM-3 (Witham, Essex, UK) and tap water ad libitum. Animal experiments were approved by the Ethics Committee for Animal Experimentation of the State Provincial Office of Southern Finland (ESAVI/3324/04.10.07/2014).

4.2 Human tissue samples (III)

Snap frozen (n=11) and formalin fixed paraffin blocks (n=13) from human adrenocortical carcinomas or healthy adrenal samples (n=3) were obtained from the Department of Pathology, Charité Berlin, Germany archive. The clinical information (age, sex and ENSAT score) was received from the Department of Clinical Endocrinology, Charité Berlin, Germany. The patients have provided a written consent and the Ethic Committee of the Charité University Hospital, Germany has approved the studies (No. EA1/169/08).

4.3 Clinical diagnostic evaluations (IV)

The subject of our case report is a 22-year old pregnant woman with Cushing syndrome (CS) symptoms. To find the mechanisms underlying her CS, she was subjected to a series of clinical evaluations (Table 1). A detailed description of clinical methods is available in the original publication IV.

4.3.1 In vivo tests

Table 1. The clinical differential diagnostic tests performed for the CS patient (detailed in publication IV).

CRH and dexamethasone suppression test
Aberrant hormone receptor testing *in vivo*
Stimulation with recombinant human chorionic gonadotropin (rhCG)

4.3.2 Gene mutation analysis

Potentially mutated regions were sequenced using gDNA and/or cDNA templates from adrenal (somatic) and lymphocyte (familial) samples according to the description given by the authors listed in Table 2:

Table 2. Sequencing methods

Gene name	**Described by**
CTNNB1	(Teo et al. 2015)
PRKAR1A	(Groussin et al. 2006)
ARMC5	(Assié et al. 2013)
PRKACA	(Thiel et al. 2015)

4.4 Gene expression analysis

4.4.1 Microarray and in silico analyses (I, III)

Total RNA from adrenal glands of 7 mo GDX WT, inhα/Tag and cetrorelix acetate (CTX, Sigma-Aldrich, Saint Louis, MO, USA) treated inhα/Tag mice was

isolated using RNeasy Mini Kit (Qiagen, Germantown, MD, USA). RNA was resuspended in 50 µl of nuclease-free water (Promega, Madison, WI, USA), quantified (NanoDrop; Thermo Fisher Scientific, Waltham, MA, USA) and quality-controlled using Bioanalyzer nano kit (Agilent Technologies, Santa Clara, CA, USA). The stock of RNA was divided into two volumes in order to use the same template for both microarray and gene expression validation by qPCR. RNA was transcribed (n = 4/group) by MessageAmp II aRNA Amplification Kit (Thermo Fisher Scientific) and applied to Agilent whole mouse genome oligo microarrays 4 × 44K (#GPL7202, Agilent Technologies) according to a provided protocol. The data was loaded into the Iimma R/Bioconductor package, normalized between arrays using the quantile normalization, and after performing a row-wise t-test, fold changes were log2-transformed. For identification of potential novel genes involved in adrenocortical tumorigenesis (I) the fold change higher than 1.5 fold and p-value lower than 0.05 were considered as differently expressed. Heat maps were generated using the gplot package in R. The list of significantly differentially expressed genes were uploaded to GOrilla (Eden et al. 2009) and separate, process-based enrichment lists for up- and down-regulated genes were generated. For identification of genes affected in adrenocortical tumors by CTX treatment (III) fold change higher than 1.5- and p-value lower than 0.10 were considered significant. Significantly altered targets for males and females were compared in Venn diagram (http://bioinfogp.cnb.csic.es/tools/venny/). The list of treatment-affected targets was uploaded to the PANTHER classification system (Mi et al. 2013) and statistical overrepresentation test was performed. Genes were classified using PANTHER GO-Slim Biological Processes and PANTHER pathways annotation data sets.

4.4.2 RNA isolation

If not stated otherwise, total RNA was isolated using TRIsure (Bioline Reagents Ltd., London, UK) reagent accordingly to the provided protocol, RNA was quantified using Nanodrop (Thermo Fisher Scientific, Waltham, MO) and quality-controlled by gel electrophoresis. RNA was treated with Amplification Grade DNase I (Thermo Fisher Scientific [I]; Sigma-Aldrich [II, III, IV]) and transcribed (1h in 48°C) using DyNAmo™ cDNA Synthesis Kit (#F470, Thermo Fisher Scientific) (I, IV) or SensiFAST™ cDNA Synthesis Kit (#BIO-65053, Bioline Reagents Ltd.) (II, III).

4.4.3 Quantitative real-time PCR (qPCR) (I, II, III, IV)

qPCR experiments were carried out on a CFX96 or CFX384 Real Time PCR Detection System (BioRad, Vienna, Austria), using the DyNAmo™Flash SYBR® Green qPCR Kit (#F415, Thermo Fisher Scientific) in conditions accordingly to the protocol. Primer sequences and their annealing temperature (determined empirically by gradient qPCR) are listed in Supplemental data of the publications (I, II, II, IV). Each reaction was run in duplicates with 15 ng of cDNA template in total reaction volume of 20 µl (I, IV) or with 7.5ng cDNA in volume of 10µl (II, III). Additionally, each sample was run with primers for potential reference genes: cyclophilin A (*Ppia*, *PPIA*), β-glucuronidase (*Gusb*, *GUSB*), hypoxanthine phosphoribosyltransferase (*Hprt1*), peptidylprolyl isomerase B (*Ppib*) and hydroxymethylbilane synthase (*Hmbs*), 18s RNA subunit (*18sRNA*), or actin beta (*ACTB*) that were tested using Bio-Rad CFX Manager software (BioRad). Treshold cycle (Ct) of gene of interest was normalized by the geometric mean of 2-4 reference genes using qBase MSExcel VBA applet (Hellemans et al. 2007).

4.5 Protein and RNA localization

4.5.1 Immunohistochemistry (I, II, III, IV)

PFA (mouse tissues) or formalin (human tissues) -fixed paraffin embedded tissues were sectioned 5±1 µm and stored in darkness at +4 °C. Prior to the staining, sections were deparafinized and hydrated. Antigens were retrieved in 10 µM citrate buffer (pH6), washed in TBS with 0.1% Tween20 (#P1379, Sigma-Aldrich, Saint Louis, US-MO) or PBS with 0.1% Tween20 (PBS-T). Primary and secondary antibodies used are listed in the original publications I, II, III, IV. Horseradish peroxidase signal was visualized by 10 min incubation with Liquid DAB + Substrate Chromogen System (Dako, Glostrup, Denmark). Slides were scanned by Pannoramic 250 Slide Scanner (3DHISTECH Ltd., Budapest, Hungary) and pictures were taken using Pannoramic Viewer (3DHISTECH Ltd.).

4.5.2 Multichannel immunofluorescence (IV)

PFA-fixed paraffin sections (5±1 µm) were deparaffinized, hydrated and boiled in 10 µM citrate buffer (pH 6.0) to retrieve antigens, washed in PBST. Sections were blocked for 1 hour with 3% BSA, 10% normal donkey serum in

PBST. Sections were incubated at 4 °C overnight with the combination of primary antibodies diluted in blocking buffer (IV, Supplemental Table 7A). As secondary antibodies we used a 1:250 dilution of donkey-anti mouse, rabbit or goat IgG conjugated with Alexa488, 546, 594 or 647 dyes (Life technologies, Carlsbad, CA, USA). The slides were mounted in mounting medium with DAPI (SantaCruz Biotechnology, Dallas, TX, USA) and pictures were taken with Zeiss Axioimager M1 microscope (Zeiss, Jena, Germany).

4.5.3 RNAscope in situ hybridization (I, III, IV)

RNAscope® 2.0 HD (IV) or 2.5 HD Reagent Kit-BROWN (I, III) (Advanced Cell Diagnostics, Newark, CA, USA) was used for *in situ* hybridization (ISH) (Wang et al. 2012) with predesigned probes for *Lhcgr*, peptidyl-prolyl cis-trans isomerase (*Ppib*, positive control probe, # 313911), *GNRHR* (#407999), *LHCGR* (#300031), *FSHR* (#408101), *PPIB* (positive control probe, #313901), *POLR2A* (positive control probe, #310451) and nonsense dapB (from Bacillus S., #310043). Hybridization was performed according to the manufacturer's protocol in HybEZ (TM) Oven (Advanced Cell Diagnostics). Slides were scanned by Pannoramic Midi FL slide scanner (3DHISTECH Ltd.) and pictures were taken using Pannoramic Viewer (3DHISTECH Ltd.).

4.6 Hormone analysis

Hormone concentrations were measured from the blood plasma or cell culture media.

4.6.1 Luteinizing hormone determination (I, II, III)

LH concentrations were measured by immunofluorometric assay (DELFIA; Perkin Elmer, Waltham, MA, USA) as described previously (Haavisto et al. 1993). The detection limit was 0.0075µg/l, whereas intra- and inter- assay coefficients of variation (CV) were below 10%.

4.6.2 Testosterone and progesterone measurements (I, II, III)

Progesterone and testosterone concentrations were analyzed by Elecsys® Progesterone II and Testosterone II immunoassays (Roche Diagnostics, Basel,

Switzerland), using Cobas e411 immunoanalyzer (Roche Diagnostics). The detection limits for progesterone and testosterone were 0.10 nmol/l and 0.09 nmol/l, whereas intra- and inter- assay CV did not exceed 10%.

4.6.3 cAMP production (II, IV)

Extracellular cAMP concentrations were measured by a standard radioimmunoassay method after sample acetylation according to the method of Harper and Brooker (Harper and Brooker 1975, Brooker et al. 1979). Cell culture medium was collected after 1 h stimulation, mixed 1:1 with 2 mM teophylline (Sigma-Aldrich), boiled in a water bath for 5 minutes and frozen in -20 °C. Pellet radiation was read using γ-counter and automatically subtracted to a standard curve (Wallac 1470 Wizard Gamma Counter, Perkin Elmer).

4.6.4 Cortisol, ACTH and hCG measurements (IV)

Immunoassays to determine plasma levels of cortisol (CLIA, Bayer Diagnostics, München, Germany), free urinary cortisol (CMIA, Abbot, Düsseldorf, Germany), ACTH (Immulite 2000, Siemens Medical, Llanberis, UK) and hCG (Cobas, Roche Diagnostics, Mannheim, Germany) were performed accordingly to manufacturers' protocol in duplicates. Detection limits were as follows, cortisol, 25.6 nmol/liter; free urinary cortisol, 2.21 nmol/liter; ACTH, 5 ng/L; hCG, <0.6 IU/liter. Intra- and inter- assay CV for these assays were below 8%.

4.7 *In vitro* studies

4.7.1 Cell lines and cultures

Mycoplasma-free murine Cα1 (II, III), Y-1 and human H295R (III) adrenocortical tumor cell lines were used. Cells were cultured in DMEM/F12 (#D2906, Sigma-Aldrich) culture media containing 5U/ml of penicilin/streptomycin (#15140-122, Gibco), supplemented for each cell line as follows, Cα1 10% fetal bovine serum (FBS); Y-1 15% fetal horse serum (FHS) and 2.5% FBS; H295R 2.5% NuSerum (#355100, Corning, New York, NY, USA) and 1x Corning™ ITS Premix Universal Culture Supplement (#354352, Corning).

4.7.2 Viability test (III)

Viability of the cells was assessed using MTS CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega). Cells were seeded on 24 well plates and after 48h of incubation, medium was changed for DMEM/F12 medium containing MTS reagent. After 240 min (4h) of incubation in 37°C absorbance at 495nm was read using Wallac 1420 Victor2 Microplate Reader (Perkin Elmer, Turku, Finland). After the blank was subtracted, the treatment groups were normalized by average control well values and presented as the percent of control.

4.7.3 Proliferation test (II, III)

Cells were seeded on 96 well plates and after 48h of incubation medium was decanted and plates frozen at -80°C overnight. After thawing, CyQUANT® Cell Proliferation Assay Kit (#C7026, Life technologies) was applied and incubated for 5 min at room temperature in the darkness. Cell line-specific DNA standard curves were prepared according to the manufacturer's protocol. Absorbance was read at Ex/Em=480nm/520nm using Wallac 1420 Victor2 Microplate Reader (Perkin Elmer). Blank values were subtracted from the readouts and DNA concentration was determined based on DNA standard curve, normalized to the control group for every cell line and presented as a percent of control.

4.7.4 Caspase 3/7 activity assay (II, III)

Caspase 3/7 activity was assessed using Caspase Glo 3/7 kit (Promega) according to the provided protocol. In brief, cells were seeded on a 96-well plate in full culture media and allowed to attach overnight. At the next day medium was changed for fresh culture medium (II) or medium with treatment substances (III) and the assay was performed after 16 h (II) or 24h (III) of incubation. Absolute luminescence was normalized by control values and presented as fold change of control.

4.8 CRISPR/Cas9 (II)

All-in-one pLV-U6gRNA-Ef1aPuroCas9GFP plasmids with targeting *Lhcgr* (MM0000334875, MM0000334894), *Gata4* (MM0000234246, MM0000234282) and non-targeting guide sequence (#CRISPR12-1EA, control)

were obtained as custom-made plasmids (Sigma-Aldrich). Packaging cell line 293FT was transfected with a mixture of MISSION® Lentiviral Packaging Mix (Sigma-Aldrich) and plasmid using FuGENE® 6 Transfection Reagent (#E2691, Promega) according to the provided protocol. After 72 h, viral particles were concentrated by centrifugation at 25.000 rpm for 2 h and snap frozen in liquid nitrogen. Cα1 cells were seeded on a 24-well plate, 50000 cells/well and allowed to attach overnight. Next day, low volume of lentiviral stocks in culture media were added and cells were incubated for 16 h. Single green fluorescent protein (GFP) positive cells were isolated by a fluorescence-activated cell sorter (FACS) (BD FACSaria II, Becton Dickinson, Franklin Lakes, NJ, USA) into 96-well plates to establish clonal cell populations.

4.8.1 Genotyping of knockout clones (II)

Clones were cultured in 10% FBS medium, and genotyped using PCR with primers flanking gRNA sequences (II, Supplemental table S1). Amplicons were separated by 4% agarose gel electrophoresis (#17856, Thermo Fischer Scientific) and amplicons with significantly altered size were isolated from the gel using QIAquick Gel Extraction Kit (#28704, Qiagen) and Sanger sequenced (The Finnish Microarray and Sequencing Centre, Turku, Finland). Three clones harboring different mutations for *Lhcgr* and *Gata4* were selected for further experiments (sequences in II, Supplemental Figure S4), analyzed separately and results from 3 independent clones were pooled for *Lhcgr*-ko or *Gata4*-ko graphs.

4.9 Statistical analysis (I, II, III, IV)

To detect differences between experimental groups on the basis of sample number, distribution and variance parametric or non-parametric tests were used. For comparing 2 groups t-test or Mann–Whitney U test was used. Differences between more than 2 experimental groups were tested by one-way analysis of variance (ANOVA) or Kruskal-Wallis with Dunnet's test or multiple comparison of mean range as post hoc tests respectively. Numerical data were presented as mean ± standard error of the mean (SEM). Statistical analyses and graphs were done using Graph Pad Prism 5 or 6 (GraphPad Software, San Diego, CA, USA), and $P<0.05$ values of were considered significant.

5 RESULTS

5.1 Novel biomarkers for adrenocortical tumorigenesis

5.1.1 Identification of genes and biological processes in adrenocortical tumorigenesis (I)

To identify novel genes participating in the adrenocortical tumorigenesis in GDX inhα/Tag mice we performed cDNA microarray analysis. Detailed microarray analysis data is accessible through the ArrayExpress number E-MTAB-5310. We found 1810 up-regulated and 1606 down-regulated genes that using Gene ontology (GO) analyses were classified accordingly to biological processes (I, Fig 2). Among the significantly up-regulated processes were: cellular response to stimulus, G-protein coupled receptor (GPCR) signaling, cellular component organization or biogenesis, gene expression, cell-cell signaling, regulation of hormone levels, steroid biosynthetic and metabolic process, and mitotic cell cycle processes. Among down-regulated biological processes were: animal organ development, proteolysis, negative regulation of cell death, ion homeostasis, extracellular matrix organization and cellular response to gonadotropin stimulus.

5.1.2 qPCR validation and protein localization of potential biomarkers (I)

We further validated the differentially expressed genes found in microarray data with qPCR. Within significantly up-regulated genes were estrogen receptor alpha (*Esr1*) and long isoform of prolactin receptor (*Prlr-rs1*) (I, Figure 3A). We confirmed the significant down-regulation of extracellular matrix regulating compounds, such as sarcoglycan delta (*Sgcd*) and matrix metallopeptidase 24 (membrane-inserted) (*Mmp24*). Moreover, down-regulated cell growth related genes such as insulin-like growth factor 2 (*Igf2*), E3 ubiquitin-protein ligase NEDD4 (*Nedd4*), growth factor receptor-bound protein 10 (*Grb10*), RAS-like estrogen-regulated growth inhibitor (*Rerg*) and gonadotropin-releasing hormone receptor (*Gnrhr*). Also, we found down-regulation of downstream mediators of G-protein coupled receptor signaling, guanine nucleotide binding protein (G protein) alpha subunit (*Gnas*) and nuclear factor of activated T cells 2 (*Nfatc2*) (I, Figure 3A). Interestingly, gene expression pattern was consistent in both males and females (I, Figure S1B). Therefore protein validation was performed on adrenal

glands of male mice. The expression of ESR1 was localized in zona glomerulosa of the normal adrenal, whereas it was abundant in almost all tumor cells (I, Figure 3B and C). We localized abundant expression of GRB10 (I, Figure 3D–E), RERG (I, Figure 4A–B), GNAS (I, Figure 4C–D), NFATC2 (I, Figure 4E–F) in the normal adrenal (zona glomerulosa), whereas faint or absent expression was observed within the adrenocortical tumor foci of inhα/Tag mice. We localized also the same proteins in intact WT male adrenals and found that GDX had no impact on cellular localization of the analyzed biomarkers in WT adrenals (I, Figure S1C-G).

5.1.3 Expression and localization of Lhcgr, GATA4 and GATA6 in adrenal tumors (I)

We revisited gene expression of previously described adrenocortical tumor biomarkers and found significant up-regulation of *Lhcgr* (I, Figure 5A) and *Gata4* (I, Figure 5B), and down-regulation of *Gata6* (I, Figure 5C) in the tumorous adrenals *vs*. GDX WT mouse adrenals. *Lhcgr* transcripts were found in the foci of normal adrenocortical cells scattered in GDX WT adrenal cortex (I, Figure 5D), in most of A cells of GDX WT (I, Figure 5E) and abundantly present within the tumor foci of inhα/Tag mice (I, Figure 5F). *Lhcgr* localization in the adrenal gland was similar in both intact and GDX WT mice (I, Figure S1H-I). GATA4 was not found in the adrenal glands of GDX WT (I, Figure 5G), but was abundantly expressed in most of the neoplastic A (I, Figure 5H) and adrenocortical tumor cells (I, Figure 5I). GATA6 was localized abundantly in the GDX WT adrenal gland (I, Figure 5J), was missing in A cells (I, Figure 5K) and faint in the tumor foci (I, Figure 5L).

5.1.4 Adrenal tumor bearing inhα/Tag mice present with an inverted adrenal-to-gonadal steroidogenic enzyme expression profile (I)

We decided to further investigate the dysregulation of steroid biosynthetic process indicated by the microarray. Analysis of hormone levels showed significantly increased LH (I, Figure 6A) and progesterone (I, Figure 6B) concentrations in GDX inhα/Tag when compared with WT mice, as well as detectable levels of testosterone in GDX inhα/Tag male mice (I, Figure 6C). Gene expression profiling showed significant down-regulation of adrenal (*Cyp21a1*, *Cyp11b1*, *Cyp11b2*; Figure 6D) and up-regulation of gonadal steroidogenic enzyme genes (*Srd5a1*, *Cyp19a1*; I, Figure 6E) in GDX inhα/Tag *vs*. GDX WT mice. Moreover, we found that *Cyp17a1* expression in the adrenal gland was significantly increased in GDX *vs*. intact WT (C57Bl/6) male mice (I, Figure S2).

5.2 Ontogeny of the adrenocortical tumors in inhα/Tag mice

5.2.1 Appearance of the adrenocortical tumors in inhα/Tag mice (II)

To follow the ontogeny of the tumors, we macroscopically and histologically analyzed adrenal glands of female mice at 2, 4, and 6 months of age. We found that total (II, Figure 1A) and normalized adrenal tumor weights of GDX inhα/Tag females (II, Figure 1B; calculated by subtraction of the average total adrenal weight of age-matched GDX WT from total adrenal weights of inhα/Tag mice) were significantly increased at 4-mo and 6-mo *vs*. age-matched GDX WT mice. Using immunohistochemical SV40Tag protein localization, we identified two distinct types of neoplastic cells that expressed SV40Tag at 2-mo of age (II, Figure 1C, Figure S1A). The first type was the spindle-shaped neoplastic A cells (II, Figure 1D, Figure S1C) that originated from the sub-capsular region and migrated towards adrenal medulla of intact and GDX inhα/Tag mice. Similar to WT mice, they did not form tumors (II, Figure S1B, S1D). The second abnormal cells had round nuclei (II, Figure 1E) and formed hyperplasia in the topmost layer of zona fasciculata exclusively in GDX inhα/Tag mice already at 2-mo, and subsequently led to the tumor development. Moreover, both A and hyperplastic cells were expressing GATA4 and MKI67 (II, Figure 1D-E).

5.2.2 Gradual decrease of GATA6 and enhancement of GATA4 expression during adrenocortical tumor ontogeny (II)

A reciprocal expression of GATA6 and GATA4 in the adrenocortical tumors has been previously shown in both mice and human (Kiiveri et al. 1999, Kiiveri et al. 2002, Rahman et al. 2004, Looyenga and Hammer 2006). The above-mentioned findings were suggested to be responsible for the adrenal-to-gonadal phenotype transition observed in adrenocortical tumors (Looyenga and Hammer 2006). In general, the GATA6 and GATA4 localization was shown at the end-point when discernible tumors had already developed. Therefore, we decided to evaluate the GATA6 and GATA4 expression in an age-dependent ontogeny of adrenocortical tumors. We found that GATA6 expression was abundant in the normal adrenal cortex of GDX WT mice (II, Figure 2A) and hyperplastic cells of 2-mo GDX inhα/Tag (II, Figure 2B), and depleted in most cells within the tumor foci of 4-mo-old (II, Figure 2C) and 6-mo-old (II, Figure 2D) GDX inhα/Tag mice. On the other hand, normal adrenal cortex of GDX WT mice was devoid of GATA4 expression (II, Figure 2F), whereas in GDX inhα/Tag mice GATA4 expressing cells could be observed at 2-mo (II, Figure 2G), and increased in abundance within

tumor foci at 4-mo (II, Figure 2H) and 6-mo (II, Figure 2I) time points. Analysis of the staining density of the same image region (n=4/ group; 5 images/specimen), revealed significantly decreased GATA6 staining (II, Figure 2E) in 4-mo and 6-mo GDX inhα/Tag *vs*. 6-mo GDX WT adrenals. Moreover, we observed a significant gradual increase in GATA4 staining density at 2-mo, 4-mo and 6-mo GDX inhα/Tag *vs*. 6-mo GDX WT mice (II, Figure 2J).

5.2.3 LHCGR- depletion prevents GATA4 induction and subsequent adrenocortical tumor formation (II)

We evaluated the role of LHCGR in the adrenocortical tumor induction by intercrossing inhα/Tag with *Lhcgr* knockout (LuRKO) mice (Zhang et al. 2001) (inhα/Tag/LuRKO mice). A significant decrease in total adrenal (II, Figure 3A) and tumor weights (II, Figure 3B), as well as down-regulation of the tumor markers *SV40Tag* and *Gata4* (II, Figure 3C), when compared 7-mo GDX inhα/Tag/LuRKO with their GDX inhα/Tag mice littermates were observed. Plasma levels of LH were similar in all the groups (II, Figure S2A), whereas progesterone was increased in GDX inhα/Tag, and decreased in both GDX inhα/Tag/LuRKO and GDX LuRKO, in comparison to GDX WT female mice (II, Figure S2B). Also, the neoplastic cell biomarker *Gata4* expression was significantly decreased in GDX LuRKO mice in comparison to GDX WT (II, Figure S2C). The histopathological analysis of 7-mo inhα/Tag/LuRKO adrenals (II, Figure 3D) identified the presence of hyperplastic cells (II, Figure 3E), resembling those previously found in 2-mo inhα/Tag mice (II, Figure 1E). Further immunohistochemical characterization of hyperplastic cells in 7-mo GDX inhα/Tag/LuRKO mouse adrenals showed presence of SV40Tag and cleaved-CASP3 but absence of GATA4 (II, Figure 3E).

5.2.4 Tumorous phenotype of Cα1 cells is induced by GATA4, but steroidogenesis is controlled by LHCGR and GATA4 (II)

In order to further characterize and compare the phenotype of adrenocortical tumors in inhα/Tag (6-mo) mice and tumor derived Cα1 cell line, we analyzed *Gata4* and *Lhcgr* as tumor markers, and the genes encoding main steroidogenic enzymes. The expression of *Lhcgr* and *Gata4* was increased in both 6-mo GDX inhα/Tag mice and Cα1 cells *vs*. 6-mo GDX WT mice (II, Figure S3A). The dose dependent hCG stimulation of cAMP production proved the functionality of LHCGR in Cα1 cells (II, Figure S3B). However, hCG stimulation had no effect on proliferation of Cα1 cells (II, Figure S3C). Gene expression profiling of steroidogenic enzymes in inhα/Tag mice and Cα1 cells revealed up-regulated

proximal *Star* (only in Cα1 cells) and *Cyp11a*, as well as gonad-specific *Srd5a1* and *Cyp19a1*, and down-regulated adrenal-specific *Cyp21a1*, *Cyp11b1* and *Cyp11b2* when compared to GDX WT mice (II, Figure S3D).

The impact of *Lhcgr* and *Gata4* on adrenocortical tumor progression was analyzed by CRISPR/Cas9-mediated mutagenesis. The genotyping workflow included PCR amplification of the region flanking gRNA sequence, electrophoretic separation (II, Figure S4A, Figure S4C) and sequencing of the mutated clone's DNA (II, Figure S4B, Figure S4D). Functionality of *Lhcgr* knockout was confirmed by lack of hCG-mediated cAMP production in *Lhcgr*-ko clones (II, Figure S4E). Results from 3 different *Lhcgr*-ko or *Gata4*-ko clones (with DNA sequences indicated in II, Figure S4B, Figure S4D) were pooled and compared with a mock-transfected (control) cell line. *Lhcgr*-ko cells had similar proliferation (II, Figure 4A), expression of anti-apoptotic *Bcl2* and proliferation markers *Pcna* and *Mki67* (II, Figure 4B) or caspase 3/7 activity (II, Figure 4C) in comparison to control cells. Adrenocortical tumor biomarker gene expression experiment showed down-regulation of *Esr1*, whereas expression of *Inha*, *SV40Tag* and *Gata4* was similar in *Lhcgr*-ko Cα1 cells *vs*. control (II, Figure 4D). In contrast, *Gata4*-ko Cα1 cells had reduced proliferation rate (II, Figure 4F) reflecting down-regulated expression of anti-apoptotic *Bcl2* and proliferation markers *Mki67* and *Pcna* (II, Figure 4G). Increased activity of caspase 3/7 in *Gata4*-ko Cα1 cells (II, Figure 4H) indicated active apoptosis. Tumor biomarkers, *Inha*, *SV40Tag*, *Lhcgr* and *Esr1* were also down-regulated in *Gata4*-ko Cα1 cells *vs*. control (II, Figure 4I).

The basal progesterone production was decreased in both *Lhcgr*-ko (II, Figure 4E) and *Gata4*-ko (II, Figure 4J) cells as compared to control. Moreover, *Lhcgr*-ko and *Gata4*-ko Cα1 cells presented with significantly down-regulated *Nr5a1*, *Star*, *Cyp11a1*, *Hsd3β1* and *Cyp19a1* steroidogenic genes (II, Figure 4K). However, only *Gata4* knockout resulted in increase of adrenal steroidogenic enzymes *Cyp21a1* and *Cyp11b2*, and decrease of gonadal steroidogenic enzymes *Cyp17a1* and *Srd5a* (II, Figure 4K), showing enhanced adrenal steroidogenic phenotype *vs*. control.

5.3 GnRH antagonist treatment of adrenocortical tumors

5.3.1 mRNA expression of GNRHR, LHCGR and FSHR in human and mouse adrenocortical tumors and cell lines (III)

Thirteen ACC patients samples, 7 females and 6 males, between 30 and 72 years of age, displaying diverse hormone excess symptoms and Ki67 proliferation

index spanning 2-50% (III, Table 1), were classified accordingly to the European Network for the Study of Adrenal Tumors (ENSAT) scale from 2 to 4. We found *GNRHR* in 54%, *LHCGR* in 77% and *FSHR* in 0% of ACC patients (III, Table 1). Representative images of hematoxylin and eosin, MKI67 immunohistochemical staining, and transcript localization of *GNRHR*, *LHCGR*, *FSHR* and *POL2A* (reference control probe) are shown in III, Figures 1A-F.

Transcript levels of *GNRHR*/*Gnrhr* and *LHCGR*/*Lhcgr* from fresh-frozen samples of human and mouse adrenal tumor tissues and cell lines in comparison to healthy adrenal controls were assessed using qPCR. We found *GNRHR* (III, Figure 2A) and *LHCGR* (III, Figure 2B) expression in all normal human adrenal (ADR) samples and the human H295R cell line, and in 73 and 100% of the 11 studied ACC samples, respectively. *Gnrhr* (III, Figure 2C) and *Lhcgr* (III, Figure 2D) were expressed in all murine healthy adrenals (ADR), ACTs and Cα1 cell line, whereas mouse Y-1 cell line only expressed *Gnrhr*.

5.3.2 CTX decreased cell viability and proliferation in vitro and tumor size in vivo (III)

The Cα1, Y-1 and H295R cells treated with CTX alone or in combination with hCG (CTX+hCG) displayed decreased viability (III, Figure 3A) and proliferation (III, Figure 3B) as well as increased activity of caspase 3/7 *vs*. CT (III, Figure 3C). Treatment with hCG alone, affected H295R only by increasing their viability and proliferation (III, Figure 3A-B). *In vivo*, treatment with CTX or CTX+hCG decreased adrenocortical tumor size in males (Figure 4A) and females (Figure 4B) as well as declined LH (III, Figure 4C-D) and progesterone (III, Figure 4E-F) plasma levels *vs*. CT. However, treatment with hCG alone did not affect the tumor size nor plasma hormone concentrations (III, Figure 4 C-F).

5.3.3 Global gene expression changes of adrenocortical tumors in inhα/Tag mice (III)

cDNA microarray analysis was run to identify the potential biological processes and pathways that were affected by CTX. Analysis of microarray data comparing CTX *vs*. CT treated ACTs of inhα/Tag mice, revealed that 1714 (918 up- and 796- down-regulated) and 4390 (2506 up- and 1884 down-regulated) genes were differently expressed in males and females respectively (Figure 5A). Enrichment analysis of the male dataset using PANTHER classification system clustered genes into biological processes and pathways. Among dysregulated biological processes the most curious (marked with arrowheads) are growth,

biological adhesion, immune system process, developmental process, response to stimulus (III, Figure 5B). Among altered pathways the most interesting (marked with arrowheads) are p53, apoptosis signaling, EGF receptor signaling, FGF signaling, gonadotropin-releasing hormone receptor, G-protein signaling pathways, angiogenesis, inflammation mediated by chemokine and cytokine signaling, and Wnt signaling (III, Figure 5C).

5.3.4 Expression of genes related to normal adrenal function gets up-regulated after the CTX treatment in vivo (III)

qPCR validation of selected candidate genes in murine ACTs treated with CTX *vs*. CT showed down-regulated expression of GATA binding protein 4 (*Gata4*) (III, Figure 6A), *Lhcgr* (III, Figure 6B), Cyclin A1 (*Ccna1*) (III, Figure 6C). We also detected up-regulation of extracellular matrix compounds such as sarcoglycan delta (*Sgcd*) (III, Figure 6D), matrix metallopeptidase 24 (*Mmp24*) (III, Figure 6E); genes related to cell growth suppression, growth factor receptor-bound protein 10 (*Grb10*) (III, Figure 6F), RAS-like, estrogen-regulated, growth inhibitor (*Rerg*) (III, Figure 6G); G0/G1 Switch 2 (*G0s2*) (III, Figure 6H); Tumor Suppressor Candidate 5 (*Tusc5*) (III, Figure 6I) and GPCR mediated Ras Protein Specific Guanine Nucleotide Releasing Factor 1 (*Rasgrf2*) (III, Figure 6J). Moreover, we found up-regulated expression of *Gnrhr* (III, Figure 6K) and the downstream mediators of GPCRs, *Nfatc2* (III, Figure 6L) and *Gnas* (III, Figure 6M). Interestingly, none of these genes were altered in *in vitro* treated cell lines (III, Figure 6A-M) suggesting they are not genomic targets of CTX.

5.4 Case report of a gonadotropin-regulated adrenal tumor phenotype in a pregnancy-induced Cushing syndrome

5.4.1 Functional characterization of the mechanisms in the case report (IV)

A 22-year-old woman at 21 weeks gestational age (GA) of the first pregnancy, presented with symptoms of CS (IV, Figure 1). The patient presented with an increased 24-hour urinary free cortisol excretion, lack of cortisol suppression by dexamethasone, low ACTH plasma concentration [<5 pg/mL (<1.1 nmol/L)] (IV, Table 1, as well as lack of responsiveness to CRH suggesting ACTH-independent CS. The patient had no family history of endogenous hypercortisolism. Computed tomography revealed bilaterally enlarged adrenals

without nodular structures [IV, Figure 1C and 1D]. Among other symptoms were hyperandrogenism resulting in acne (IV, Figure 1A; IV, Supplemental Table 1], insulin-dependent diabetes mellitus, as well as hypokalemic hypertension. Preterm labor at 25 weeks GA, resulted in the vaginal delivery of a male infant (with sex chromosomes XY) (IV, Figure 1B) that died on day 3. The symptoms of CS receded within a week postpartum. Two weeks after delivery, peripheral and adrenal vein concentrations of aldosterone were still suppressed [aldosterone <2.3 ng/mL (<63.8 pmol/L); normal range supine, 2.3 to 16 ng/mL (63.8 to 440.0 pmol/L)] and were not stimulated by ACTH. Adrenal volume as well as biochemical changes normalized 49 days after the delivery (IV, Table 3; Figure 1C-D).

5.4.2 Aberrant hormone receptor analysis in vivo (IV)

At 19 weeks post-delivery *in vivo* tests for aberrant hormone receptors showed normal costisol secretion after ACTH stimulation (IV, Table 2; Supplemental Table 2). On the other hand, orthostasis, a standard meal, injections with GnRH, thyrotropin-releasing hormone, glucagon, or oral metoclopramide administration, did not result in a positive cortisol response. Moreover, angiotensin vasopressin injection resulted in a 30-fold increase in cortisol, although basal plasma cortisol concentration was low due to the preceding dexamethasone administration (IV, Table 2; IV, Supplemental Table 2). Long-term stimulation with hCG induced a peak in plasma hCG concentration of 809 IU/L (day 6, hCG 10,000 IU/d) that represented 3.2% of the hCG plasma concentration at week 26 of GA. The plasma cortisol concentration peak increased to 144% [15.1 to 21.7 mg/dL (386.4 to 555.2 nmol/L)] at day 2 on hCG 5000 IU/d and to 133% [16.0 to 21.3 mg/day (409.4 to 545.0 nmol/L)] at day 2 on hCG 10,000 IU/d, while ACTH levels were suppressed from 11.0 pg/mL to <5 pg/mL (2.4 to <1.2 nmol/L) at day 2 on hCG 5000 IU/d. Moreover, hCG stimulation induced a significant increase in testosterone levels (IV, Table 3). Dexamethasone treatment did not affect cortisol and androgen secretions during the hCG stimulation (10,000 IU/d) (IV, Table 3), indicating that hCG was causing CS and hyperandrogenism. At week 51 postpartum (IV, Figure 1B) the patient presented with recurrent CS symptoms that presented with an increased 24-hour urinary free cortisol excretion [999.4 mg/24 h (2757 nmol/d); upper limit of normal, 176 mg/24 h (485.6 nmol/d)] and a coinciding hCG plasma concentration of 2634 IU/L, suggesting pregnancy. After the localization and termination of an ectopic pregnancy, both urinary free cortisol excretion as well as hCG concentrations normalized. After four weeks (IV, Figure 1B), on the basis of the patient's request, both adrenal glands were surgically

removed and they were functionally characterized with *in vitro* and histological studies.

5.4.2.1 Histopathological analysis of surgically removed adrenals

Histopathological analysis of the patient's adrenal glands exhibited adrenocortical hyperplasia (IV, Figure 1E). Two types of abnormal cells were detected. The first atypical cells were large spongiocytic and lipid-laden (IV, Figure 1F) that formed hyperplasia within the adrenal cortex. The second type were small compact cells forming clusters in the central medulla (IV, Figure 1G), which morphologically resembled the cells of zG.

5.4.2.2 Pregnancy-induced Cushing syndrome was not associated with known mutations (IV)

To exclude genetic background of the pregnancy-induced primary bilateral macronodular adrenal hyperplasia (BIMAH), we sequenced regions of genes known to be related to CS, *CTNNB1*, *PRKAR1A*, *ARMC5*, and *PRKACA* and no such mutations were found.

5.4.2.3 In vitro culture of isolated adrenal cells (IV)

Stimulation of isolated hyperplastic adrenal cells with ACTH or rhCG resulted in an increased cAMP production by 158.6% ($P = 0.048$) or 25.4% ($P = 0.011$), respectively, in comparison to non-stimulated cells (IV, Figure 2G). Moreover, ACTH and rhCG stimulation of dispersed and cultured adrenal cells resulted in a significant increase in corticosterone, 11-deoxycortisol, cortisol, and androstenedione production corresponding to the positive *in vivo* cortisol and testosterone response to long-term hCG stimulation (IV, Figure 2H; Table 3). This functional response to hCG stimulation provoked/encouraged the molecular characterization of hCH/LHCGR signaling in the hyperplastic adrenal gland.

*5.4.2.4 LHCGR/*LHCGR *expression and localization (IV)*

The mRNA levels for *LHCGR* (IV, Figure 2A)*, GATA4* and zinc finger protein multitype 2 *(ZFPM2)* (IV, Figure 3A) were up-regulated in hyperplastic adrenals as compared with normal healthy female adrenal, 4-fold, 8-fold and 2.8-

fold respectively. Histopathological analysis demonstrated adrenocortical hyperplasia (IV, Figure 1E) with the presence of large lipid-loaded spongiocytic cells (IV, Figure 1F) and clusters of small compact cells in the central medulla (IV, Figure 1E, Figure 1G) that resembled zR cells. In the undifferentiated subcapsular cells we localized *LHCGR*/LHCGR (IV, Figure 2B, Figure 2E) and GATA4 (IV, Figure 3B), in zG *LHCGR*/LHCGR (IV, Figure 2C, Figure 2E), GATA4 and ZFPM2 (IV, Figure 3C, Figure 3F), whereas hyperplastic cells of the cortex were expressing *LHCGR*/LHCGR (IV, Figure 2D, Figure 2F, Supplemental Figure 1) but not GATA4 and ZFPM2 (IV, Figure 3D, Figure 3G). Moreover, hyperplastic cells expressed steroidogenic enzymes CYP11A1, 3β-HSD, CYP21A1, CYP17A1 and CYP11B1 (IV, Figure 4A-F) enabling the production of cortisol.

6 DISCUSSION

6.1 Novel biomarkers and ontogeny of adrenocortical tumors of inhα/Tag mice

Abundant expression of ESR1 observed in tumor foci *vs*. healthy adrenal gland suggest ESR1 as a novel biomarker for adrenocortical tumorigenesis in inhα/Tag mice. Up-regulated expression of *Esr1*/ESR1 seems to be common for models susceptible to GIACT since it could be also found in GDX NU/J mice (Bielinska et al. 2005) as well as in a subpopulation of adrenocortical tumor cells in domestic ferrets (Newman et al. 2004, Bielinska et al. 2006). Moreover, in functional human ACCs the ESR1/ESR2 ratio is increased (Barzon et al. 2008) and 17β-estradiol directly stimulates the proliferation of H295R cells (Montanaro et al. 2005). In our studies, the upregulated *Esr1* but no change in *Esr2* in GDX inhα/Tag *vs*. GDX WT mice indicates increased *Esr1/Esr2* ratio. Therefore, it is logical to suggest that estrogens could affect the progression of adrenocortical tumors in inhα/Tag mice.

On the other hand GRB10, RERG, GNAS and NFATC2 were abundantly localized in the healthy adrenal gland suggesting their role in the maintenance of murine adrenal physiology. The lower abundancy of GNAS and NFATC2 in tumor foci may suggest GPCR signaling suppression (Hill et al. 2001) in comparison to healthy adrenal glands. The role of GRB10 and RERG remains unclear as their expression have never been analyzed in the adrenal gland. Since both of these proteins inhibit cell proliferation (Liu and Roth 1995, Finlin et al. 2001), one of the possibilities is that they prevent the overgrowth of subcapsular and zG regions that are crucial for the adrenal gland regeneration (Pihlajoki et al. 2015). Moreover, by localization of the GRB10, RERG, GNAS and NFATC2 proteins we excluded GDX impact on their localization.

6.1.1 Lhcgr expression in the adrenal gland

The expression of *Lhcgr* in the healthy mouse adrenal gland has been considered ectopic since it does not produce androgens (Pihlajoki et al. 2015). However, PCR-based methods showed detectable *Lhcgr* mRNA levels in intact adrenals (Looyenga and Hammer 2006, Chrusciel et al. 2013) that, due to the lack of available quality antibodies against LHCGR, could not be confirmed at protein level. In this study we observed *Lhcgr* transcripts in normal adrenocortical cells indicating eutopic expression of *Lhcgr* in the adrenal cortex. It has been

hypothesized that chronically elevated LH levels, e.g. due to GDX or LH overproduction in bLHβ-CTP mice, could induce ectopic overexpression of *Lhcgr* in the adrenal gland (Kero et al. 2000, Rahman et al. 2001, Mikola et al. 2003) and in GIACT models result in the tumor development (Matzuk et al. 1994, Kananen et al. 1996b, Kumar et al. 1996, Kumar et al. 1999, Bielinska et al. 2003, Bielinska et al. 2005, Krachulec et al. 2012, Chrusciel et al. 2013, Basham et al. 2016).

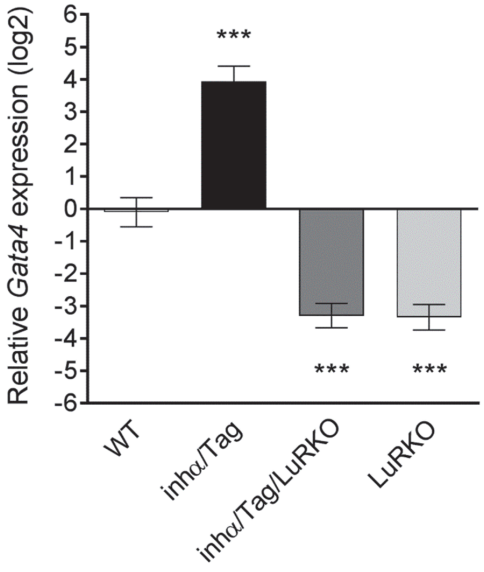

Figure 5. *Gata4* expression in the adrenals of mouse models used in our studies. The expression of neoplastic cell biomarker *Gata4* in GDX WT, tumor bearing inihα/Tag, and LHCGR knockout GDX inihα/Tag/LuRKO and LuRKO mice.

Besides normal adrenocortical localization, *Lhcgr* was also found in adrenal A cells of 6-mo intact and GDX C57BL/6N mice that are not genetically susceptible for GIACT. The *Lhcgr* transcripts were previously shown in A cells when accompanied by tumorous B cells in the adrenal gland of GDX DBA/2J mice (Bielinska et al. 2003). Many studies showed up-regulation of *Lhcgr* in aging mice following GDX (Kero et al. 2000, Looyenga and Hammer 2006, Chrusciel et al. 2013), which coincides with the increased abundance of A cells (Bandiera et al. 2013). Decreased expression of A cell biomarker *Gata4* in mice lacking functional LHCGR (GDX LuRKO and GDX inhα/Tag/LuRKO) as compared with GDX WT (Figure 5), suggests that the number of A cells is lower when LHCGR is depleted. Therefore, LH/LHCGR signaling might be involved in the propagation of A cells, whereas their ontogeny is gonadotropin independent as suggested before (Chrusciel et al. 2013).

6.1.2 LHCGR is a prerequisite for hyperplasia-adenoma transition but does not affect the tumor progression

Tumor constituting SV40Tag expressing hyperplastic cells were localized already at the age of 2-mo in zF. This SV40Tag localization is consistent with the highest activity of *Inha* 6kb promoter in young and adult mouse adrenal glands (Hsu et al. 1995). Thus, we suggest zF as the primary localization of adrenocortical tumors in the inhα/Tag model, not xZ (Kananen et al. 1996b) or the stem/progenitor cell niche (Looyenga and Hammer 2006) as previously suggested. However, SV40Tag expression is not sufficient enough for the hyperplasia-adenoma progression for which LH/LHCGR downstream targets are indispensable as shown in inhα/Tag/LuRKO mice. Interestingly, in a mouse model expressing SV40Tag under a zona fasciculata-specific *Akr1b7* promoter (AdTAg mice) that develop adrenocortical tumors (Batisse-Lignier et al. 2017), SV40Tag expression was also implicated into tumor initiation, whereas promotion of the malignant progression was attributed to a spontaneous activation of WNT/β-catenin signaling (Batisse-Lignier et al. 2017).

We found that lack of *Gata4* in the adrenocortical tumor cells is the leading cause of decreased proliferation, and increased cell survival, whereas abrogation of *Lhcgr* had rather modest effects. The important pro-survival role of GATA4 has also been shown in gonadal Leydig and granulosa cells (Kyrönlahti et al. 2008, Schrade et al. 2015). Conversely, the lack of proliferative effect of hCG stimulation and *Lhcgr* knockout on Cα1 cells, as well as that of hCG stimulation of adrenocortical tumors *in vivo* (III, Figure 4) suggest that the progression of the adrenocortical tumors becomes independent on LH/LHCGR signaling in this model.

6.1.3 Reciprocal expression of GATA6 and GATA4 in adrenocortical tumors

The reciprocal expression of GATA6 and GATA4 in mouse and human adrenocortical tumors has been reported previously (Kiiveri et al. 1999, Kiiveri et al. 2005, Looyenga and Hammer 2006, Vuorenoja et al. 2007). In our studies we localized GATA6 uniformly distributed along all adrenocortical zones as shown in previous *in situ* experiments (Kiiveri et al. 1999), not only in the capsular/subcapsular region as suggested in a recent immunolocalization study (Pihlajoki et al. 2013). We confirmed that GATA6 staining can be a good marker of healthy adrenal cells, whereas GATA4 a neoplastic cell biomarker (Looyenga and Hammer 2006, Krachulec et al. 2012, Chrusciel et al. 2013). During the age-dependent ontogeny of adrenocortical tumors we found a gradual decrease in GATA6 expression and increase in GATA4 expression in GDX inhα/Tag *vs*. GDX

WT mouse adrenals. The expression of GATA4 is attributed to B cell onset and gonadal-type gene expression resulting in a gonadal-like phenotype of adrenal tumors (Looyenga and Hammer 2006, Krachulec et al. 2012, Chrusciel et al. 2013). On the other hand, lack of GATA6 in the steroidogenic cells of the adrenal gland correlates with an increased abundancy of neoplastic A cells (Pihlajoki et al. 2013) suggesting that lack of GATA6 expression allows neoplastic transformation. It has been suggested that malignant transformation of adrenocortical cells in *Inha*$^{-/-}$ mice is an effect of phenotype reprogramming, namely a decrease in native GATA6 expression and induction of ectopic GATA4 (Looyenga and Hammer 2006). According to these authors this transcription factor switch is mediated by FSH (Looyenga and Hammer 2006). This assumption stays in opposition to the previous data about *Inha*$^{-/-}$ mouse model, showing that *in vivo*, FSH is not able to trigger tumor formation which is clearly dependent on LH/LHCGR signaling (Kumar et al. 1999, Beuschlein et al. 2003). In our studies, the hyperplastic cells in inhα/Tag/LuRKO mice expressed SV40Tag and GATA6 (Figure 6) but were lacking GATA4, supporting the notion that cell reprogramming by GATA4 expression induction and sequential tumorigenesis are down-stream effects of LH/LHCGR signaling. It would be interesting to investigate in the future if GATA6-defficient mice would develop adrenocortical tumors faster in one of the GIACT susceptible models.

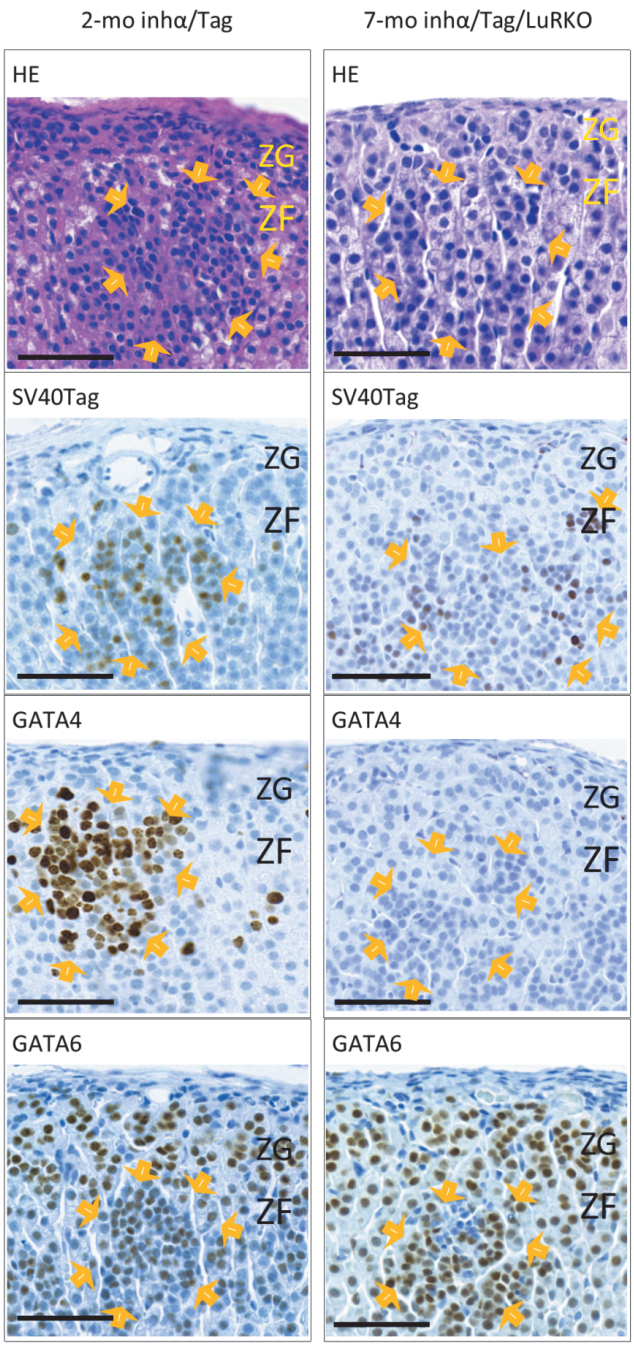

Figure 6. Hyperplastic cells in the adrenal cortex of inhα/Tag/LuRKO mice lack GATA4.

Comparison of histology, and expression of GATA4 and GATA6 in adrenals of 2-mo inhα/Tag and 7-mo inhα/Tag/LuRKO mice. Yellow arrows, hyperplastic cells; inhα/Tag, transgenic mice expressing SV40Tag oncogene under inhibin α promoter; inhα/Tag/LuRKO, inhα/Tag mice crossbred with LHCGR deficient mice (LuRKO); ZG, zona glomerulosa, ZF, zona fasciculata

6.1.4 Steroidogenic profile of adrenocortical tumor cells

The gene expression profiling of steroidogenic enzymes genes in GDX inhα/Tag male and female mice adrenals and Cα1 cells revealed up-regulation of gonadal-type at the expense of reduced adrenal-type steroidogenic enzymes. Interestingly, expression of *Cyp17a1* was not altered when comparing tumor bearing GDX inhα/Tag with GDX WT male mouse adrenals. However, it is significantly up-regulated in both GDX inhα/Tag and GDX WT *vs*. intact WT mouse adrenals. Recently it was shown that indeed increase of adrenal CYP17A1 expression in a C57Bl/6 background is rather correlated with GDX than tumorigenesis (Bandiera et al. 2013). Thus, we suggest that in C57Bl/6 background, *Cyp17a1* could be demethylated as a consequence of GDX, not due to adrenocortical tumor presence as it was suggested in GDX DBA/2J mice (Bielinska et al. 2003, Krachulec et al. 2012). The expression of gonadal-type steroidogenic enzymes in adrenal tumor bearing GDX inhα/Tag mice was manifested by detectable testosterone levels in males (Figure 7A) and larger uteri in females (Figure 7B) *vs*. GDX WT mice. Our data corresponds to the estrogen levels found in tumor bearing GDX DBA/2J mice (Bielinska et al. 2003, Bielinska et al. 2005), suggesting that the sex steroid production is a common feature of GIACT models (Bielinska et al. 2003, Bielinska et al. 2005, Krachulec et al. 2012).

Our *in vitro* knockout studies revealed that both *Lhcgr* and *Gata4* regulate expression of the genes encoding steroidogenic enzymes manifested by decreased progesterone production by *Lhcgr*-ko and *Gata4*-ko mice. However, the clear inversion of gonadal-type (*Cyp17a1*, *Srd5a1*, *Cyp19a1*) to adrenal-type (*Cyp21a1*, *Cyp11b2*) steroidogenic enzymes expression was observed only in *Gata4*-ko cells. These findings support earlier reports that gonadal-like steroidogenic phenotype in GIACT models is a consequence of GATA4 expression (Looyenga and Hammer 2006, Krachulec et al. 2012). We confirmed the previously suggested assumptions that LH/LHCGR signaling is a steroidogenesis enhancer in the adrenal gland (Kero et al. 2000). However, the gonadal-like steroidogenic phenotype of the adrenocortical tumor cells is induced by GATA4 expression (Looyenga and Hammer 2006).

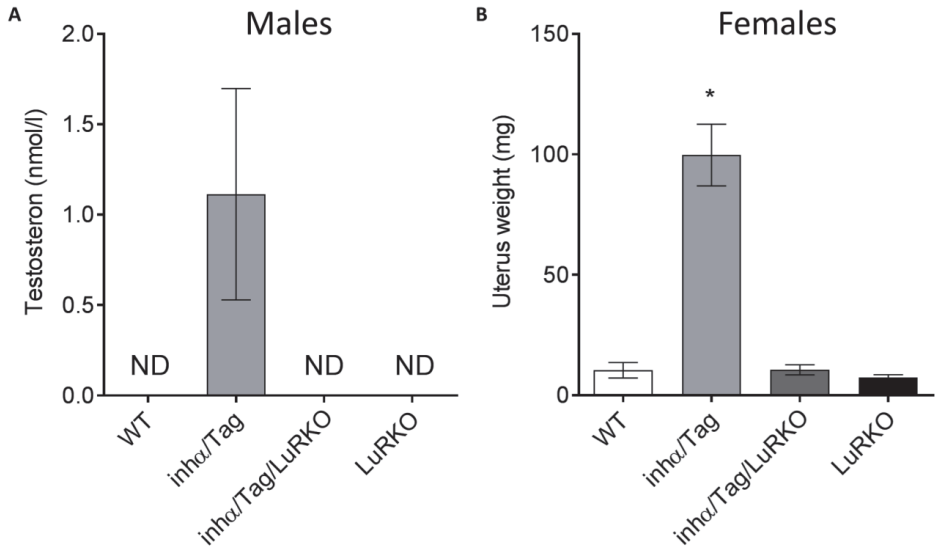

Figure 7. Manifestations of gonadal-type steroid production by adrenocortical tumors in gonadectomized mice. Comparison of testosterone levels (A) and uteri weight (B) between GDX WT, tumor bearing GDX inhα/Tag, and LHCGR lacking GDX inhα/Tag and GDX LuRKO mice.

6.2 Direct and systemic effects of GnRH-antagonist treatment on adrenocortical tumors

Here, we showed the expression of GNRHR and LHCGR in a subgroup of human ACC specimen, qualifying them as systemic or direct targets for GnRH antagonist treatment. Moreover, in our *in vivo* and *in vitro* models CTX directly targeted human and mouse adrenocortical tumor cells resulting in the tumor regression. Similar to previous studies on breast, uterine, lung (Ghanghoria et al. 2016), prostate (Sakai et al. 2015), and adrenocortical (Ziegler et al. 2009) cell lines, CTX decreased cell viability, proliferation and increased the number of apoptotic events *in vitro*. Interestingly, we found that H295R cells that express functional LHCGR (Rao et al. 2004) responded to hCG with increased proliferation. *In vivo*, CTX treatment decreased tumor size and ablated LH and progesterone plasma levels, whereas hCG treatment had no effect on these parameters. The up-regulation of healthy adrenal gland-related *Rerg*, *Grb10*, *Nfatc2* and *Gnas* (see the paragraph 6.1) in CTX *vs.* CT treated adrenocortical tumors *in vivo* could be a manifestation of regenerative processes to restore physiological adrenal gland functions. On the other hand, down-regulation of tumor biomarkers *Lhcgr* and *Gata4* in CTX *vs*. CT treated tumor bearing mice can be explained by the regressing tumor cells. Since these genes were not affected by CTX treatment *in vitro*, they are most likely not the direct targets of CTX. Thus,

we suggest that in certain cases of *GNRHR* and *LHCGR*-positive ACCs, CTX could be utilized for both, suppression of tumor growth-stimulating gonadotropins and/or steroid hormones, and direct tumor growth inhibition (summarized in Figure 8).

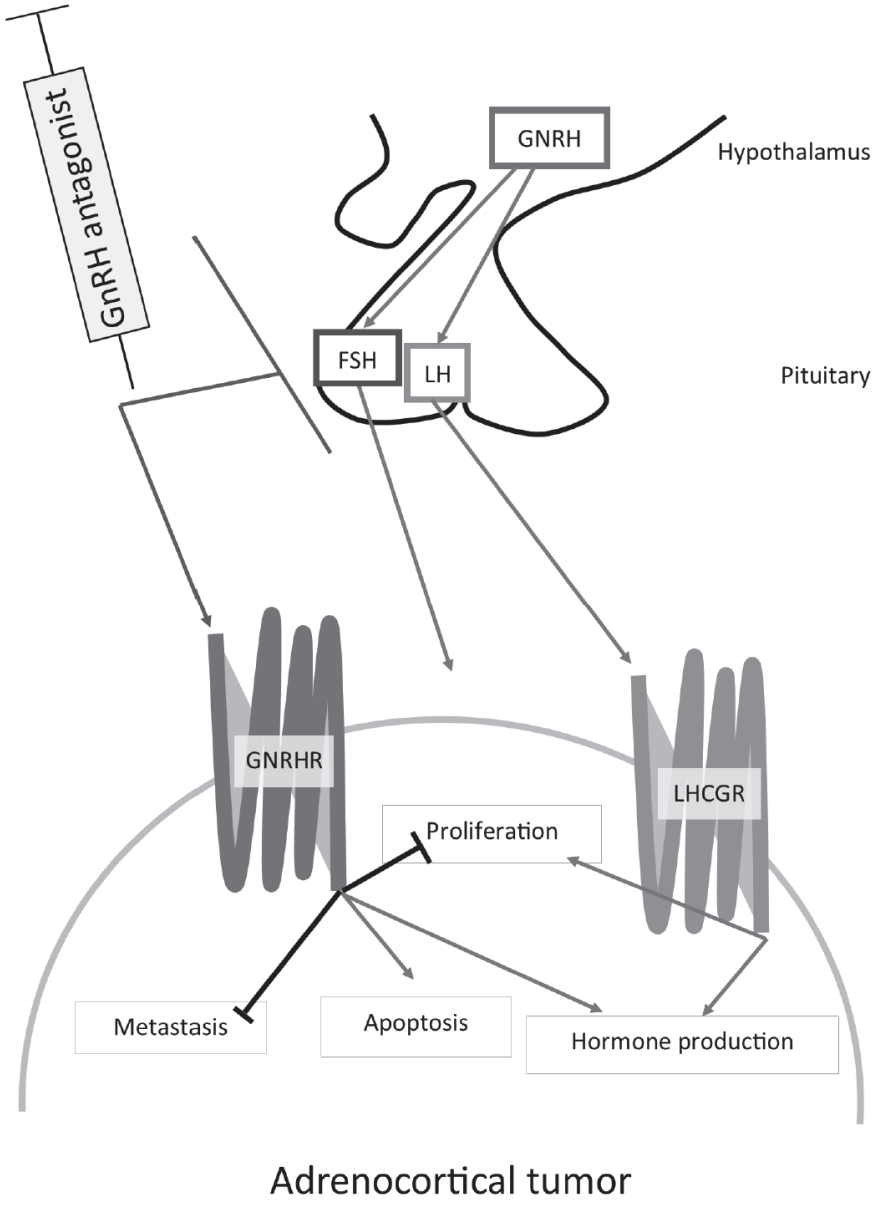

Figure 8. Summary of the tumor growth suppression by GnRH antagonist in adrenocortical tumor cells. GnRH antagonist by acting systemically blocks the release of gonadotropins, ablating LHCGR-mediated steroid hormone production and proliferation of adrenocortical cells. By acting directly on adrenocortical cells, GnRH antagonist decreases proliferation and metastatic abilities, as well as induces apoptosis.

6.3 LHCGR implications in pregnancy-induced Cushing syndrome

Diagnosis of hypercortisolism during pregnancy is challenging due to dysregulation of HPA axis by CRH and ACTH produced by the placenta (Lindsay and Nieman 2005). Prominent signs and symptoms of CS in our patient, high urinary free cortisol as well as suppressed ACTH plasma levels led to the diagnosis of primary adrenal hypercortisolism (Lindsay and Nieman 2005). Moreover, the remission of CS after the pregnancies indicated transient pregnancy-induced hypercortisolism related to hCG secretion.

The negative results of *CTNNB1*, *PRKAR1A*, *ARMC5*, and *PRKACA* familial and somatic mutation screening excluded a possibility that CS was due to genetic background (Groussin et al. 2006, Assié et al. 2013, Beuschlein et al. 2014, Teo et al. 2015). The analysis of the resected adrenals showed up-regulated expression of *LHCGR, GATA4 and ZFPM2*. Moreover, we co-localized *LHCGR* mRNA, LHCGR and GATA4 proteins in undifferentiated subcapsular and zG cells, whereas hyperplastic cells only expressed *LHCGR*/LHCGR. The presence of LHCGR in the subcapsular cells suggests that they may belong to a pool of progenitor cells that have a capacity to develop into hyperplastic adrenocortical cells. Therefore, the sequence of events, up-regulation of LHCGR and induction of GATA4 could be similar to GIACT models discussed above. However, during the differentiation from zG hyperplastic cells acquire zF-like steroidogenic enzyme profile, therefore stop expressing GATA4 and ZFPM2.

7 SUMMARY AND CONCLUSIONS

Despite broad and long lasting research novel therapies for adrenocortical tumors are needed. Here, we characterized the tumorigenic processes related to LH/LHCGR signaling and GATA4 expression, identified dysregulated genes/pathways and unraveled direct effects of GnRH antagonist treatment on inhα/Tag mouse model for adrenocortical tumorigenesis. We also characterized the involvement of LH/LHCGR signaling in the pregnancy-induced Cushing syndrome. The main findings of the projects included in this doctoral thesis are:

1. Mouse healthy adrenals and neoplastic A cells express *Lhcgr*.

2. ESR1 is a potential novel biomarker of adrenocortical tumors in inhα/Tag mice presenting with an inverted adrenal-to-gonadal steroidogenic gene expression profile.

3. Adrenocortical tumors of inhα/Tag mice originate from zona fasciculata.

4. LH/LHCGR signaling is a prerequisite for adrenal tumor formation in inhα/Tag mice and is most probably needed for GATA6 to GATA4 transcription factor switch in adrenal cells.

5. GATA4 drives induction and progression of adrenocortical tumors displaying gonadal phenotype in inhα/Tag mice.

6. GnRH antagonist Cetrorelix acts directly on adrenocortical tumor cells decreasing their viability, proliferation and inducing apoptosis.

7. Adrenocortical tumor progression in inhα/Tag mice is gonadotropin independent.

8. Persistently high LH/hCG levels may stimulate transformation of *LHCGR*-positive progenitor cells into LH/hCG-responsive adrenocortical cells which further differentiate into functional hyperplastic cells.

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