Regulation of Normal and Neoplastic Steroidogenic Cell Differentiation in the Adrenal Gland and Ovary

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ACADEMIC DISSERTATION

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To my family

Abstract

Two of the main steroidogenic organs, the adrenal cortex and ovary, arise from a common pool of progenitors in the developing embryo, and similar signaling pathways regulate the differentiation, growth, and survival of cells in these tissues. Proper development of the adrenal cortex and ovary requires precise spatiotemporal control of gene expression and apoptosis; disruption of these processes may lead to congenital disorders or malignant transformation. This dissertation project focuses on the molecular mechanisms that regulate normal and neoplastic steroidogenic cell development in the adrenal gland and ovary.

Earlier *in vitro* studies demonstrated that GATA6, a member of the GATA family of transcription factors, regulates the expression of multiple steroidogenic genes in the adrenal cortex. To show that GATA6 is a crucial regulator of adrenocortical development and function *in vivo*, we generated a mouse model in which *Gata6* is conditionally deleted in steroidogenic cells using Cre-Lox recombination with *Sf1*-cre. These mice exhibited a complex adrenal phenotype that includes cortical thinning, blunted aldosterone production, lack of an X-zone, impaired apoptosis, and subcapsular cell hyperplasia with increased expression of gonadal-like markers. These results offer genetic proof that GATA6 regulates the differentiation of steroidogenic progenitors into adrenocortical cells.

Ovarian granulosa cell tumors (GCTs), the most common sex-cord stromal tumors in women, are thought to be caused by aberrant granulosa cell apoptosis during folliculogenesis. A somatic missense mutation in *FOXL2* (402C→G) is present in vast majority of human GCTs. FOXL2 is a transcription factor that plays a key role in the development and function of normal granulosa cells. Wild type (wt) FOXL2 induces GCT cell apoptosis, while mutated FOXL2 is less effective. To clarify the molecular pathogenesis of GCTs, we investigated the impact of FOXL2 and two other factors implicated in granulosa cell function, GATA4 and the TGF-β mediator SMAD3, on gene expression and cell viability in GCTs. Expression of these factors positively correlated with one other and with their common target gene *CCND2*. Furthermore, we showed that GATA4, FOXL2, and SMAD3 physically interact and that GATA4 and SMAD3 synergistically induce *CCND2* promoter transactivation, which is reduced by both wt and mutated FOXL2. Finally, we demonstrated that GATA4 and SMAD3 protect GCT cells from wt FOXL2 induced apoptosis without affecting the apoptosis induced by mutated FOXL2. These findings underscore the anti-apoptotic role of GATA4 in GCTs, and suggest that mutated *FOXL2* gene disrupts the balance between growth and apoptosis in granulosa cells, leading to malignant transformation.

The treatment of recurrent or metastatic GCTs is challenging, and biologically targeted treatment modalities are urgently needed. Tumor necrosis factor-related apoptosis inducing ligand (TRAIL), a member of TNF ligand superfamily, activates the extrinsic apoptotic pathway. Interestingly, TRAIL is able to induce apoptosis in malignant cells without affecting normal cells. Vascular endothelial growth factor (VEGF) is one of the

key regulators of both physiological and pathological angiogenesis. Cancer cells often express VEGF receptor, and an autocrine VEGF/VEGFR signaling loop has been shown to exist in several types of cancer cells. We found that GCT cells express functional TRAIL receptors and activated VEGF receptors, and that treatment with TRAIL and the VEGF-binding antibody bevacizumab induce GCT cell apoptosis. These findings establish a preclinical basis for targeting these two pathways in the treatment of GCTs.

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List of original publications

This thesis is based on the following publications, which are referred to in the text by their roman numerals:

I **Pihlajoki M**, Gretzinger E, Cochran R, Kyrönlahti A, Schrade A, Hiller T, Sullivan L, Shoykhet M, Schoeller EL, Brooks MD, Heikinheimo M, Wilson DB. Conditional mutagenesis of *Gata6* in SF1-positive cells causes gonadal-like differentiation in the adrenal cortex of mice. *Endocrinology*, 154:1754-67, 2013.

II Anttonen M, **Pihlajoki M***, Andersson N*, Georges A*, L'Hôte D, Vattulainen S, Färkkilä A, Unkila-Kallio L, Veitia RA, Heikinheimo M. FOXL2, GATA4, and SMAD3 co-operatively modulate gene expression, cell viability and apoptosis in ovarian granulosa cell tumor cells. *PLoS One*, 9;9(1):e85545, 2014.

III Kyrönlahti A*, **Kauppinen M***, Lind E, Unkila-Kallio L, Bützow R, Klefström J, Wilson DB, Anttonen M, Heikinheimo M. GATA4 protects granulosa cell tumors from TRAIL-induced apoptosis. *Endocr Relat Cancer*, 17:709-17, 2010.

IV Färkkilä A*, **Pihlajoki M***, Tauriala H, Bützow R, Leminen A, Unkila-Kallio L, Heikinheimo M, Anttonen M. Serum Vascular Endothelial Growth Factor A (VEGF) Is Elevated in Patients with Ovarian Granulosa Cell Tumor (GCT), and VEGF Inhibition by Bevacizumab Induces Apoptosis in GCT in Vitro. *J Clin Endocrinol Metab*, 96:1973-81, 2011.

* The authors contributed equally to the study.

Abbreviations

ACTH	Adrenocorticotrophic hormone
AGP	Adrenogonaldal primordium
AMH	Anti-Müllerian hormone (also termed as Müllerian Inhibiting Substance)
AMHR2	Anti-Müllerian hormone receptor 2
BCL2	B cell lymphoma 2
BMP	Bone morphogenetic protein
BVZ	Bevacizumab
CCND2	CyclinD2
CYP19	Cytochrome P450 19A1 (also termed as aromatase)
DAPI	4',6-diamino-2-phenylindole hydrochloride
DAX-1	Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1
DcR	Decoy receptor
DR	Death receptor
Dz	Definitive zone
ELISA	Enzyme-linked immunnosorbent assay
FOXL2	Forkhead box protein L2
Fz	Fetal zone
GCT	Granulosa cell tumor
GDF	Growth/differentiation factor
GDX	Gonadectomy
HPA	Hypothalamic-pituitary-adrenal axis
Inhα	Inhibin α
LH	Luteinazing hormone
LMD	Laser microdissection
PKA	Protein kinase A
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RIA	Radioimmunoassay
SEM	Strandard error of mean
SF1	Steroidogenic factor 1
SHH	Sonic hedgehog
shRNA	Small hairpin RNA
SRY	Sex-determining region of the Y chromosome
StAR	Steroidogenic acute regulatory protein
TGF	Transforming growth factor
TRAIL	Tumor necrosis factor-related apoptosis inducing ligand
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
WNT	Wingless type MMTV integration site
zF	Zona fasciculata
zG	Zona glomerulosa
zR	Zona reticularis

Review of the literature

1. The development of adrenal cortex and gonads

1.1 The common origin of adrenal cortex and gonads

The main steroidogenic organs, the adrenal cortex and gonads, arise from a common precursor, the adrenogonadal primordium (AGP) (1). The AGP is derived from a specialized region of coelomic epithelium called the urogenital ridge, which also gives rise to the kidney (Figure 1). During embryogenesis, progenitors of the adrenal cortex and the bipotential gonad separate and begin to differentiate into their final forms. Adrenal precursors combine with neural crest cells to form the nascent adrenal gland, while gonadal progenitors combine with primordial germ cells to form a bipotential gonad (Figure 1).

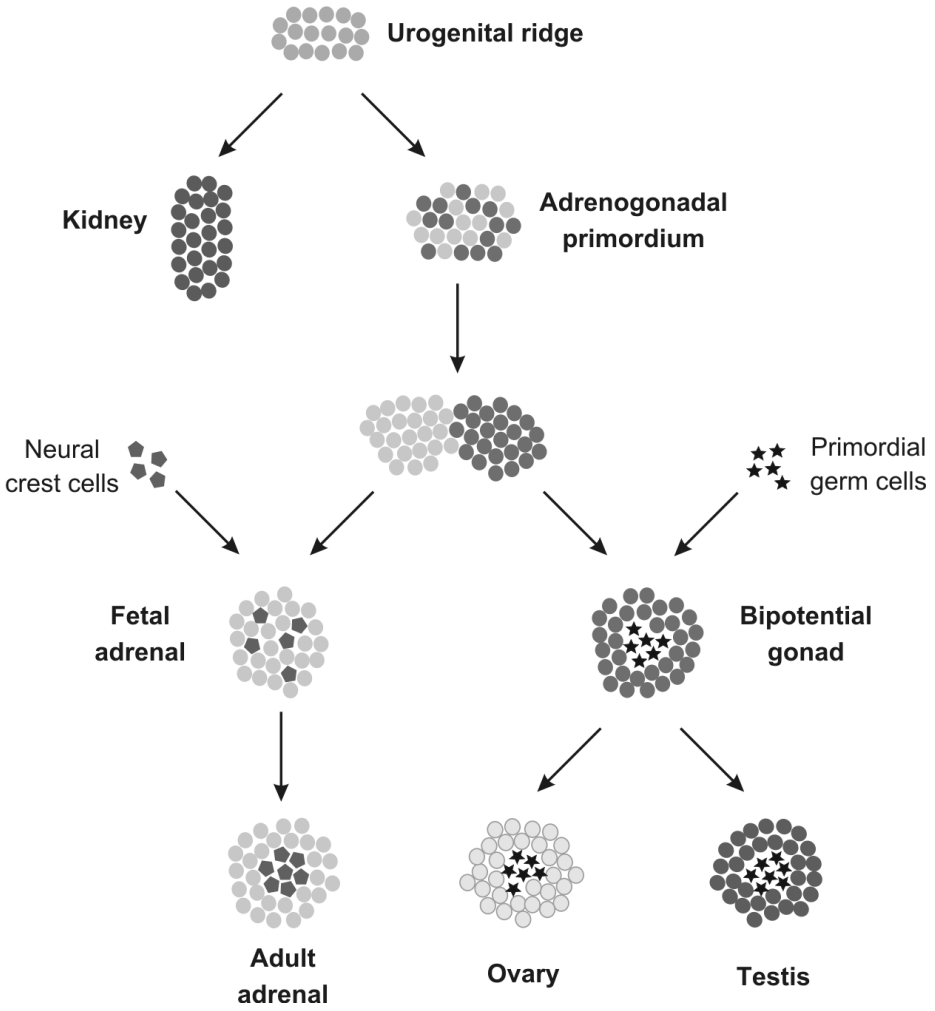

Figure 1 *A schematic drawing of the development of urogenital ridge derivatives.*

1.2 Development of the adrenal cortex

In humans, the AGP separates from the gonadal anlage at 33 days post-conception (dpc). By the 8th week of gestation the fetal adrenal consists of two distinctive layers: the inner fetal zone (Fz) and the outer definitive zone (Dz). The Fz is relatively thick and contains large eosinophilic cells, whereas the Dz is a thin band of small basophilic cells (2). Cells in both Fz and Dz show characteristics of steroidogenic capability (1). At the 9th week of gestation, mesenchymal cells surround the developing fetal adrenal and form a protective capsule. Shortely thereafter, neural crest cells, the progenitors of the adrenal medulla, migrate inside the nascent adrenal gland. During gestation, the adrenal medulla consists of small clusters of chromaffin cells scattered around the fetal adrenal cortex. After birth, these clusters coalesce to form a structurally discrete medulla (2). Postnatally, the morphology of adrenal gland changes dramatically when Fz undergoes apoptosis and Dz forms the adult adrenal zones; zona glomerulosa (zG) and zona fasciculata (zF) (2). In addition to zG and zF, adult human adrenal cortex contains also a third layer, zona reticularis (zR), which starts to develop between zF and medulla at the age of four, and continues to differentiate until the age of fiveteen (3).

Mouse adrenal gland development differs somewhat from that of human. As in humans, the mouse fetal adrenal gland consists of an inner Fz and an outer Dz. During late gestation, the Dz becomes thicker and forms the zF and zG while the Fz becomes thinner and its cells sporadically distribute in the medulla. After birth Fz cells coalesce and form a new layer between medulla and zF. This layer, termed the X-zone, disappears at the puberty in males and during the first pregnancy in females (4, 5).

1.3 Gonadal development

Gonadal development starts at week four of gestation when primordial germ cells migrate from the extraembryonic mesoderm of yolk sac to the AGP. Primordial germ cell arrival induces the proliferation of epithelial cells in the AGP, which leads to formation of the gonadal primordium. Proliferating epithelial cells extend into adjacent mesenchymal tissue and form sex cords. Primordial germ cells migrate into the developing gonad and are surrounded by the sex cord cells that differentiate into Sertoli cells in testis and granulosa cells in ovary. Adjacent mesenchymal cells differentiate into testosterone producing Leydig cells in male and ovarian androgen producing theca cells in female. The developing gonad remains sexually indifferent until gestation week seven. Genetic sex determinates whether the bipotential gonad develops into a testis or an ovary.

Sex-determining region of the Y chromosome (*SRY*) gene on Y chromosome of male genome expressed in somatic cells of developing testis is responsible for triggering the male sexual differentiation (6). SRY activates transcription factor SOX9, which in turn activates the molecular cascade leading to a male phenotype. One of the target genes of SOX9 is *Anti-Müllerian hormone* (*AMH;* also termed as *Müllerian inhibiting substance*,

MIS), a member of TGF-β superfamily ligands. The main function of AMH is to cause the regression of Müllerian ducts, but it also regulates testosterone production by Leydig cells. Testosterone, in turn, causes the formation of secondary structures of male reproductive tract.

In the past, female sex differentiation was thought to be a passive process that occurs in the absence of Y chromosome and *SRY* gene. Recently, evidence has accumulated indicating that certain signaling molecules are essential for the proper female sex differentiation. These factors include Wingless type MMTV integration site family, member 4 (WNT4), R-spondin1 (RSPO1), and forkhead transcription factor L2 (FOXL2) (7). All of these factors prevent male sexual differentiation by inhibiting *SOX9* expression. These factors also promote female reproductive development by sustaining Müllerian duct differentiation. Studies with transgenic mouse models support the importance of these factors in female sex differentiation. *Wnt4*-deficient female mice are masculinised, lacking Müllerian ducts while the Wolffian ducts continue to develop, and expressing male spesific steroidogenic enzymes 3β-hydroxysteroid dehydrogenase and 17α-hydroxylase (8, 9). RSPO1 synergises with WNT4 in stabilization of β-catenin in ovarian somatic cells, and the ovarian phenotype of female *Rspo1*$^{-/-}$ mouse largely resembles that of *Wnt4*-deficient mouse (10). FOXL2, in turn, is important for granulosa cell differentiation and maintenence of the ovarian phenotype. In adult ovary, FOXL2 prevents the transdifferentiation of granulosa cells into testicular Sertoli cells (11).

In developing ovary the primordial germ cells surrounded by a layer of squamous granulosa cells form primordial follicles that proliferate mitotically until the mid gestation. After the last mitotic division primordial germ cells enter meiosis and are thereafter called oocytes. By the 20th week of gestation the number of oocytes reaches the maximum 6-7 million. Over the ensuing weeks the number of oocytes decreases rapidly as most of the primordial follicles undergo the degenerative process called atresia. At birth about 1-2 million and by the puberty only 300 000 primordial follicles remain, of which only ~400 are ovulated during the reproductive life of a woman (12). Several genes are linked to the primordial follicle formation, including transcription factor *FIGα* (13), *Nerve growth factor* (14), and zinc-finger protein *ZFX* (15).

2. Adult adrenal cortex

2.1 Structure and function

Adult human adrenal cortex consists of three functionally distinct layers: zona glomerulosa (zG), zona fasciculata (zF), and zona reticularis (zR) (Figure 2) (16). The zG is the outermost layer and is composed of a thin region of columnar cells. The middle layer, the zF, is the thickest zone comprising more than 70% of the cortex, and it is composed of columns of polyhedral shaped secretory cells separated by capillaries. zR is the innermost layer also consisting of polyhedral cells organized in round clusters of cells (17). In adult murine adrenal cortex the zG and zF are well defined, but the zR is hardly recognizable. Instead, mouse adrenal cortex contains a transient zone between zF and medulla, termed the X-zone (Figure 2)(18).

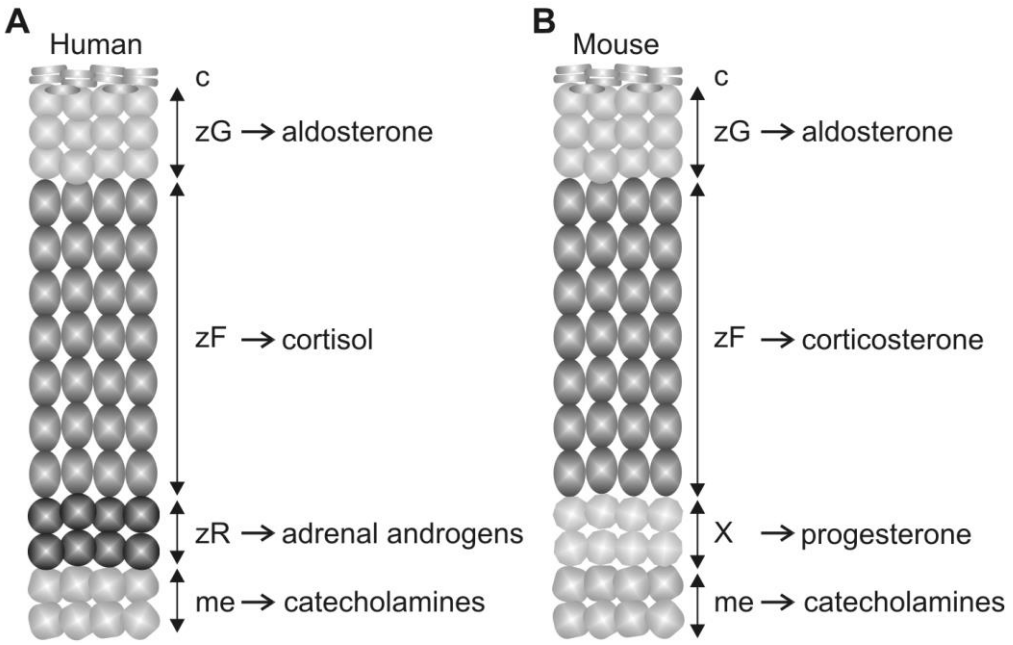

Figure 2 *A schematic depiction of the structure of adult human (A) and mouse (B) adrenal cortex, and the hormones produced by each zone. Abbreviations: c, capsule; zG, zona glomerulosa; zF, zona fasciculata; zR, zona reticularis; me, medulla; X, x-zone.*

Adult adrenal cortex is a dynamic organ that undergoes constant turnover. Stem and progenitor cells residing under the capsule differentiate and move centripetally to repopulate the cortex (19). The main function of adrenal cortex is to produce steroid hormones. In humans, zG secretes mineralocorticoids (mainly aldosterone), zF secretes glucocorticoids (cortisol), and zR is responsible for adrenal androgen production. Mouse

adrenal cortex lacks one of the steroidogenic enzymes, P450 17α-hydroxylase/17,20-lyase (P450c17), which is required for cortisol production. Thus, the main glucocorticoid produced by mouse zF is corticosterone. Adrenal corticosteroid production is controlled by hypothalamic-pituitary-adrenal (HPA) axis. After certain stimulus (e.g. stress), corticotropin-releasing hormone (CRH) is secreted from hypothalamus. This promotes the anterior pituitary to produce ACTH that induces adrenocortical cells to secrete corticosteroids. Corticosteroids in turn act back on hypothalamus and pituitary to suppress excess CRH and ACTH production in a negative feedback loop (17).

2.2 Regulation of adrenocortical function

Signaling pathways

Various endo- and paracrine factors, such as adrenocorticotrophic hormone (ACTH), luteinizing hormone (LH), activins, inhibins, and components of the WNT and Sonic hedgehog (SHH) signaling pathways, regulate the homeostasis of adrenocortical steroidogenic cells (20, 21).

The WNT signaling pathway is highly conserved phylogenetically and regulates a vast array of cellular functions, including proliferation, differentiation, and apoptosis. WNT-ligands exert their effects through three different WNT pathways, of which canonical β-catenin pathway is the most prominent in adrenocortical function. As the name implies, activation of canonical β-catenin pathway activates the cytoplasmic β-catenin leading to its translocation to the nucleus, where it acts as transcription factor activating the target gene expression. Two of the WNT-ligand family members, WNT4 and WNT11, are expressed in adrenal cortex (22, 23). Transgenic mouse studies have revealed that complete inactivation of β-catenin causes a drastic decrease of adrenocortical cell proliferation and differentiation at early stages of development leading to complete absence of adrenal gland. On the other hand, partial inactivation of β-catenin does not affect the development of the fetal adrenal cortex but has effects on adult cortex causing cortical thinning, disorganisation, increased apoptosis, and lack of differentiation of adrenocortical cells (24). This finding indicates that β-catenin signaling is required for normal adrenocortical renewal. Mice deficient for *Wnt4* have impaired zG differentiation and decreased CYP11b2 (aldosterone synthase) expression coupled with lower plasma aldosterone levels (25).

Another evolutionary conserved signaling pathway important for adrenocortical development and function is Sonic Hedgehog (SHH) signaling. SHH ligand is a secreted protein that by binding to its receptor Patched (PTCH) activates the signaling cascade leading to target gene activation. In mice, SHH is secreted by the non-steroidogenic cells of adrenal subcapsule, and capsular cells expressing PTCH transduce the signal and subsequently upregulate transcription factor Gli1. These Gli1 positive cells migrate into

the cortex and differentiate into steroidogenic cells (26). *Shh* null mice have small adrenals with thin capsule/subcapsule. Altough SHH does not directly signal to the cortical cells, the cortical growth of the *Shh* null mice is largely impaired, whereas the adrenal zonation and hormone production in these mice are normal (20). The expression patterns of SHH and its receptor in the human adrenal cortex has not yet been described.

Gene regulation

Precise spatiotemporal gene regulation is essential for proper function and homeostasis of the adrenal cortex. Several key transcription factors have been implicated in regulation of adrenocortical steroidogenic cell function.

Steroidogenic factor 1 (SF1; also termed as NR5A1, AD4BP) is an orphan member of the nuclear receptor superfamily. SF1 is expressed in the steroidogenic cells of adrenal cortex as well as in gonadal somatic cells both during development and in adult organs. *In vitro* and *in vivo* studies have shown that SF1 is a key regulator of steroidogenesis by activating the expression of steroidogenic enzymes (27, 28). It has also been shown to promote adrenocortical cell growth and limit apoptosis (29, 30). *Sf1* null mice develop no adrenals and die shortly after birth (30, 31). In humans, various heterozygous mutations in *SF1* gene have been associated with adrenal failure (32).

Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1 (*Dax1;* also termed as *Nr0b1*), another member of the nuclear receptor family, is an X-linked gene whose expression is mainly restricted to steroidogenic tissues. In adult adrenal cortex, DAX1 is expressed in the adrenal progenitor cells located in subcapsular region, where it has been shown to inhibit the steroid production stimulated by SF1 (33), and maintain the progenitor cell pool by inhibiting the differentiation of steroidogenic cells (34). DAX1 deficiency in mice and mutations in *DAX1* gene in humans cause the similar phenotype of adrenal dysplasia and early adrenocortical failure (34).

Transcription factor GATA6 is one of the six GATA factors that play crucial roles in development, differentiation, and function of diverse organs. GATA factors recognize and bind to the (A/T)GATA(A/G) sequence on their target gene promoter, and trigger the gene transcription. GATA6 is expressed in various tissues including heart, lung, liver, gonads, pancreas, placenta, and adrenal cortex (35). In adrenal cortex GATA6 is expressed both during development and in adult organ in all cortical zones (36). Transactivation studies have demonstrated that GATA6, in concert with SF1, regulates the expression of multiple steroidogenic genes including *steroidogenic acute regulatory protein* (*StAR*), *CYP11A1*, *CYP17A1*, *HSD3B2*, *CYB5*, and *SULT2A* (37-40). *Gata6* null mice die early during development due to defects in yolk sac endoderm function preventing the use of this model for studying the *in vivo* role of GATA6 in adrenocortical cells (41). In humans, no adrenocortical defects caused by mutations in *GATA6* gene have been found so far, but its expression has shown to be downregulated in human adrenocortical carcinomas (42).

Another GATA family member, GATA4, is implicated in fetal adrenocortical development, but its expression is downregulated soon after birth (43).

2.3 Adrenocortical tumors

Adrenocortical tumors (ACT) are fairly common in humans and some domestic animals including mice, dogs, ferrets, and goats. In humans, the most common ACT is the so-called incidentaloma, found in ~5 % of people older than 50 years old. Incidentalomas are devided in non-secreting and hormone-secreting tumors, of which the former are usually asymptomatic and do not require treatment. Hormone-secreting tumors include aldosterone- and cortisol-producing adenomas. These benign tumors can cause Cushing syndrome and other complications (44). Malignant adrenocortical carcinoma (ACC) is more rare with incidence of 1 case per million people per year. ACCs are also classified as secreting and non-secreting tumors, and they possess high metastatic potential with 5-year survival rate of 16-38 % (patients with metastatic ACC) (45).

The molecular pathogenesis of ACT is still largely unknown. Some mutations causing loss or gain of chromosomes have been found from ACT patients (45). Furthermore, several studies have revieled abnormalities in the expression of *INHA* (TGF-β signaling mediator), *IGF2* (growth factor), *CTNNB1* (gene coding for β-catenin), and *TP53* (tumor suppressor gene) in most ACCs (46-49). Moreover, silencing of β-catenin pathway has been shown to decrease ACC cell proliferation and increase apoptosis *in vitro*, as well as attenuate tumor formation in mouse xenograft model *in vivo* (50). The expression of transcription factor GATA6 has been shown to be downregulated in human ACCs compared to normal adrenal cortex and adenomas, whereas GATA4, which is not expressed in normal adrenal cortex, is highly expressed in both adenomas and ACCs (42, 51). Furthermore, GATA6 expression in human ACCs is shown to correlate with poor outcome (52).

Gonadectomy-induced adrenocortical neoplasms

Subcapsular adrenocortical neoplasms that histologically resemble ovarian stroma have been reported in postmenopausal women and men with testicular atrophy (53, 54). Elevated LH levels and decreased sex steroid levels of these patients have been suggested to cause tumor formation (21). The similar kind of phenotype is found in certain inbred mouse strains (such as DBA/2J, C3H, and CE/J) after gonadectomy (GDX). GDX-induced adrenocortical neoplasms arise in subcapsular region and invade deeper in the cortex, forming wedge shaped areas of tumor tissue. These sex steroid producing tumor cells express gonadal specific markers including transcription factor GATA4, AMH and its receptor, CYP17, as well as LH receptor (LHR), whereas transcription factor GATA6, which is normally expressed in adrenocortical steroidogenic cells, is downregulated (21).

The molecular mechanisms behind GDX-induced adrenocortical neoplasms are still poorly understood. Recently, DNA methylation is shown to play a role in formation of these neoplasms. It is speculated that because of the common origin of adrenocortical and gonadal cells the changes in DNA methylation status of the adrenocortical progenitor cells induced by the elevated serum LH levels could lead to cell fate conversion. It has been shown that the genes expressed in tumor versus adjacent normal tissue are differentially methylated leading to differential gene expression (55). Transcription factor Wilms tumor 1 (WT1) is also connected to the pathogenesis of these neoplasms. Bandiera *et al*. showed that the adrenal capsule contains a pool of progenitor cells that express AGP markers WT1 and GATA4 (56). Under normal conditions these cells differentiate into adrenocortical cells, but GDX triggers the differentiation of these AGP-like cells into gonadal-like cells. Furthermore, transcription factor GATA4 has been shown to act as a dose-dependent key modifier of these neopleasms. Mice heterotzygous for *Gata4* show attenuated tumor formation in susceptible mouse strains and reduced expression of gonadal specific genes (57), while transgenic expression of *Gata4* induces adrenocortical neoplasia in a non-susceptible strain (58).

3. Adult ovary

3.1 Structure and function

Adult ovaries are paired oval-shaped organs located on either side of the uterus and surrounded by the surface epithelium. Ovary consists of a number of vesicular follicles that are imbedded in the ovarian stroma. Stroma is composed of blood vessels and interstitial cells, mostly connective tissue cells (59).

Adult ovaries have two main functions: 1) to secrete sex steroids (mainly estrogen) and 2) to produce fertile gametes for reproduction, which can be fertilized. The process of ovarian follicle development from primordial follicle to ovulation is called folliculogenesis. Different phases and regulation of folliculogenesis are discussed in more detailed in the next chapter.

3.2 Regulation of follicular development

Initial recruitment

The first phase of folliculogenesis is formation of primordial follicles during gestation and right after birth (see chapter 1.3). Primordial follicles remain in a quiescent phase until they are recruited into the primary stage for growth. This process, called initial recruitment, starts already in fetal life and continues postnatally over the whole reproductive life until the ovarian reserve is depleted. Initial recruitment of primordial follicles is gonadotropin-independent unlike the later stage of folliculogenesis, the cyclic recruitment of antral follicles from puberty onwards. During initial recruitment the size of the oocyte increases and granulosa cells around the oocyte change their shape from squamous to cuboidal (Figure 3). After this morphological transformation, granulosa cells start to proliferate forming two or more layers of cells around the oocyte. This stage is called secondary follicle (Figure 3). At the secondary stage a layer of theca cells is recruited from interstitial stromal cells to surround the follicle. As folliculogenesis proceeds and granulosa cells accumulate, a fluid filled space, termed the antrum, forms within the granulosa cell layers. Antrum formation divides granulosa cells into two distinct compartments, cumulus cells surrounding the oocyte and mural cells lining the follicle wall. This stage is called antral follicle (Figure 3) (reviewed in (60)).

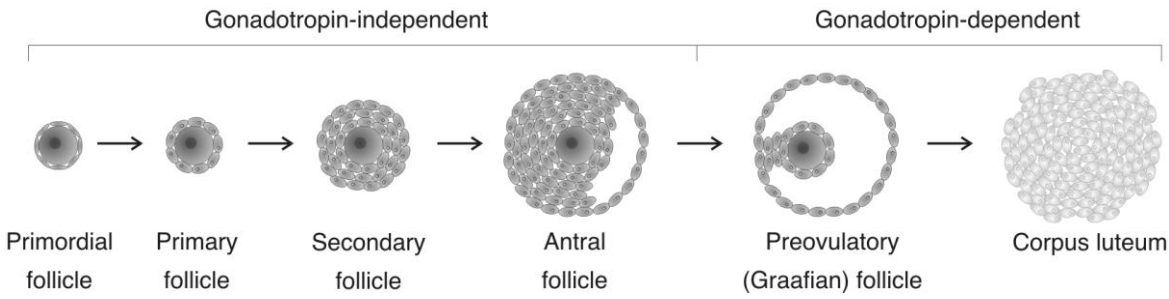

Figure 3 *A schematic drawing of folliculogenesis.*

Initial recruitment and follicle growth from primordial to antral follicle stage are regulated by the complex interplay between the oocyte and somatic granulosa and theca cells. Certain extra-cellular matrix components as well as paracrine and autocrine growth factors play roles in this regulation. Some of these factors, including tumor suppressors TSC-1 and PTEN, forkhead transcription factors FOXO3a and FOXL2, cyclin-dependent kinase inhibitor p27, and TGF-β member AMH inhibit the activation of follicular growth. In mouse models, loss of these factors leads to premature activation of the primordial follicle pool and premature ovarian failure (POF) (61-66). In addition to its inhibitory role, transcription factor FOXL2 has been shown to be important for the differentiation of granulosa cells from squamous to cuboidal state. Mice with mutated *Foxl2* gene show normal primordial follicle development but granulosa cells fail to complete the squamous-to-cuboidal transition leading to the absence of secondary follicles (67). In humans, mutations in *FOXL2* gene have been associated with Blepharophimosis-ptosis-epicanthus inversus (BPES) syndrome causing premature ovarian failure (POF) (68, 69).

Other important regulators of initial recruitment are the Transforming growth factor (TGF)-β superfamily members. Growth/differentiation factor (GDF) 9 is secreted from oocytes where it is expressed from primary follicles until ovulation (70). *Gdf9* deficient mice are infertile and the follicle development is arrested at the primary stage. These mice also show impaired recruitment of theca cells around the developing follicle (71). *In vitro* studies have demonstrated increased number of primary and secondary follicles in human and rodent ovarian cortical samples cultured with oocyte-derived recombinant GDF9 (72-74). These findings suggest that GDF9 is an important positive regulator of follicle development. Another oocyte derived TGF-β family member, Bone morphogenetic protein (BMP) 15, positively regulates follicle growth by stimulating granulosa cell proliferation (75). BMP4 and BMP7, derived from theca cells, also promote the follicle growth from primordial stage onwards (76, 77). Granulosa cell derived activin has been shown to promote pre-antral follicle growth and granulosa cell proliferation through autocrine and paracrine effects in rodent models (78-80). TGF-β is secreted from granulosa and theca cells during pre-antral follicular growth. In rodents TGF-β has shown to promote granulosa cell proliferation (79).

Cyclic recruitment

At the puberty, when the levels of circulating follicle-stimulating hormone (FSH) rise, a few developing antral follicles are rescued from atresia during each menstrual cycle. This process is called cyclic recruitment, and unlike initial recruitment it is gonadotropin dependent. The pool of recruited follicles continues growing as the granulosa and theca cells proliferate further and the antrum increases in size. At this stage theca cells begin to express LHR and produce androgens stimulated by anterior pituitary-secreted LH. Granulosa cells express both FSH receptor (FSHR) and LHR. Furthermore, *Cyp19* expression of granulosa cells enables the estrogen production from thecal androgens. One of the recruited follicles is chosen as the dominant follicle through a very comlex and incompletely understood chain of events. This dominant follicle grows faster and develops into a Graafian follicle competent for ovulation and fertilization (Figure 3). The rest of recruited follicles undergo atresia. The LH surge triggers ovulation and shifts granulosa cell steroid production from estogen to progesterone. During ovulation the follicle wall ruptures, and the oocyte is released into the peritoneal cavity. The remaining follicle degenerates and forms the corpus luteum whose main function is to produce progesterone (reviewed in (60)).

The growth of recruited follicles, their steroidogenic activity and responsiveness to gonadotropins, as well as prevention of premature luteinisation is controlled by endo-, para-, and autocrine factors. FSH is one of the two gonadotropin hormones secreted by anterior pituitary. It exerts its effects by binding to its receptor FSHR, which then activates the protein kinase A (PKA) pathway. Activated PKA pathway in granulosa cells, in turn, activates genes important for follicle development, including inhibin α and β, Cyp19, and cell cycle regulator CyclinD2. The importance of FSH for proper follicle growth and development has been demonstrated in studies using mice lacking either FSH or its receptor. Both of these mutants are infertile, and the folliculogenesis is blocked at antral stage (81, 82).

Activin, a member of TGF-β family, has shown to induce the FSHR expression in granulosa cells *in vitro* (83). It also suppresses the growth of primary follicles while promoting follicular growth at later stages, and positively regulates Cyp19 and LHR expression (80, 84, 85). Activin receptor deficient mice show arrested follicle development (86). Inhibin A and B, expressed in granulosa cells of antral follicles, oppose the effects of activin. While activin is shown to stimulate the FSH production of pituitary, inhibin A and B decreases it (87, 88). Inhibins have also shown to decrease the growth of developing follicles (89). Another TGF-β member, AMH, inhibits cyclic recruitment by reducing responsiveness of antral follicles to FSH (90). Oocyte-derived factors BMP6, BMP15, and GDF9, as well as granulosa cell derived BMP4 and BMP7 inhibit premature luteinisation by suppressing gonadotropin-driven progesterone synthesis (89, 91).

The ligands of TGF-β family exert their effects through their serine/threonine kinase receptors and intracellular signaling molecules, called SMADs that act as transcription

factors regulating the target gene expression. Precise spatiotemporal regulation of expression of these downstream effectors is essential for proper course of folliculogenesis. Eight different SMAD molecules have been identified, of which SMAD2 and -3 are activated by TGF-β, GDF9, and activin, whereas BMPs and AMH activate SMAD1, -5, and -8. In addition, SMAD6 and -7 act as inhibitory molecules and SMAD4 as a common co-activator (reviewed in (92)). In the ovary, mice deficient for both *Smad2* and *Smad3* are infertile having defects with follicular development and ovulation (93). Double knockout mice of *Smad1* and *-5*, or triple knockouts of *Smad1*, *-5*, and *-8* are infertile as well, but they also develop metastatic granulosa cell tumors (Discussed in more detailed in chapter 4.3) (94).

Another important regulator of ovarian function is transcription factor GATA4. This member of the GATA transcription family is expressed in both fetal and postnatal ovarian granulosa and theca cells (95, 96). In adult ovary, GATA4 is expressed in proliferating granulosa cells but its expression is downregulated before ovulation and luteinisation (95-97). FSH has been shown to positively regulate the expression and intrinsic activity of GATA4 (96, 98). *In vitro* studies have demonstrated that GATA4 activates genes important for steroidogenesis, such as *Star*, *Cyp11a1*, and *Cyp19* (98-100). GATA4 heterozygous mice have delayed puberty and their responsiveness to exogenous gonadotropins is decreased, while mice conditionally deleted GATA4 in granulosa cells show impaired fertility with cystic ovarian morphology and attenuated response to gonadotropin stimulation (101-103).

3.3 Apoptosis in the ovary

Apoptosis is the process of programmed cell death that occurs in every multicellular organism and plays a crucial role in shaping organs during development and controlling homeostasis and proper function of various tissues in adult organisms, including the human reproductive system. Unlike necrosis, apoptosis is an energy-requiring and well co-ordinated process that results in the formation of apoptotic bodies that are engulfed by the neighboring cells or macrophages without causing an inflammatory response.

The default pathway of ovarian follicles is to undergo apoptosis, as only ~400 follicles ovulate during a female's reproductive life, while the rest of the developing follicles become atretic and die during folliculogenesis. During the fetal period, the main cell type undergoing apoptosis are the germ cells. Oocytes that fail to become surrounded by somatic granulosa cells during primordial follicle formation degenerate and undergo apoptotic demise. In the postnatal ovary, apoptosis is prominent in the granulosa cells of the growing follicles during the cyclic recruitment. Furthermore, apoptosis is responsible for *corpus luteum* regression if pregnancy does not occur (104).

Regulation of ovarian apoptosis

Gonadotropins are important regulators of postnatal ovarian apoptosis. *In vivo* rodent studies (105, 106) and *in vitro* studies utilizing the cultured follicles (107) have demonstrated that the decrease in gonadotropin levels causes follicular atresia, while the early apoptotic follicles can be rescued by exogenous gonadotropins. Locally produced paracrine growth factors (e.g. Insulin-like growth factor 1, IGF1; Epidermal growth factor, EGF; Basic fibroblast growth facor, FGF; and Interleukin-1β, IL-1β) as well as hormones (e.g. estrogen and progesterone) also play a role in the regulation of ovarian apoptosis by acting as prosurvival factors of granulosa cells and inhibiting apoptosis (107-111).

In addition to the aforementioned prosurvival factors, two cellular apoptotic pathways, the extrinsic and intrinsic pathways, also regulate ovarian apoptosis. The extrinsic pathway is activated by binding of extracellular protein ligands to the proapoptotic death receptors (DR) located on the cell surface, whereas the intrinsic pathway (also termed as mithocondrial pathway) is activated in response to intracellular signals, including cellular stress and DNA damage. Both of these pathways lead to the activation of cystein-aspartic protease (caspase) cascade. Caspases are proteases that execute the cellular processes during apoptosis. After the apoptotic signal, the initiator caspases (caspase-2, -8, -9, -10) are activated, which in turn activate the downstream effector caspases (caspase-3, -6, -7). These effector caspases cleave various cellular proteins leading to the characteristic morphological changes of apoptotic cell, including blebbing of the plasma membrane, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (reviewed in (112)). An overview of the extrinsic and intrinsic pathways is presented in Figure 4.

BCL2 protein family members are important mediators of intrinsic apoptotic pathway. These proteins are devided into anti- and pro-apoptotic factors based on their function. Anti-apoptotic members of BCL2 family include BCL2, BCL-XL, BCL-W, A1, and Mcl-1, while pro-apoptotic members include BAX, BAK, BOK, BID, BAD, PUMA, and NOXA (112). The balance between these factors sets the threshold of apoptosis for intrinsic pathway.

One of the extracellular ligands that activate the extrinsic apoptosis pathway is Tumor Necrosis Factor (TNF)-Related Apoptosis Inducing Ligand (TRAIL) that belongs to the TNF superfamily. TRAIL acts through its receptors DR4 (TRAIL-R1) and DR5 (TRAIL-R2), whose activation leads to caspase activation and apoptosis (113). In addition to its death receptors, TRAIL is also capable of binding to two decoy receptors DcR1 (TRAIL-R3) and DcR2 (TRAIL-R4), which are lacking the intracellular death domain (114, 115), and thus modulate the TRAIL pathway activity by competing the ligand binding with DR4 and DR5 (116).

TRAIL and its receptors are widely expressed in variety of tissues, including liver, lung, prostate, and myometrium (117). In addition to its ability to induce apoptosis,

TRAIL has also been shown to have other functions, including the control of hematopoiesis, prevention of autoimmunity, and regulation of endothelial cell physiology (118-120). In human fetal ovary, TRAIL and its receptors DR5 and DcR2 are expressed both in oocytes and granulosa cells, whereas in postnatal ovary, TRAIL, DR5, as well as both DcR1 and DcR2, but not DR4, are expressed in oocytes and granulosa cells of small primary and secondary follicles (121). TRAIL and its receptors are also expressed in porcine ovaries, where the expression of TRAIL has been shown to increase and the expression of DcR1 to decrease during follicular atresia (122, 123). Furthermore, TRAIL has been shown to induce the apoptosis of primary cultured porcine granulosa cells *in vitro*, and eliminating the DcR1 from these cells results in increased number of apoptotic cells (123). These findings suggest that TRAIL has apoptosis-inducing activity in granulosa cell, and that decoy receptors can inhibit this ability.

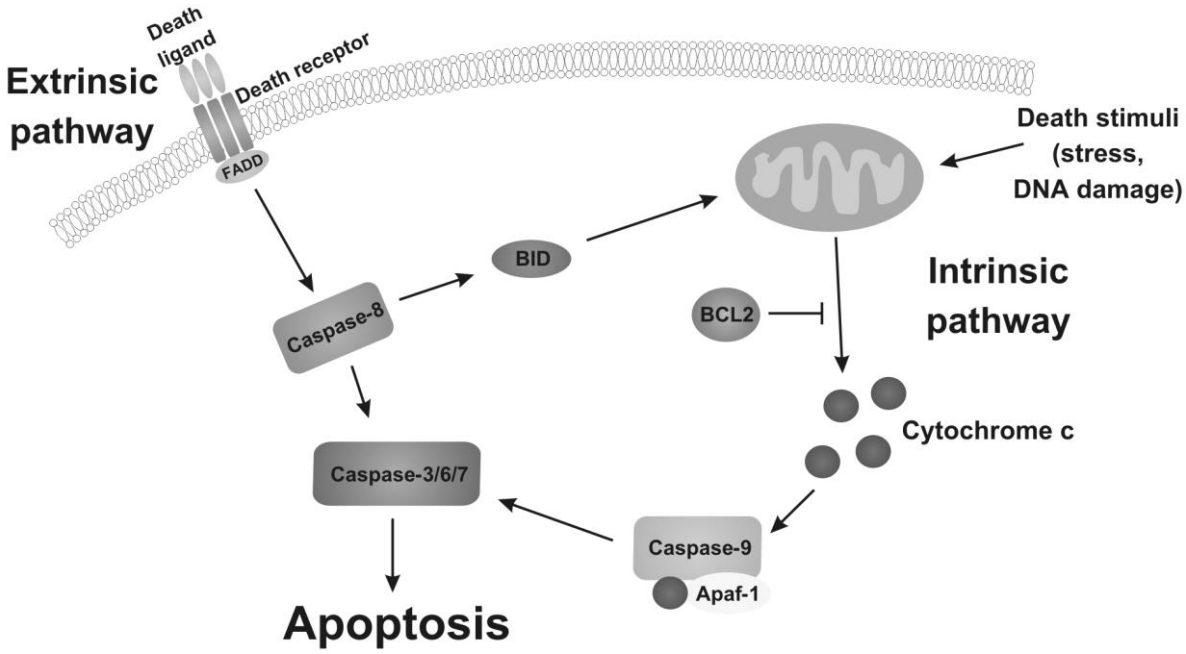

Figure 4 *Apoptotic signaling pathways. Extrinsic pathway: the binding of extracellular death ligand to its plasmamembrane receptor (death receptor) activates the intracellular Fas-associated protein with death domain (FADD). FADD, in turn, recruits caspase-8, which activates the effector caspases (caspase-3, -6, -7) leading to cell death. Intrinsic pathway: death stimulus (e.g. cellular stress or DNA damage) induces the release of cytochrome c from mitochondria. Cytochrome c catalyzes the oligomerization of Apoptosis protease activating factor-1 (Apaf-1), which recruits and promotes the activation of procaspase-9. This, in turn, activates caspase-3, -6, and -7 leading to apoptosis. B cell lymphoma 2 (BCL2) inhibits the intrinsic apoptotic pathway by controlling the mitochondrial membrane permeability and thus preventing the release of cytochrome c. Activation of extrinsic pathway can also trigger the intrinsic pathway through activation of BH3 interacting domain death agonist (BID), which in turn causes the release of cytochrome c.*

4. Steroidogenesis in the adrenal cortex and ovary

One of the main functions of adult adrenal cortex and gonads is the production of steroid hormones. Adult human adrenal cortex produces mineralocorticoids (aldosterone) secreted by zG cells, glucocorticoids (cortisol) secreted by zF cells, and adrenal androgens secreted by zR cells (17). Adult human ovarian granulosa cells in cooperation with theca cells, in turn, secrete sex steroids (mainly estrogen) (59).

Cholesterol is the precursor for all steroid hormones. Cholesterol is converted to steroid hormone intermediates and mature hormones by oxidative cytochrome-450 enzymes in the mitochondria and smooth endoplasmic reticulum. ACTH in adrenal cortex, and FSH and LH in ovarian cells regulate the uptake and storage of cholesrerol. The pathways of adrenal cortex and ovarian steroid biosynthesis use the same enzymes for the first steps of steroidogenesis, but the final active product of each pathway depends on the enzymes present in a given cell type (Figure 5). This explains the differences in steroid hormone production among the steroidogenic tissues.

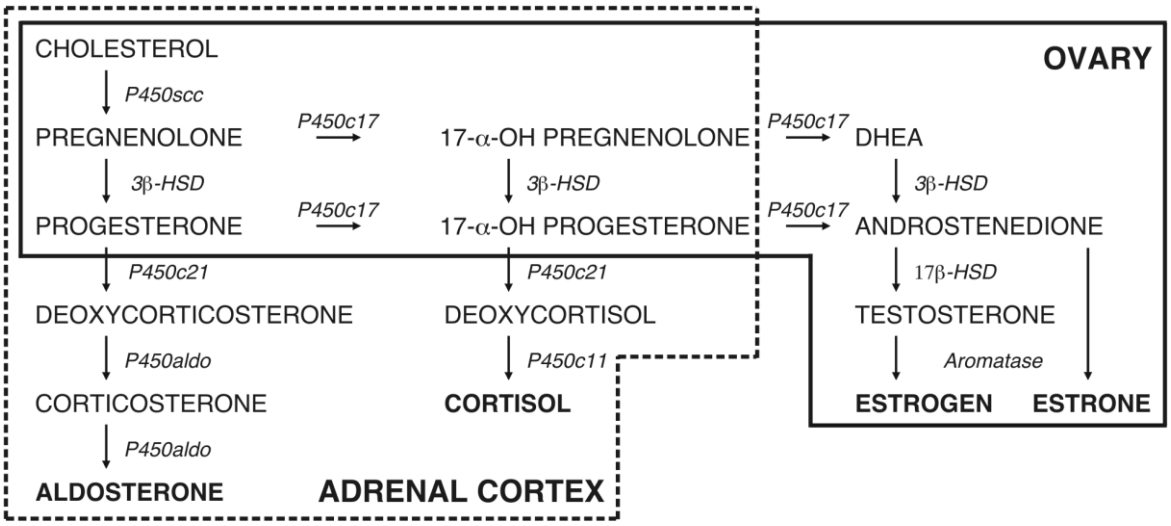

Figure 5 *Steroidogenic pathways in the human adrenal cortex and ovary*.

5 Ovarian granulosa cell tumors

5.1 Overview

Invasive ovarian tumors are the most common lethal gynecological malignancy. Ovarian tumors are classified in three groups based on their histopathological patterns reflecting the various cell types in the ovary. Epithelial ovarian tumors, representing 80-90 % of all ovarian cancers, are derived from the ovarian surface epithelium. Germ cell tumors, accounting 1-2 % of the ovarian malignancies, are thought to be derived from the fetal primordial germ cells, and are much more common among children and adolescents than older women. The third group, sex cord-stromal tumors, arising from the sex cord and stromal components of the ovary represent approximately 8 % of the ovarian cancers. These tumors include granulosa cell tumors (GCTs), thecoma-fibromas, Sertoli-Leydig cell tumors, and sex cord-stromal tumors of mixed or unclassified cell types (reviewed in (124)).

GCT is the most common sex-cord stromal tumor accounting for 90 % of the tumors within this subgroup. Based on the clinical behavior and histopathological charasteristics GCTs are further classified in two subgroups, juvenile (more common among children and young adults) and adult (more common among postmenopausal women) form, of which adult GCTs account for 95 % of the cases. The annual incidence of GCT is 0.4-1.7 cases per 100 000 women (125). Unless otherwise stated, the chapters below will focus on the adult subtype of GCT.

Adult GCT is commonly diagnosed in peri- or postmenopausal women with the median age of 50-54 yrs (125). The most common symptoms of GCT include acute abdominal pain caused by tumor rupture, swelling due to a large tumor mass, and postmenopausal bleeding or irregular menstruation caused by excessive hormone production by the tumor cells. Size of the tumor varies from microscopic lesions to large abdominal masses, the average size being 12 cm (124). These tumors are often cystic and hemorrahagic, and microscopic examination shows poorly and well differentiated histologies, both of which contain characteristic round to oval shaped cells with “coffee-bean grooved” nuclei. Low mitotic index and mild nuclear atypia are also characteristic for GCTs (125).

Surgery is the primary treatment option for GCT, but the surgical treatment of metastatic or recurrent disease is challenging (126, 127). For these cases radiation and conventional chemotherapy have been used, but the efficacy of chemotherapeutic regimens, which were developed for epithelial ovarian cancer, is poor, highlighting the need for new treatment modalities, including biologically targeted therapies.

GCTs are considered to have low malignant potential with tendency of late recurrence after primary diagnosis. Tumor stage at time of diagnosis is the only prognostic factor

with clinical significance. The 5-year survival rate for patients with stage I disease ranges from 75-95 %, while the 5-year survival rate for stage II is 55-75 %, and for stages III and IV only 22-50 % (125, 126). Recurrences may appear many years, even decades, after the primary tumor has been removed, and with the recurrent tumor the mortality rises up to 80 % (128).

5.2 Pathogenesis

The molecular pathogenesis of GCT is still largely unknown. Compared with other ovarian malignancies, GCTs exhibit a relatively stable karyotype without chromosomal aberrations. Furthermore, no activating mutations in known oncogenes (129, 130), or loss of heterozygosity or mutations in tumor suppressor genes (129) have been found. GCT exhibits many features of normal proliferating granulosa cells of the preovulatory follicles, including estrogen and inhibin B production (131, 132), as well as expression of FSH receptor (133) and transcription factor GATA4 (97). Therefore it is suggested that the molecular pathogenesis of GCT involves developmentally abnormal or disrupted expression of essential signaling pathways that function during folliculogenesis and regulate proliferation and apoptosis of normal granulosa cells.

Transcription factor FOXL2 mutation

Recently, a huge step towards understanding the mechanisms behind GCT pathogenesis was taken, when the whole transcriptome RNA sequencing study identified a somatic missense mutation in gene coding for transcription factor *FOXL2* (402C→G) in four adult GCTs (134). This mutation leads to the substitution of a tryptophan residue for cysteine residue at amino acid position 134 (C134W). Subsequent studies confirmed this finding in larger patient cohorts, the average of mutation positive GCTs being 94 % (134-140). Interestingly, another GCT subtype, juveline GCT lacks this mutation and the expression of FOXL2 in this GCT type has been shown to negatively correlate with tumor aggressiveness (141).

Although the vast majority of GCTs bear the C134W mutation in FOXL2, the mechanistic explanation of the effect of this mutation is still lacking. FOXL2 has been shown to negatively regulate cell cycle progression (142) and promote apoptosis (143) in granulosa cells, whereas C134W mutated FOXL2 disturbs this balanced regulation by upregulating genes involved in the control of cell cycle, and downregulating genes involved in apoptosis (144, 145) leading to a less effective induction of granulosa cell apoptosis (143). Furthermore, mutated FOXL2 inhibits the activin and GDF9 induction of anti-proliferative follistatin, which consequently may lead to increased cell proliferation and enhanced tumor formation (146).

Transcription factor GATA4 in GCT biology

The majority of GCTs express transcription factor GATA4 at comparable levels to normal preovulatory granulosa cells (97), and GATA4 expression in these tumors has been shown to correlate with tumor aggressiveness and increased risk of recurrence (147). GATA4 expression also correlates with the intrinsic apoptotic pathway inhibitor BCL2 and proproliferative CyclinD2 (CCND2) expression both at mRNA and protein level (148). *In vitro* studies have revealed that GATA4 activates the expression of *Bcl2* and *Ccnd2* in murine GCT cells (148). Furthermore, GATA4 has been shown to protect cardiomyocytes from apoptosis (149, 150). Taken together, these findings suggest that GATA4 may act as an anti-apoptotic factor also in GCTs.

SMAD3 in GCT pathogenesis

As discussed in chapter 3.2, SMAD proteins are the essential intracellular mediators of TGF-β signaling in normal granulosa cells. Activin A and TGF-β signaling are mediated by SMAD3, which is an important regulator of *Ccnd2* in rat granulosa cells (151). Moreover, in human GCT cell lines SMAD3 drives cell viability by activating NF-κB, a transcription factor regulating a vast array of stimuli related to biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis, and apoptosis. NF-κB in turn up-regulates SMAD3 expression; this positive feedback loop activates the ERK1/2 pathway leading to increased GCT cell survival (152). Deficiency of inhibin-α subunit, or SMAD1/5 in mice has been shown to promote GCT formation, and SMAD3 is upregulated and activated in these murine GCTs (discussed in more detail below) (94, 153, 154). Collectively these findings suggest a role for SMAD3 in GCT pathogenesis.

5.3 Transgenic mouse models

A number of transgenic mouse models have been generated to shed light on the possible mechanisms behind GCT pathogenesis, as detailed below.

Mouse with simian virus 40 T-antigen (SV40 TAg) driven by inhibin α subunit promoter

In order to generate *in vivo* gonadal tumor model and establish immortalized gonadal somatic cell line, Kananen *et al*. developed transgenic mice in which the SV40 TAg was overexpressed under the inhibin α promoter (inhα/SV40TAg) (155). These inhα/SV40TAg mice were infertile and developed GCTs by the age of 5-7 months with 100 % penetrance. The features of these animals resemble those in human GCT patients, including elevated serum inhibin levels, continued folliculogenesis, depressed serum gonadotropins, and similar histopathlogical alterations (156). Moreover, the suppression

of circulating gonadotropin levels in these animals prevented tumor formation, and the constitutively overexpression of LH stimulated tumor formation, suggesting a tumor promoter role for LH (157, 158). More studies are, however, needed to assess the role of LH in human GCT pathogenesis.

Inhibin α knockout mouse

Complete deletion of inhibin α (*Inhα*) gene in mouse results in the formation of bilateral, mixed, or incompletely differentiated sex cord-stromal tumors with 100 % penetrance in both sexes at age of 4 weeks (159). These mice suffer from severe cancer cachexia-like syndrome that was caused by the significant increase in circulating activin A levels (160). These findings suggest that inhibin α acts as a tumor suppressor in these mice. Subsequent studies, in which the Inhα$^{-/-}$ mice have been crossbred with other conditional mouse models, have generated more valuable data to help understanding the modifiers of gonadal tumorigenesis. For instance, when the mice lacking FSH or LH were crossed with *Inhα*$^{-/-}$ mouse, the resulting mice exhibited delayed onset of tumor formation and absence of the cancer cachexia-like syndrome (161, 162). In contrast, when the *Inhα*$^{-/-}$ mice were crossed with *estrogen receptor α* deficient mice, tumor development was more rapid and the cancer cachexia-like syndrome started earlier compared to *Inhα*$^{-/-}$ mice (163).

Interestingly, human GCTs express and secrete inhibin B, and it is widely used as a diagnostic and surveillance marker in clinical practice (132, 164). Thus, further studies are needed to unravel the role of inhibins in human GCT pathogenesis.

SMAD knockout mice

Several mouse models have been generated to investigate the role of TGF-β family signaling, especially the intracellular signaling mediators (SMADs) in GCT pathogenesis. As stated in chapter 3.2, the mice deficient for both *Smad2* and *-3* are infertile but do not get tumors (93), whereas the granulosa cell specific douple KO mice of *Smad1* and *-5*, or triple knockouts of *Smad1*, *-5*, and *-8* are infertile as well, but also develop poorly differentiated, metastatic, uni- or bilateral GCTs by the age of 3 months with 100 % penetrance (94). More detailed analyses of the *Smad1/5* double-knockout mice revealed several histological and physiological similarities to human juveline GCT (154). These studies indicated that BMP and AMH signaling, and their mediators SMAD1, -5, and -8 act as tumor suppressors in mice. Interestingly, SMAD2 and -3 as well as some of their downstream target genes were shown to be upregulated in *Smad1/5* and *Smad1/5/8* deficient mice, which may mean that in normal granulosa cells SMAD1/5/8 inhibit SMAD2/3 signaling (94). *Inhα*$^{-/-}$ mice also show the upregulation of SMAD3 (153). This hyperactivity of the TGF-β signaling pathway is shown to stimulate tumor invasion and metastasis formation in many cancers. Thus based on the abovementioned findings it is plausible that this is the case also in GCT pathogensis.

Mouse with constitutively activated Wnt/β-catenin pathway

Misregulation of Wnt/β-catenin pathway is a common hallmark of several types of cancer. To investigate the role of this pathway in GCT pathogenesis Boerboom *et al*. generated a mouse model with granulosa cell specific constantly active β-catenin (*Catnb*$^{flox(ex3)/+}$; *Amhr2*$^{cre/+}$ mice) (165). These mice were subfertile and developed ovarian lesions resembling disorganized follicles, which later evolved into GCT. At 7.5 months of age 57 % of these mice had tumors that shared several histopathological features with human GCT (165) suggesting that the overexpression of the Wnt/β-catenin pathway may play a role also in human GCT pathogenesis. In the same study, Boerboom *et al*. also showed the nuclear localization of β-catenin in 15/24 human and equine GCTs. However, this finding was not supported by another study with 32 human GCT samples, where none of the examined tumors showed nuclear localisation of β-catenin (166).

Table 1 *GCT mouse models. dKO, double knockout; tKO, triple knockout.*

Genotype	Phenotype	Tumor penetrance (%)	Reference
Inhα/SV40TAg	Infertile, GCT formation at age of 5-7 mo, serum inhibinB↑, serum gonadotropins↓	100	(155)
Inhα$^{-/-}$	Bilateral, mixed, or incompletely differentiated sex cord-stromal tumors coupled with cachexia-like syndrome at age of 4 wk	100	(159)
Smad1/5 dKO	Infertile, poorly differentiated, metastatic, uni- or bilateral GCT at age of 3 mo, histopathological features similar to human juvenile GCT	100	(94) (154)
Smad1/5/8 tKO	Infertile, poorly differentiated, metastatic, uni- or bilateral GCT at age of 3 mo	100	(94)
Catnb$^{flox(ex3)/+}$; *Amhr2*$^{cre/+}$	Subfertile, follicle-like lesions that evolve into GCT at 7.5 mo of age	57	(165)

5.4 Tumor angiogenesis

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is an essential process during development and also postnatally, e.g. in wound healing and formation of placenta during pregnancy. In addition to these physiological phenomena, angiogenesis

plays a pivotal role in several pathological conditions, including tumorigenesis, since the proliferation and metastatic spread of cancer cells are dependent on an adequate supply of oxygen and nutrients and the removal of waste products.

Vascular endothelial growth factor-A

Vascular endothelial growth factor-A (VEGF, also referred as VEGFA), a member of platelet-derived growth factor superfamily, is one of the key regulators of both physiological and pathological angiogenesis. It is a secreted, soluble growth factor that regulates endothelial cell proliferation, migration, vascular permeability, secretion, and other endothelial cell functions. Both *Vegf* homozygous knockout mice and heterozygous ($Vegf^{+/-}$) mice die early during development due to immature blood vessel formation (167, 168). Several VEGF subtypes are generated through alternative splicing (169). These subtypes differ from each other in their biological activity and binding affinity to receptors (170).

In cancerous tissue, VEGF production and secretion are stimulated by hypoxia and several growth factors, such as EGF, TGF-β, IGF1, FGF, and platelet-derived growth factor (PDGF), as well as oncogenic mutations of the Ras pro-oncogene (170).

VEGF exerts its effects through binding to its two tyrosine kinase receptors, VEGFR-1 and VEGFR-2 (also termed as Flt-1 and KDR/Flk-1, respectively) (171, 172). These receptors are mainly expressed in endothelial cells, but inflammatory cells, osteoblasts, and hematopoietic stem cells express them as well (170). During early embryogenesis VEGFR-1 and VEGFR-2 have opposite roles in angiogenesis: VEGFR-2 is a positive signal transducer, whereas VEGFR-1 supresses VEGFR-2 signaling (173, 174). In adult organs and cancer, VEGFR-2 has shown to be the major mediator of the mitogenic and angiogenic effects of VEGF. VEGFR-2 consists of an extracellular ligand-binding domain organized into seven immunoglobulin-like folds, a single transmembrane domain, and an intracellular tyrosine kinase domain (170). The binding of VEGF causes dimerization of VEGFR-2 and autophosphorylation of several tyrosine residues of the intracellular tyrosinekinase domain (175). Upon activation, VEGFR-2 activates multiple intracellular signaling cascades resulting in mitogenic, chemotactic, and prosurvival signals (Figure 6) (170).

VEGF signaling in tumor cells

VEGF is expressed in and secreted by a majority of solid tumors, and serum VEGF levels are often elevated in cancer patients (176-178). Moreover, elevated serum VEGF levels are associated with poor prognosis (179). In addition to endothelial cells, VEGFR-2 has been shown to be expressed in several tumor types (180). In normal endothelial cells, VEGF secretion leads to downregulation of VEGFR-2, whereas in tumor cells this

regulation is lost (180). Previously, an autocrine VEGF/VEGFR-2 signaling loop has been shown to exist in breast cancer and ovarian carcinoma cells (181, 182), and it has been proposed that this autoloop promotes cancer cell growth and survival by phosphorylation and activation of VEGFR-2. VEGF and its receptors are also abundantly expressed in both primary and recurrent human GCTs (183). Furthermore, VEGF expression has been shown to correlate with that of VEGFR-2 at both the mRNA and protein level suggesting an autoregulatory VEGF/VEGFR-2 loop in GCTs (183).

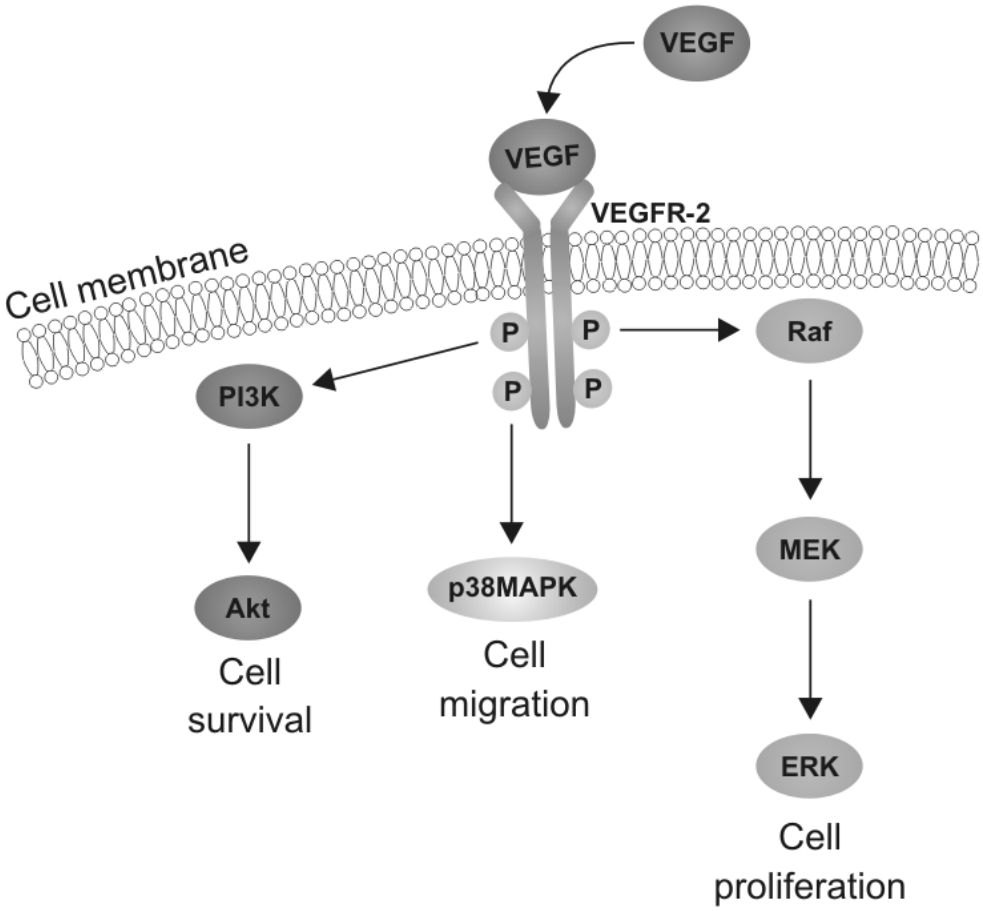

Figure 6 *Overview of VEGFR-2 intracellular signaling. VEGF binding to the extracellular domain induces dimerization and autophosphorylation of intracellular tyrosine residues. Several intracellular messangers bind to the tyrosine residues leading to the phosphorylation and activation of these proteins. Activation of PI3K/Akt signaling leads to increased cell survival, p38MAPK signaling leads to enhanced cell migration, and Raf/MEK/ERK signaling activates cell proliferation. Modified from (184).*

Owing its importance for tumor angiogenesis, growth, and metastasis formation, VEGF/VEGFR-2 system has become an attractive target for cancer treatment. It has been shown that an anti-human VEGF antibody efficiently suppresses the growth of human tumor xenograft transplanted in immunedficient mouse (185). Currently several drugs

targeting either VEGF ligand or its receptors have been generated, and some of them have been approved for clinical use. One of these is Bevacizumab (BVZ), a recombinant humanized monoclonal antibody that efficiently inhibits VEGF/VEGFR-2 system by binding to the soluble VEGF. BVZ is used in the treatment of breat, lung, renal, colorectal, and epithelial ovarian cancers (186, 187). There are also small retrospective clinical studies and a case report showing that BVZ is effective in treatment of recurrent GCTs (188-190), but the *in vitro* evidence of its actions in GCT cells is lacking.

Aims of the study

This dissertation project focuses on the molecular mechanisms that regulate normal and neoplastic steroidogenic cell development in the adrenal gland and ovary.

The specific aims of the research are:

1) Study the *in vivo* role of GATA6 in adrenocortical development and function.

2) Investigate the impact of FOXL2, GATA4, and SMAD3 on GCT cell survival and apoptosis.

3) Evaluate the potential of TRAIL and VEGF pathways as novel targets for GCT treatment.

Materials and methods

1. Mice

1.1 Experimental mice

Procedures involving animal experiments were approved by the institutional committee for laboratory animal care and were conducted in accordance with National Research Council's (NRC) publication *Guide for Care and Use of Laboratory Animals*. All mice had free access to water and standard rodent chow, and were exposed to 12 h light/12 h dark photoperiods.

1.2 Generation of *Gata6* conditional knockout mice

Adrenocortical cell specific *Gata6* knockout (*Gata6* cKO) mice were generated using the Cre-loxP recombination system. Mice bearing a floxed *Gata6* allele ($Gata6^{F/F}$; $Gata6^{tm2.1Sad}$/J)(The Jackson Laboratory) were mated with mice harboring the Cre recombinase under the control of *Sf1* gene (*Sf1*-Cre; (FVB-Tg(Nr5a1-cre)2Lowl/J)(The Jackson Laboratory). The resultant $Gata6^{F/+}$;*Sf1*-cre mice were mated with $Gata6^{F/F}$ mice to produce $Gata6^{F/F}$;*Sf1*-cre mice. The mice were genotyped as described earlier (191, 192).

1.3 Mouse gonadectomy

Female (3-4 wk of age) or male (2 mo of age) mice were anesthetized with ip administration of ketamine (75 mg/kg) and subjected to gonadectomy or sham surgery. Females were killed 3 mo and males 30 d after gonadectomy by CO_2 inhalation.

1.4 Assessment of adrenal and reproductive function

For plasma corticosterone and ACTH measurements 8-week-old male mice were decapitated at 8:00 to 9:00 $_{AM}$ with minimum stress and handling, and the trunk blood was collected in EDTA. For restrained stress experiment the mice were kept in 50 ml falcon tubes for 30 min after which blood was collected in EDTA. For the ACTH stimulation experiment the hypothalamic-pituitary-adrenal axis was suppressed by subcutaneous injection of 5 mg/kg dexamethasone at 6:00 $_{PM}$ 1 day before and at 8:00 $_{AM}$ on the day of testing. Mice were anesthetized with 1.5% isoflurane in 50% nitrogen/50% oxygen, and an external jugular venous catheter was placed. At 10:00 $_{AM}$, 1 mg/kg $ACTH_{1-24}$ was injected

ip, and 40 µl blood samples were drawn 15, 30, and 60 min after the injection for corticosterone measurements. The blood Na^+ measurements were made on heparinized blood drawn from the tail vein using a Radiometer ABL90 Flex blood gas analyzer. Fertility was evaluated by housing male and female mice with wild type C57BL/6 mice and measuring the litter sizes. Sperm motility was assessed using Computer Assisted Sperm Analysis (Hamilton Thorne Biosciences IVOS, Beverly, MA, USA) with parameters optimized for detection of mouse sperm as described in (193).

1.5 Electron microscopy

Mice were anesthetized and perfused with Karnovsky's fixative. Adrenal glands were postfixed with 2% OsO_4 and embedded in epon. The sections were stained with uranyl acetate and lead citrate and examined by transmission electron microscopy.

2. Cell culture

2.1 Cell lines and primary hGCT cells

The cells were grown at 37 °C in humidified atmosphere containing 5% CO_2. Human GCT cell line KGN (a gift from Dr Toshihiko Yanase, Kyushu University, Fukuoka, Japan) was cultured in DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml). KGN cells harbor the 402C→G mutation in FOXL2. Another human GCT cell line, COV434, that has a wild type FOXL2 genotype, was cultured in DMEM supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 U/ml). COS-7 cells (originating from African green monkey kidney fibroblast-like cells) were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 U/ml).

Following the intraoperative diagnosis of GCT, fresh tumor sample was obtained straight from the operation room for cell culture. GCT tissue was mechanically minced and treated with 0.5% kollagenase (Sigma-Aldrich® Corporation, St Louis, MO, USA) in DMEM/F12 containing 0% FBS for 2 h, filtered through 140 µm filter mesh to obtain single cells, washed twice with 0% FBS culture medium, counted, and plated for experiments in DMEM/F12 containing 10% FBS, penicillin (100 U/ml) and streptomycin (100 U/ml). All the primary GCT cell cultures harbored the 402C→G mutation in FOXL2.

2.2 Transfections

For adenoviral transfections the following replication deficient adenoviral constructs used: wild type rat GATA4 (G4wt) (194) and dominant negative GATA4 (G4dn), construct that produces a fusion protein of GATA4 and engrailed domain tagged with flag epitope (195). 5×10^5 KGN cells were seeded in 6-well plates 24 h before transfection. The cells were infected by incubating them with viruses in 1 ml DMEM containing 0% FBS. After 1 h, 2 ml of DMEM/F12 containing 10% FBS was added to stop the infection.

For lentiviral transfections the lentiviruses expressing small hairpin RNA (shRNA) were generated using constructs and protocols provided by the Biomedicum Virus Core Facility (196). pLKO.1 shRNA targeting GATA4 (shGATA4)-containing constructs were obtained from Sigma (Sigma-Aldrich® Corporation) and were as follows: shGATA4-1 (TRCN0000020426) 5'-GAGGAGATGCGTCCCATCAA-3' and shGATA4-2 (TRCN0000020427) 5'-CTGAATAAATCTAAGACACCA-3'. 2×10^5 KGN cells were seeded in 6-well plates 24 h before transfection. The cells were transduced with 750 µl of lentiviral particles in 1.5 ml DMEM/F12 containing 10% FBS for 10 min at 37 °C, centrifuged for 30 min at 500 *g*, and further incubated for 2 h at 37 °C. Puromycin (1 µg/ml) was used to select the tranduced cells.

The overexpression and control plasmids used for the plasmid transfections were: pMT2-GATA4-V5 (V5-tag amino acid sequence GKPIPNPLLGLDST), untagged, V5-tagged, or GFP-tagged pcDNA3.1-FOXL2-WT, pcDNA3.1-FOXL2-C134W, and pCDNA3.1-SMAD3. Twenty thousand KGN cells/well were transfected with the overexpression plasmids by electroporation using Neon transfection system (Invitrogen Corporation, Carlsbad, CA, USA) according to manufacturer's instructions. Following settings were used: 1400V, 20 ms, and 1 pulse.

2.3 Treatments/stimulations

To induce apoptosis, the KGN/primary hGCT cells were incubated with recombinant human TRAIL (Millipore, Bedford, MA, USA) at concentrations of 10, 50, and 100 ng/ml or with BVZ (Genetech/Roche, San Francisco, CA, USA) at concentrations of 1 and 10 µg/ml.

2.4 Promoter activity assays

KGN cells were plated 16 h before transfection at a density of $4x10^4$ cells/cm^2. The plasmid used for promoter transactivation assay was pGL3-680bp *CCND2*-luciferase (151). Transfection was done by using the calcium-phosphate method (Invitrogen Corporation, Carlsbad, CA, USA) and the luciferase activity was measured using dual-Luciferase® Reporter Assay System (Promega, Madison, WI, USA) as described in (197).

2.5 Apoptosis and cell viability assays

For 4',6-diamino-2-phenylindole hydrochloride (DAPI) staining 20,000 KGN/primary hGCT cells were seeded on 8-well chamber slides. Prior to staining the cells were fixed with formalin for 10 min at room temperature followed by cold methanol for 10 min at -20 °C. The cells were incubated for 30 min at room temperature with 0.4 μg/ml of DAPI in PBS, dehydrated, and mounted with UltraKitt (J T Baker, Deventer, Holland). The percentage of apoptotic cells from total cell count was analyzed with ImageJ version 1.42q software (National Institutes of Health, Bethesda, MD, USA).

Caspase 3/7 activation was measured using Caspase-Glo® 3/7 Assay (Promega Corporation, Madison, WI, USA) according to manufacturer's instructions. Luminescence was measured using a luminometer (Labsystems Luminoscan RS, Helsinki, Finland).

Cell viability was assessed by either tetrazolium dye (MTT)-base cell growth determination kit (Sigma-Aldrich Corporation) or Cell Proliferation Reagent WST-1 kit (Roche Applied Science, Indianapolis, IN, USA) according to the instructions of manufacturer.

3. Tissue and serum samples

3.1 Murine tissues

Mice were killed with CO_2 inhalation. The adrenal glands, ovaries or testes were harvested and fixed overnight in 4% paraformaldehyde, Bouin's solution, or Müller's fixative (198) and embedded in paraffin. For cryosections the adrenals were embedded to OCT and frozen in –80°C.

3.2 Human normal ovary, hGCT tissue microarray, and serum samples

Normal adult ovary samples obtained from three premenopausal women undergoing ovariectomy due to cervical cancer were used as reference tissue to compare antigen expression between normal and malignant granulosa cells. A tumor tissue microarray consisting of 80 primary and 13 recurrent GCTs from 90 patients was previously assembled (147). Paraffin-embedded sections of the tumor tissue microarray consisted of quadruple core samples of 93 GCT on a single slide. Seventy-four serum samples were collected from 54 GCT patients between August 2007 and November 2011 and stored at -80°C until analysis.

4. mRNA expression

4.1 Laser microdissection

Adrenal cryosections (10 µm) were cut on PEN-Membrane slides (Leica Microsystems, Wetzlar, Germany). Sections were fixed with ice-cold ethanol for 1 min, stained with Gram's crystal violet, and dehydrated. Laser microdissection (LMD) was performed using Leica LMD6000 microscope. Dissectates were collected in RNA extraction buffer (RNeasy Mini Kit, Qiagen) for qRT-PCR analysis.

4.2 Real time RT-PCR

The RNA was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA, USA)) according to instructions. First-strand cDNA synthesis was performed from 0.5 µg of total RNA using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen Corporation, Carlsbad, CA, USA). The primers listed in Table 2 were designed using NCBI/Primer-BLAST tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) or retrieved from the literature. Analysis was performed with SYBR® GreenER™ qPCR SuperMix reagents (Invitrogen Corporation) and light cycler (Stratagene Mx3005, Agilent, CA, USA) according to manufacturer's instructions. The relative expression of target genes was calculated using the relative standard curve method. Target gene expression was normalized to the expression of β-Actin and GAPDH.

Table 2 *qRT-PCR primers*

Gene	Reference	Oligonucleotide sequence (5' → 3')
Gata6	NM_010258.3	F: CGGCCGCTGAACGGAACGTA R: TCCAACAGGTCTGTGCTGGGG
Lhcgr	NM_013582.2	F: CGCCCGACTATCTCTCACCTA R: GACAGATTGAGGAGGTTGTCAAA
Cyp17	NM_007809.3	F: CCAGATGGTGACTCTAGGCCTCTTGTC R: GGTCTGTATGGTAGTCAGTATCG
Amhr2	NM_144547.2	F: GGGGCTTTGGACACTGCTT R: GTCTCGGCATCCTTGCATCTC
Gata4	NM_008092.3	F: CCCTACCCAGCCTACATGG R: ACATATCGAGATTGGGGTGTCT
Tcf21	NM_011545.1	F: CAGTCAACCTGACGTGGCCCTTT R: GGGAAGGGCAGGGGTCGTCA
Tyrosine hydroxylase	NM_009377.1	F: ACCTGGACCATCCGGGCTTCT R: GGCCCTTCAGCGTGGCGTAT
Akr1c18	NM_134066.2	F: TCCCATCGTCCAGAGTTGGTCA R: TCCATGGCCTCCCATGTGTCA
Cyp11b2	NM_009991.3	F: GCACCAGGTGGAGAGTATGC R: CCATTCTGGCCCATTTAGC
Pik3c2g	NM_011084.2	F: GTGGACCCAGGTGAGAACT R: GGAACACACTTTGTTTTCTTTCTC
β-actin	NM_007393.3	F: GCGTGACATCAAAGAGAAGC R: AGGATTCCATACCCAAGAAGG
Gapdh	NM_008084.2	F: GCTCACTGGCATGGCCTTCCGTG R: TGGAAGAGTGGGAGTTGCTGTTGA

4.3 Microarray analysis

RNA was extracted from whole adrenal glands with RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and amplified using the TotalPrep RNA amplification kit (Illumina, San Diego, CA, USA), and hybridized on an Illumina Mouse6v2 oligonucleotide array according to manufacturer's instructions. Hybridization was performed by the GTAC Microarray Core facility (Washington University in St Louis, MO, USA). Results can be found at the GEO database (GSE40398).

4.4 *In situ* hybridization

In situ hybridization was performed on paraffin-embedded, paraformaldehyde-fixed sections. Digoxygenin-labeled riboprobes were prepared as described in (199). First,

sections were treated with Proteinase K and pre-hybridized in 50 % formamide, 5x SSC, 2% Boehringer blocking powder, 0.1% Triton X100, 0.5% CHAPS, 1 mg/ml yeast RNA, 5 mM EDTA, 50 µg/ml heparin for 1 h at 60°C. Digoxygenin labeled probe was added to the hybridization mix and incubated overnight at 60°C. Sections were washed with 50% formamide/0.2× SSC, 2× SSC at 60°C and blocked with Boehringer blocking reagent and incubated overnight at 4°C with peroxidase-conjugated anti-digoxygenin antibody, in the presence of 0.5 mg/ml levamisole. Peroxidase activity was detected by incubation with with 0.18 mg/ml BCIP and 0.34 mg/ml NBT.

5. Protein expression

5.1 Western blotting

For western blotting, the proteins were extracted from the cell lysates using Nucleospin RNA/Protein kit (740 933.250; Macherey-Nagel, Düren, Germany), and the proteins were separated with 7.5% SDS-PAGE and transferred onto Immobilon-P membrane (Millipore). Nonfat 5% milk in 0.1% Tween-TBS buffer was used for blocking the nonspecific binding. The primary and secondary antibodies and dilutions used are listed in Table 3. After o/n incubation at 4 °C with primary antibody the secondary antibody was incubated for 1 h at room temperature. The proteins were visualized by the Enhanced Chemiluminescence Plus Kit (Amersham Biosciences Inc., Piscataway, NJ, USA). β-Actin was used as a loading control.

5.2 Immunohistochemistry and scoring of the results

Paraffin-embedded tissues were deparaffinized, rehydrated, treated by boiling for 20 min in 10 mM citric acid for antigen retrieval, and the endogenous peroxidase was quenched by 3% H_2O_2 treatment for 5 min at room temperature. Immunoperoxidase staining was performed using an avidin-biotin immunoperoxidase system (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) and DAB (Sigma) to visualize the bound antibody. Primary antibodies used are listed in Table 3. Hematoxylin was used for counterstaining the sections. Stainings were evaluated by light microscopy and the images were acquired with an LS Leica DMRXA microscope, connected to an Olympus DP70 camera and DCP controller image acquisition program.

The immunohistochemistry results of the GCT tissue microarray were scored blinded to the clinical data and separately by two researchers. Tumors were classified based on the staining intensity (cytoplasmic antigens) or the percentage of positive cells (nuclear antigens). Disagreements were resolved by a joint review.

5.3 ELISA and RIA assays

The serum, plasma, and cell culture supernatant samples were analyzed using commercial enzyme-linked immunnosorbent assay (ELISAs) or radioimmunoassay (RIA) kits according to manufacturer's instructions. The following kits were used: corticosterone and cortisol RIA kits (Siemens Healthcare Diagnostics, Erlangen, Germany), ACTH RIA kit (MP Biomedicals, Santa Ana, CA, USA), aldosterone ELISA kit (Endocrine Technologies Inc., Newark, CA, USA), VEGF and Endostatin ELISA kits (R&D Systems, Minneapolis, MN, USA).

Table 3 *Antibodies utilized. TH, tyrosine hydroxylase; IHC, immunohistochemisty; WB, western blotting.*

Antigen	Manufacturer	Catalog#	Used in	Method	Dilution
GATA4	Santa Cruz Biotechnology	Sc-1237	Mouse, human	IHC WB	1:200 1:1000
GATA6	R&D Systems	AF1700	Mouse	IHC	1:100
Tyosine hydroxylase	Chemicon	AB152	Mouse	IHC	1:500
FOXL2	Imgenex	IMG-3228	Human	IHC WB	1:400 1:1000
SMAD3	Invitrogen Corporation	#51-1500	Human	IHC WB	1:400 1:1000
CCND2	Santa Cruz Biotechnology	Sc-593	Human	IHC	1:1000
DR4	Santa Cruz Biotechnology Abcam	Sc-7863 Ab8414	Human	IHC IHC	1:100 1:400
DR5	Santa Cruz Biotechnology	Sc-7192	Human	IHC	1:50
Cleaved caspase 3	Cell Signalling Technology	#9661L	Human	WB	1:1000
p-VEGFR-2	Abcam	Y1214	Human	IHC WB	1:70 1:500
β-actin	Santa Cruz Biotechnology	Sc-1616	Human	WB	1:10000

5.4 Protein co-immunoprecipitation

COV434 or COS-7 cells were lysed in lysis buffer (50 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, pH 7.6) supplemented with protease inhibitors PMSF 1mM and Complete mini EDTA-free cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and phosphatase inhibitors (Sigma Aldrich, St Louis, MO, USA). Immunoprecipitation was

performed using Anti-V5 Agarose Affinity Gel (Sigma) according to manufacturer's instructions. Precipitated proteins were eluted in 2x-SDS sample buffer containing 100 mM Tris, 4% SDS, 20% glycerol, 0.1% bromophenol blue, and 100 mM DTT, and separated by SDS-PAGE. Primary antibodies used for detection are listed in Table 3.

6. Statistical analysis

The statistical analyses were carried out with JMP® 9.0.0 software (SAS Institute Inc., Cary, NC, USA). The statistical significance of qPCR results and hormone measurements in Study I were analyzed using Student's t-test. One-way ANOVA followed by Dunnett's or each pair Student's t-test was used for apoptosis, cell viability and proliferation experiments. The tissue microarray scoring results and categorical variables were analyzed with contingency tabling (2x2) followed by the χ^2 or Fisher's exact test and with Cox proportional hazard model. The serum data in Study IV were tested for normal distribution with Shapiro-Wilks test followed one-way ANOVA and Student's t-test in case of normal distribution, or Wilcoxon/Kruskall-Wallis and Wilcoxon matched-pairs sign-rank tests when differing from the normal distribution. A p-value <0.05 was considered significant.

Results and discussion

1. GATA6 in adrenocortical development and function (I)

Transcription factor GATA6 has been connected to normal adrenal development and function (43). It is expressed in both fetal and adult adrenal cortex, and *in vitro* studies have identified some putative target genes, including genes involved in steroid biosynthesis (37-39, 200). However, genetic proof of its importance in adrenocortical cell function has still been lacking.

1.1 Conditional deletion of *Gata6* in *Sf1*-positive cells

GATA6 KO mice die early in gestation due to defects in endoderm differentiation (201) preventing the use of this mouse model to study the role of GATA6 in adrenocortical function. To circumvent this problem we generated a cell type specific conditional *Gata6* knock out mouse model (hereafter referred to as *Gata6* cKO) using Cre-LoxP recombination system. We used a *Sf1*-cre mouse line which was mated with mice bearing a floxed allele of *Gata6* (*Gata6*$^{F/F}$) (191), resulting mice in which GATA6 is deleted specifically in *Sf1*-positive cells. Based on the *Rosa26* reporter analysis (http://cre.jax.org/Nr5a1/Nr5a1-creNano.html), *Sf1*-cre is expressed both in the fetal and adult adrenal cortex, gonadal somatic cells, hypothalamus, and pituitary gland. As a control we used either *Gata6*$^{F/F}$ or *Gata6*$^{F/+}$;*Sf1*-cre mice (neither of these strains had an abnormal phenotype). The generation of *Gata6* cKO mouse is illustrated in Figure 7A.

The deletion of *Gata6* was confirmed in the adrenal glands of cKO mice by immunohistochemistry and RNA analyses. In line with previous studies (43) the immunostaining of the control mouse adrenal gland showed nuclear GATA6 staining in capsular, subcapsular, and scattered vascular cells (Figure 7B). In cKO mouse, decreased GATA6 immunoreactivity was observed in subcapsular cells, where *Sf1*-cre is active (24), but persistent GATA6 immunostaining in capsular and vascular cells, where *Sf1*-cre is inactive (Figure 7C). *In situ* hybridization also showed reduced *Gata6* mRNA expression in the cKO adrenal glands compared to control (Figure 7D and E). Finally, qRT-PCR analysis of adrenal glands from female mice at varying ages confirmed that *Gata6* mRNA levels were significantly lower in cKO mice compared to age-matched controls (Figure 7F).

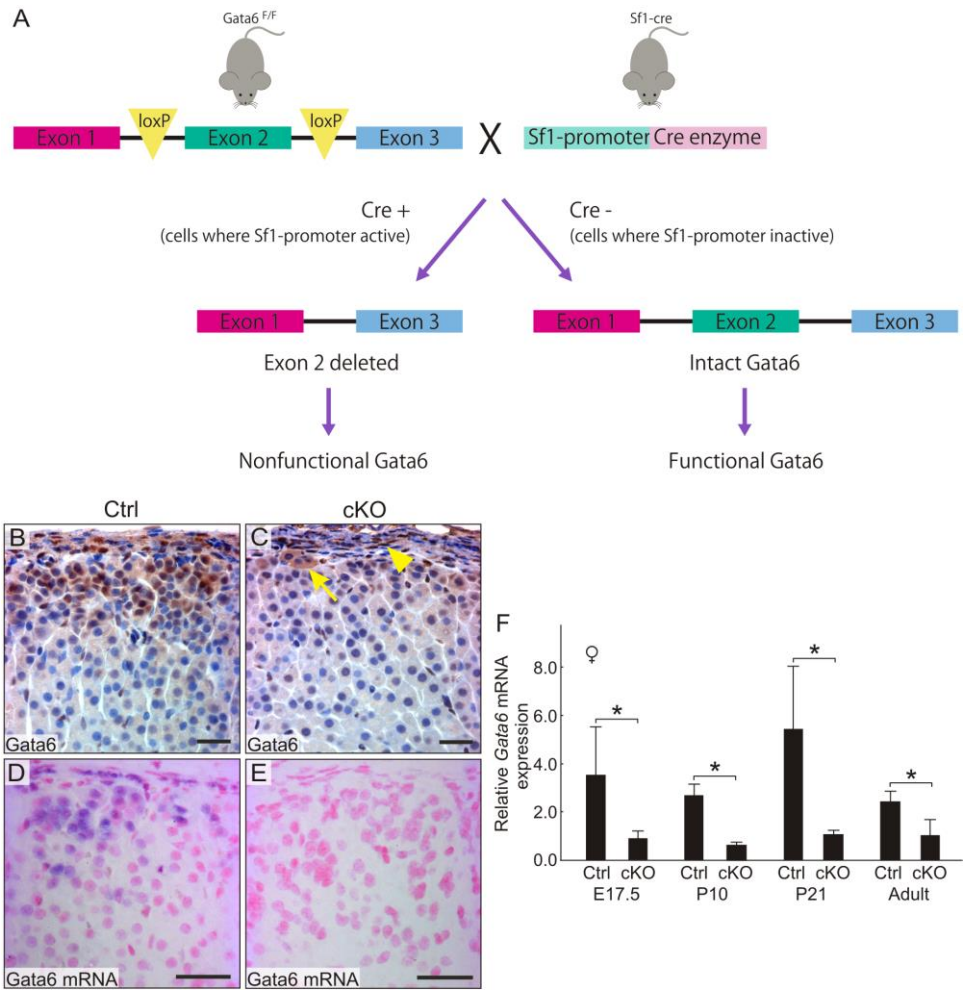

Figure 7 *Generation of Gata6 cKO mouse. A) a schematic illustration of the Cre-loxP recombination system. In cells where Sf-1 is active Cre enzyme is produced and it catalyses the recombination of loxP sites leading to a non-functional GATA6 protein. B and C) GATA6 immunostaining of 3 mo old control and cKO mice. The arrow highlights ectopic medulla cells and arrowhead points out persistent GATA6 staining in Sf-1 negative capsular cells. D and E) Gata6 in situ hybridization of 2 mo old virgin female adrenal glands. F) qRT-PCR analyses of Gata6 mRNA from whole adrenal glands. Scale bar = 30 µm. *P < 0.05.*

1.2 *Gata6* cKO mice are viable and fertile

Gata6 cKO mice were born in expected Mendelian ratio (42 cKO of 173 total ≈ 1:4) and expected sex ratio (21 male and 21 female =1:1). These mice appeared healthy and their growth curves were identical with those of control mice.

As it is known that *Gata6* is expressed in gonadal somatic cells (96, 202), and we found the *Gata6* mRNA levels to be lower in the ovaries and testes of cKO mice compared to age-matched controls (Figure 8A and B) we next assessed the reproductive

phenotype of this mouse. To our surprise, none of the examined features (plasma estrone sulfate/testosterone levels, ovarian/testicular mass, sperm count/motility, and seminal vesicle mass) was altered in the cKO mice. Moreover, the gonadal histology appeared normal. When mated with the wild-type mice, both female and male cKO mice produced viable pups at the same rate as control mice (Figure 8C and D). These findings are consistent with the recent study where GATA6 was deleted specifically in granulosa cells using *Cyp19*-cre line (102). These mice also lack the reproductive phenotype possibly due to a functional compensation by another member of GATA family, GATA4. This kind of redundancy as to GATA factors has also been reported in the cardiomyocytes (203) and in small intestine (204).

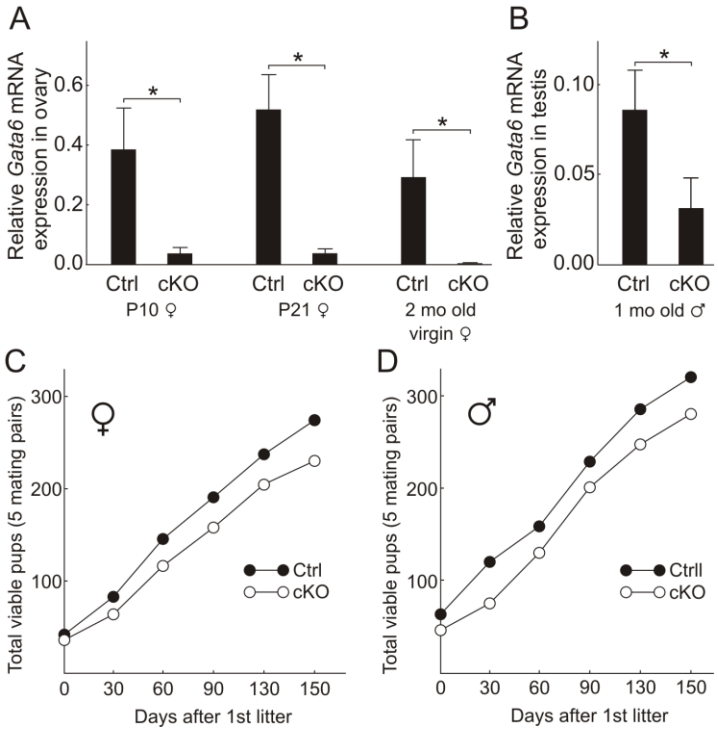

Figure 8 *Reproductive phenotype of Gata6 cKO mice. A and B) qRT-PCR analyses of Gata6 mRNA from whole ovary (A) and testis (B). *P < 0.05. C and D) Number of viable pups produced by female (C) or male (D) Gata6 cKO mice.*

1.3 *Gata6* cKO mice have small adrenal glands

Next, we evaluated the effects of *Gata6* deletion on the adrenal gland development and function. We found that the adrenal glands of *Gata6* cKO mice were significantly smaller than those of control mice at 3 months of age (Figure 9A). The reduced adrenal gland mass was already evident at 1 month of age in both male and female cKO mice, but not at the embryonic day 17.5 (Figure 9B and C).

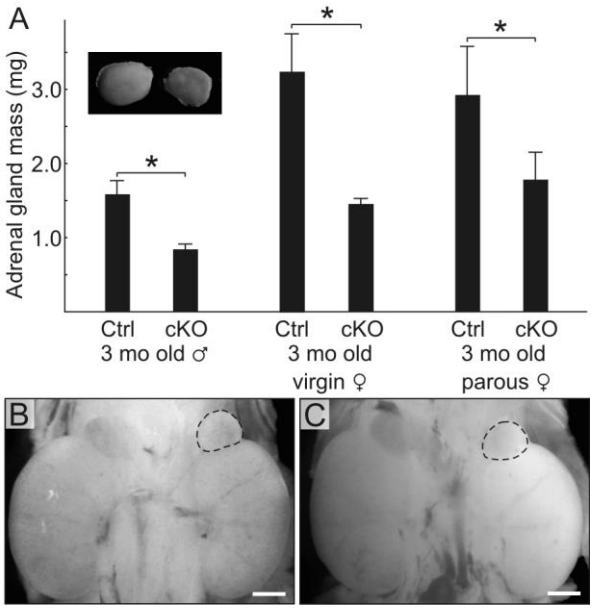

Figure 9 *Gata6 cKO mice have small adrenal glands. A) Average adrenal masses of 3 mo old male, virgin female, and parous female mice. *P < 0.05. B and C) Gross morphology of adrenal glands (sircled) from E17.5 mice. Scale bar = 0.5 mm.*

To investigate whether the reduced adrenal gland size in *Gata6* cKO mice was due to decreased proliferation of the cortical cells we stained the sections of cKO and control adrenal glands with two proliferation marker antigens, PCNA and BrdU. Unlike *Pbx1*$^{+/-}$ mice, in which reduced adrenal gland mass is associated with reduced cortical cell proliferation (205), we could not find differences in cell proliferation between *Gata6* cKO mice and controls.

1.4 *Gata6* cKO mouse adrenal glands show cytomegalic changes and ectopic chromaffin cells

Electron microscopy demonstrated normal ultrastructure of zF cells in adult *Gata6* cKO mice with typical characteristics of steroidogenic cells (17) (Figure 10B), but organization of the fascicular cells was abnormal. Normally, zF cells form columns separated by prominent capillaries (17), but in *Gata6* cKO mice zF was disordered (Figure 10C and D). Furthermore, the mutant adrenal glands showed cytomegalic changes (Figure 10C-F). Cytomegaly is a hallmark of adrenocortical dysfunction and hypoplasia, and it is connected to multiple genetic disorders (206-208). Cytomegalic cells are enlarged and have large nuclei. It is thought that cytomegaly is a compensatory mechanism to a reduced number of cortical cells, and that it ensures the sufficient hormone production of hypoplastic adrenals (206). Interestingly, cytomegalic changes were evident already in E17.5 *Gata6* cKO adrenals (Figure 10E and F) indicating that the deletion of *Gata6* in SF1-positive cells has an effect on both fetal and adult adrenocortical development.

Another abnormality in the *Gata6* cKO mouse was ectopically located medulla cells. Tyrosine hydroxylase immunostaining and Müller's chromaffin stain showed both chromaffin cell islands and finger-like projections in periphery of 3-month-old cKO adrenal gland (Figure 10G-J). Ectopic medulla cells were evident already in adrenals of E17.5 *Gata6* cKO mice (Figure 10K and L), which is further evidence of the important of GATA6 in adrenal development.

Ectopic medulla cells have also been reported in other mouse models, such as in mice with impaired SHH signaling (209), and SF-1 sumoylation (210), as well as in mice with constantly active or inactivated β-catenin signaling in adrenocortical cells (198). The latter study showed that proper β-catenin signaling is essential for normal growth and organization of the medulla (198). Since GATA6 is important for normal β-catenin signaling in other tissues (211), it is plausible that β-catenin signaling is abnormal in the *Gata6* cKO adrenals. Despite the fact that medulla cells were ectopically located in the cKO mice, the tyrosine hydroxylase mRNA levels were not changed compared to control indicating that the ablation of *Gata6* does not affect the chromaffin cell number.

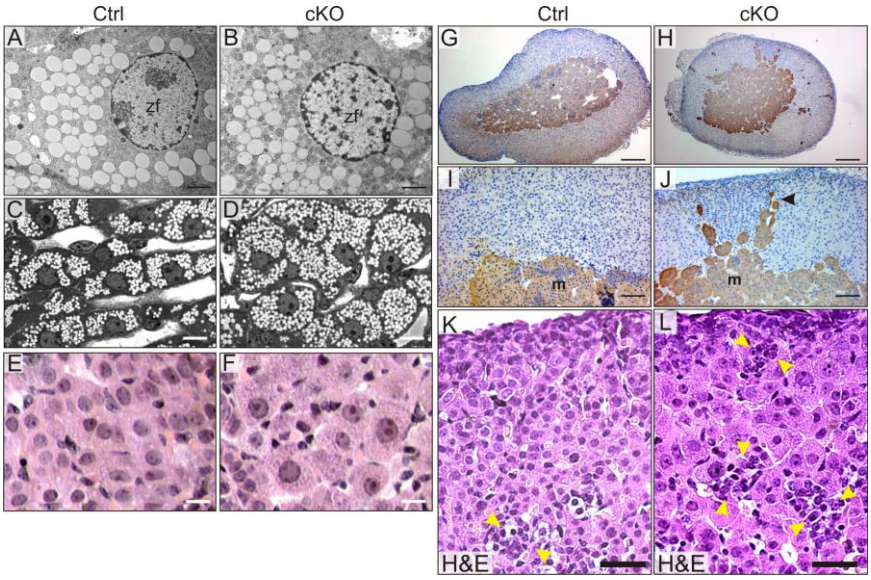

Figure 10 *Cytomegalic changes and ectopically located medulla cells in Gata6 cKO mice. A and B) Electron microscopy images from zF cells of 2 mo old mice. Scale bar = 1 µm. C and D) Semi-thin (1 µm) sections from adrenal glands of 2 mo old mice. Scale bar = 2 µm. E and F) Adrenal glands from E17.5 embryos. Scale bar = 10 µm. G-J) Tyrosine hydroxylase immunostaining of 3 mo old male adrenal glands. Scale bar = 200µm (G and H), 50 µm (I and J). K and L) Adrenal glands from E17.5 embryos. Arrowheads highlight differentiating chromaffin cells. Scale bar = 10 µm.*

1.5 Hormonal consequences of *Gata6* deletion

Next, we examined whether *Gata6* deletion in adrenocortical cells affects hormone production. We first measured the basal and stress-induced corticosterone levels from plasma. There was a trend towards blunted secretion, but the results did not reach statistical significance. Basal ACTH levels were the same in cKO and control mice. The aldosterone levels measured both from serum and whole adrenal homogenates were significantly lower in cKOs versus controls (Figure 11A and B), but the blood sodium levels were indistinguishable between these mice. Accordingly, also the mRNA expression of aldosterone synthase (*Cyp11b2*) was significantly reduced in *Gata6* cKO mice compared to controls. In contrast to our mouse model, mice with constitutively active β-catenin signaling, as well as mice with mutated β-catenin signaling inhibitor, *Adenomatous polyposis coli* (*APC*) gene exhibit hyperaldosteroism, resulting from aberrant zG differentiation (212, 213).

Finally, we performed the ACTH stimulation test, which is known to be a sensitive measure of adrenal steroidogenic capacity (214). We first suppressed the endogenous ACTH by dexamethasone, and then stimulated the adrenals with $ACTH_{1\text{-}24}$ after which the plasma corticosterone levels were measured. Interestingly, corticosterone secretion after ACTH stimulation was significantly reduced in *Gata6* cKO mice compared with controls (Figure 11C), indicating a reduced steroidogenic capacity of *Gata6* cKO mice. Normally, prolonged dexamethasone suppression induces apoptosis in the inner part of zF. Interestingly, we found a significantly decreased number of apoptotic cells in cKO mice when compared to controls after dexamethasone suppression. Similar kind of phenotype is observed in *Prkar1a* knockout mice and aged rats (215, 216).

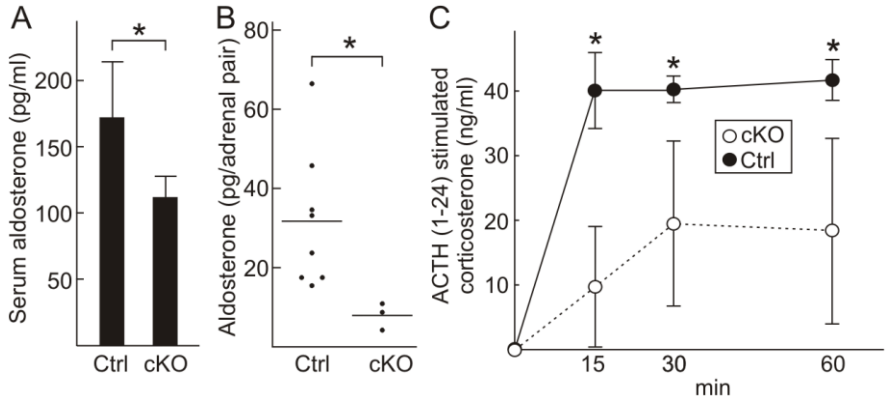

Figure 11 *Hormonal consequences of Gata6 deletion. A) Serum aldosterone levels in 8 wk old female mice. B) Aldosterone content in whole adrenal glands from 8 wk old male mice. C) Plasma corticosterone levels after overnight dexamethasone suppression and administration of $ACTH_{1\text{-}24}$ at time 0. * $P < 0.05$.*

1.6 *Gata6* cKO mice lack the X-zone

Murine adrenal X-zone is a unique zone, derived the fetal adrenal cortex (4), that forms between zR and medulla at the age of two weeks (217). In males, the X-zone soon stops growing and vanishes before puberty, but an orchiectomy-induced rise of serum LH restores it in weanling mice (218). In females, the X-zone continues growing until the first pregnancy when it rapidly disappears, or in older nulliparous females until it undergoes fatty degeneration at the age of 3 months (217).

To assess whether *Gata6* deletion has an effect on the X-zone in our mouse model we looked at the cKO and control adrenals using both light and electron microscopy. To our surprise we found that young virgin female cKO mice lacked the X-zone completely. To confirm this finding we measured expression levels of the known X-zone marker *Akr1c18* with qRT-PCR, and found it to be significantly decreased in nulliparous female cKO mice compared to controls. Furthermore, the orchiectomized male cKO mice lacked the secondary X-zone while orchiectomized controls formed it properly. We could not detect *Gata6* mRNA in the postnatal X-zone of the control mouse adrenals, which indicates that the lack of X-zone in the cKO mouse is a secondary effect caused by the adjacent zones. It has been shown that activin induces X-zone apoptosis in mice (219). Interestingly, we found the activin subunits, *Inhba* and *Inhbb*, mRNA levels to be elevated in our *Gata6* cKO mice, which might cause the early X-zone regression.

Similar to our *Gata6* cKO mice *acd*/*acd* mice that develop a severe adrenocortical dysplasia also lack X-zone (220). Other mouse models that have an abnormal X-zone phenotype are e.g. *Pre-B-cell transcription factor 1* (*Pbx1*) haploinsufficient mice in which the size of the X-zone is reduced (205) and female *Prophet of PIT1* (*Prop1*) deficient mice that has underdeveloped X-zone that undergoes early regression (221). It still remains unclear whether the lack of X-zone in *Gata6* cKO mouse is caused by early regression of a preexisting X-zone or lack of progenitor proliferation.

1.7 *Gata6* cKO mice exhibit subcapcular cell hyperplasia coupled with upregulation of gonadal-like markers

The adrenal capsule, which is normally only 3-5 cell layers thick, was expanded in *Gata6* cKO mice (Figure 12B). The subcapsule was also enlarged compared to control adrenal glands. Adrenal subcapsular cell hyperplasia is typically seen in older mice (222) and in certain mouse strains following gonadectomy (21), but it is rare in non-gonadectomized young mice. In both male and female *Gata6* cKO mice subcapsular cell hyperplasia was evident by 1 month of age. These subcapsular cells, which are also called type A cells (21), were small, spindle-shaped nonsteroidogenic cells that resemble postmenopausal ovarian stroma (21, 223). Immunohistochemistry staining (Figure 12C and D) and qRT-PCR analysis showed increased expression of type A cell marker GATA4 in the subcapsular cells of *Gata6* cKO mice. Using qRT-PCR other gonadal-like markers were

shown to be significantly upregulated in the *Gata6* cKO adrenals, including *Amhr2*, which is another type A cell marker (224), activin and inhibin subunits, as well as *Fkhl18* (gene coding for FOXS1), which is an important factor for the development of testicular vasculature (225). Also the mRNA expression of transcription factor *Tcf21*, which is expressed both in adrenal capsule and gonadal somatic cells, was increased in *Gata6* cKO mice. Microarray hybridization on adrenal glands from *Gata6* cKO versus control mice confrimed increased mRNA expression of *Amhr2* (9-fold), *Fkhl18* (2-fold), and *Tcf21* (3-fold) in cKO adrenals. Other mouse models that exhibit GATA4 positive type A cell hyperplasia are mouse with constitutive activation of β-catenin signaling in adrenocortical cells (226) and *Prkar1a* knockout mouse (227).

Gonadectomy-induced increases in serum LH levels not only induce nonsteroidogenic type A cell hyperplasia but also sex steroidogenic type B cell accumulation in the subcapsular area (21, 228). The latter cells express both *Cyp17* and *Lhcgr* and are capable of producing sex steroids, such as estrogen (228). To evaluate the effects of gonadectomy on our *Gata6* cKO mouse model, we gonadectomized both male and female mice and monitored the subcapsular cell hyperplasia one month (males) and 3 months (females) after surgery. Both male and female cKO mice showed expanded subcapsular cell compartment compared with gonadectomized controls (Figure 12E and F). These hyperplastic cells stained positive with GATA4 antibody (Figure 12G and H) and qRT-PCR showed upregulation of both type A cell (*Amhr2* and *Gata4*) and type B cell (*Cyp17* and *Lhcgr*) markers in gonadectomised cKO females, suggesting that both of these cell types were present in the gonadectomized cKO adrenals. The similar kind of gonadal-like subcapsular cell hyperplasia has also been reported in postmenopausal women (53) and men with testicular atrophy (54), as well as in ferrets, rats, guinea pigs, and hamsters (229).

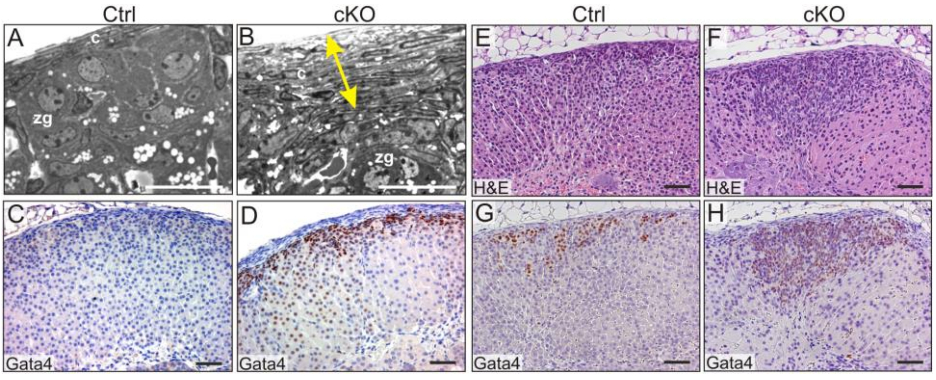

Figure 12 *Capsular and subcapsular cell hyperplasia in Gata6 cKO mice. A and B) Semi-thin sections of adrenal glands from 2mo old female mice. Yellow arrow points out the thickened capsule in mutant adrenal. Scale bar = 10 µm. C and D) GATA4 immunostaining of adrenal glands from 3 mo old female mice. Scale bar = 100 µm. E and F) Adrenal glands from gonadectomized female mice. Scale bar = 100 µm. G and H) GATA4 immunostaining of adrenal glands from gonadectomized female mice. Scale bar = 100 µm.*

Adult adrenal cortex undergo constant renewal as endogenous progenitor cells under the capsule proliferate, differentiate, and migrate centripetally to replace cells undergoing apoptosis (19). Previous studies have revealed several factors and signaling pathways that regulate this process. Transcription factor SF1 as well as SHH-, and WNT/β-catenin signaling promote steroidogenic cell differentiation, while DAX1 represses it (24, 26, 29, 34). It is proposed that adrenocortical progenitor cells are capable of differentiating into adrenocortical or gonadal-like cells, and some exogenous factors, such as gonadectomy-induced rise of serum LH levels, favor the gonaldal-like differentiation (21, 230). Based on our findings from the *Gata6* cKO mice we presume that GATA6 inhibits the differentiation of adrenal progenitor cells into gonadal-like cells favoring the adrenocortical cell differentiation, whereas GATA4 is thought to drive gonadal-like cell differentiation (57) (Figure 13). All in all, our data provide evidence that GATA6 is an important regulator of the balance between stem and progenitor cell growth and differentiation in the adrenal cortex.

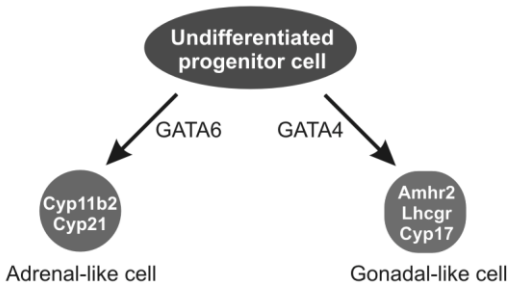

Figure 13 *Adrenocortical and gonadal cells originate from common pool of progenitor cells.*

2. GATA4, FOXL2, and SMAD3 in the regulation of GCT cell viability and apoptosis (II, III)

The balance between signals promoting cell survival and death is often disrupted in malignant cells. Transcription factors GATA4, FOXL2, and SMAD3 are all implicated in normal granulosa cell function as well as pathogenesis of adult GCT. GATA4 is abundantly expressed in GCTs and its expression correlates with tumor aggressiveness, *FOXL2* gene harbors a point mutation (C134W) in a vast majority of adult GCTs, and SMAD3 promotes GCT cell survival through NF-κB activation (134, 147, 152).

2.1 The expression patterns of GATA4, FOXL2, and SMAD3 correlate with each other in GCT tissue microarray

The expression patterns of GATA4, FOXL2, and SMAD3 overlap in developing and adult ovary, but the correlations have not been previously studied in GCTs. GATA4 expression pattern in the tumor tissue microarray containing 93 GCT samples has been previously

published (147). In brief, GATA4 was expressed at high/intermediate level in 90 % of the tumors, while only 10 % showed low/negative expression. We now analyzed the spatiotemporal protein expression patterns of FOXL2 and SMAD3 in GCT tissue microarray. Tumors were classified into three groups (high, intermediate, low) based on the staining intensity. The majority of tumors exhibited high or intermediate staining (Figure 14, Table 4).

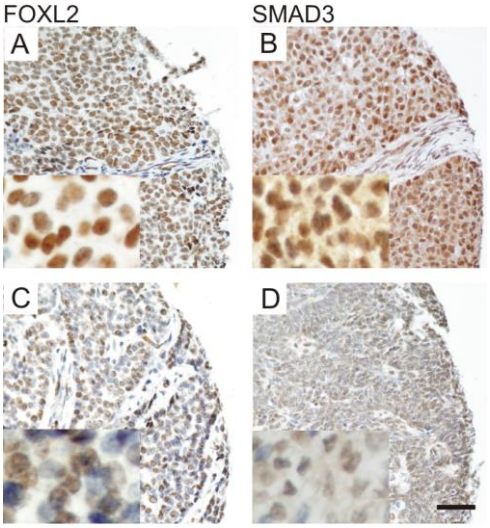

Figure 14 *FOXL2 and SMAD3 are expressed in adult GCTs. Representative immunostaining images of high/intermediate expression (A and B) and low expression (C and D) tumors. Higher magnifications are shown in insets. Scale bar = 100µm.*

The high expression patterns of GATA4, FOXL2, and SMAD3 correlated with one other, but not with any of the clinicopathological parameters analyzed (i.e. age, menopause status at diagnosis, clinical stage, tumor size, tumor subtype, nuclear atypia, and mitotic index). Interestingly, when the association analyses were done only with the larger tumors (> 10 cm in diameter, n=35) the correlations of FOXL2 and GATA4 with each other and with SMAD3 were absent. In addition, the correlation between FOXL2 and GATA4 was lost in the primary GCTs that had recurred (n=19), suggesting that the imbalances in the expression of GATA4, FOXL2, and SMAD3 might give the tumor a growth advantage and therefore lead to more aggressive tumor behavior. Furthermore, high FOXL2 expression associated with an increased 5 years risk of recurrence, while low FOXL2 expression correlated with low 5-year recurrence risk. This is in line with the previous finding that FOXL2 expression in the primary tumor associates with the risk of recurrence (231). Currently, the only prognostic factor with clinical significance is tumor stage at time of diagnosis, and molecular prognostic markers are lacking (232-234). Finding new prognostic markers is difficult due to the rarity of GCT and long follow-up time needed. Based on our data, FOXL2 expression level might serve as a new tool for

evaluating the risk of recurrence, although further studies are needed to validate this finding.

Table 4 *The protein expression patterns of FOXL2 and SMAD3 in GCT microarray*

	FOXL2	SMAD3
Low	5 (5%)	8 (9%)
Intermediate	52 (58%)	43 (50%)
High	33 (37%)	36 (41%)
Total	90	87

2.2 GATA4, FOXL2, and SMAD3 physically interact with each other

GATA4 and SMAD3 have been shown to interact with each other (235). Moreover, SMAD3 also interacts with FOXL2 (236). The physical interaction between GATA4 and FOXL2, however, has not been demonstrated before. To investigate the interactions between GATA4, FOXL2, and SMAD3 in GCT cells we overexpressed V5-tagged GATA4 with SMAD3 and either untagged, V5- or GFP-tagged wild type (wt) or C134W-FOXL2 in juveline GCT cell line COV434 cells (Figure 15). We chose to use this cell line, as the pilot experiments with adult GCT cell line (KGN) were unsuccessful probably due to their poor transfectability and low endogenous expression of transcription factors GATA4, FOXL2, and SMAD3. Protein complexes were immunoprecipitated using the V5 epitope.

Our data revealed that wt and C134W-mutated FOXL2 equally co-immunoprecipitated with both GATA4 and SMAD3, suggesting that the loss of interaction between these factors is not the cause of GCT. FOXL2 is also shown to interact with SF1 in granulosa cells, where it represses the binding of SF1 to *CYP17* promoter, and thus act as inhibitor of steroidogenesis (237). Yet another identified binding partner of FOXL2 is DEAD box-containing protein DP103. This transcription complex is able to induce granulosa cell apoptosis during follicular development (238). Altogether, our results do not directly prove that GATA4, FOXL2, and SMAD3 are all part of the same macromolecular transcription complex, but rather shows that they are capable of forming binary interactions with each other.

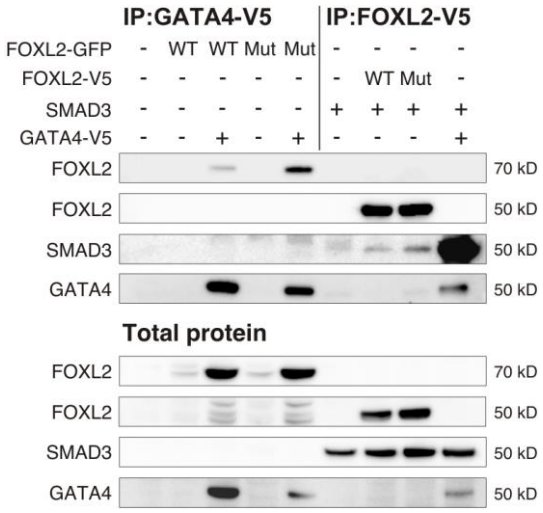

Figure 15 *GATA4, FOXL2, and SMAD3 physically interact with each other. COV434 cells were transfected with V5 tagged GATA4, SMAD3, and wild type or C134W mutated FOXL2 overexpression vectors for 48h followed by immunoprecipitation using V5 epitope. Immunoprecipitated proteins were detected using antibodies against FOXL2, SMAD3, and GATA4. Total proteins are shown as controls for transfections. Similar results were obtained in at least three independent experiments.*

2.3 GATA4, FOXL2, and SMAD3 synergistically regulate the *CCND2* promoter activation

Cell cycle regulator *CCND2*, encoding cyclin D2,is a known target gene for GATA4, FOXL2, and SMAD3. GATA4 and SMAD3 are its positive regulators, while FOXL2 inhibits its expression (148, 151, 239). Previously, CCND2 has shown to be expressed at high/intermediate levels in GCTs (148), and now we show that its expression pattern correlates with that of GATA4 and SMAD3, but not with FOXL2.

To investigate the synergistic roles of GATA4, FOXL2, and SMAD3 in the regulation of CCND2 promoter we overexpressed GATA4, wt and C134W-mutated FOXL2, and SMAD3 in KGN cells, and measured the *CCND2* promoter activity. None of these factors alone could significantly increase the promoter activity, whereas GATA4 together with SMAD3 synergistically caused a 8-fold increase in promoter activity compared to control. This finding strengthens the role of GATA4 in the TGF-β signaling in GCT cell (235). These data together with the positive correlation of expression of GATA4, SMAD3, and CCND2 in GCTs suggest that GATA4-SMAD3 co-operation is vital for *CCND2* expression and the proliferation of GCT cells. Furthermore, both FOXL2 forms decreased GATA4/SMAD3-induced *CCND2* promoter activity by 50%. In rat granulosa cells, another member of forkhead transcription factor family, FOXO1, is shown to repress the transcription of *CCND2* by binding to its promoter, and FSH signaling as well as positive signaling from activin-stimulated phosphorylation of SMAD2/3 are required to release

this repression (151). All in all, our findings imply that the disrupted functional interactions between GATA4, FOXL2, and SMAD3 with CCND2 promoter cannot explain how the mutated FOXL2 participates in GCT pathogenesis.

2.4 GATA4, FOXL2, and SMAD3 modulate GCT cell viability and apoptosis

GATA4 serves as an anti-apoptotic factor in cardiomyocytes protecting them from apoptosis induced by exogenous stimuli (149, 150). Furthermore, high GATA4 expression has been associated with more aggressive tumor behavior and increased risk of recurrence in GCTs (147).

In order to investigate the effects of GATA4 on GCT cell apoptosis *in vitro* we transfected KGN cells with adenoviral constructs expressing either wild type or dominant negative GATA4, or lentiviral vectors expressing GATA4 targeting small hairpin RNAs (shRNA), and quantified the caspase 3/7 activity as a measure of apoptosis. Overexpression of GATA4 with wild type adenovirus construct did not affect the GCT cell apoptosis (Figure 16A), while disrupting GATA4 function significantly increased the number of apoptotic GCT cells (Figure 16B and C). This finding supports the anti-apoptotic role of GATA4 in GCTs and is in line with the previous discoveries in cardiomyocytes (149, 150) and in normal ovary, in which downregulation of GATA4 expression precedes the physiological apoptosis of granulosa cells in ovulating follicles (96).

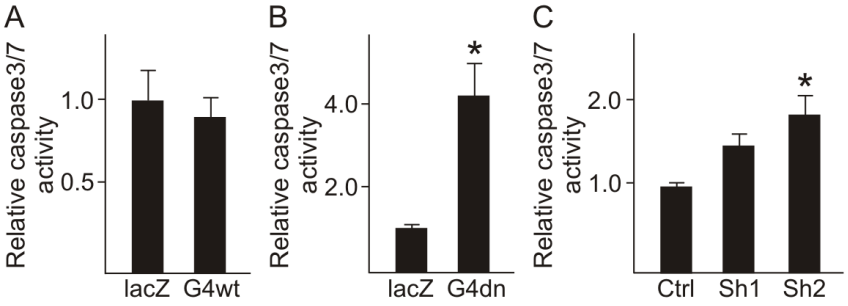

Figure 16 *Disrupting GATA4 function protects GCT cells from apoptosis. KGN cells were transfected either with A) wild type (G4wt), B) dominant negative (G4dn) GATA4 adenovirus constructs or C) lentiviral vectors expressing GATA4 targeting small hairpin RNAs (Sh1 and Sh2). Caspase3/7 was measured 6h after transfections and presented relative to control transfection as the mean ±S.E.M. of three independent experiments performed in triplicate. *P<0.05.*

In contrast to anti-apoptotic GATA4, wt FOXL2 induces GCT cell apoptosis (143, 240). Interestingly, C134W-mutated FOXL2 has been shown to be less capable of inducing apoptosis compared to the wt version (143). Furthermore, SMAD3 has shown to promote GCT cell survival by activating ERK1/2 signaling pathway (152).

To examine the synergistic effects of GATA4, wt and C134W-mutated FOXL2, and SMAD3 on cell viability and apoptosis in GCT cells, we overexpressed these factors separately and simultaneously in KGN cells. After transfection cell viability and apoptosis were measured. Wt FOXL2 overexpression alone or together with GATA4 and/or SMAD3 significantly decreased the viable cell count compared to control, while overexpression of mutated FOXL2 alone or together with GATA4 and/or SMAD3 did not affect the cell viability.

Moreover, in accordance with previous findings (143), wt FOXL2 induced a significant 3-fold increase in caspase3/7 activity, whereas mutated FOXL2 showed significantly weaker effect (Figure 17). Overexpression of GATA4 and SMAD3 alone or together did not affect the caspase activity. Interestingly, GATA4, but not SMAD3, significantly decreased wt FOXL2-induced apoptosis, but had no effect on mutated FOXL2-induced apoptosis (Figure 17) further supporting the anti-apoptotic role of GATA4 in GCTs. Interestingly, a recent study suggested that wt, but not mutated FOXL2, induces GCT cell apoptosis by increasing gonadotropin-releasing hormone receptor expression (241). Furthermore, our findings are in line with a recently published study by L'hôte *et al*., in which they identified 10 novel partners for FOXL2 (240). Partners with pro-apoptotic capability were able to increase apoptosis induction by wt FOXL2, but not by the mutated form, whereas partners with an anti-apoptotic effect decreased apoptosis induction by both FOXL2 versions, and thus promote GCT cell viability and inhibit apoptosis (240).

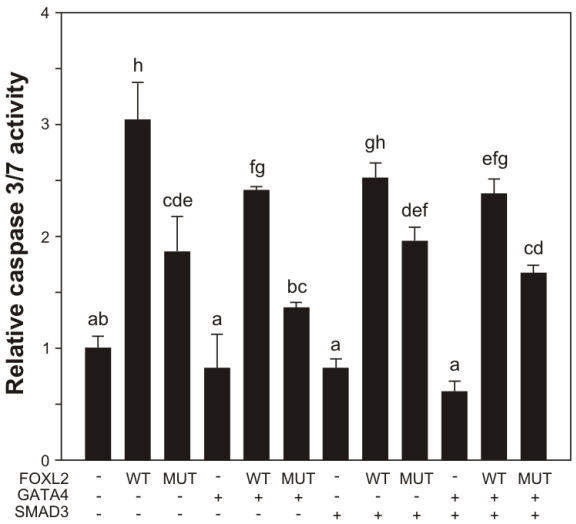

Figure 17 *GATA4 protects GCT cells from wt FOXL2 induced apoptosis. KGN cells were transfected with wild type FOXL2, C134W mutated FOXL2, GATA4, and SMAD3 expression plasmids. The activated caspase 3/7 was measured 24 h after transfection. Caspase 3/7 activity is presented relative to control transfection as the mean ±SEM of at least three independent experiments performed in triplicate. Bars not connected by the same letter are significantly different. $P < 0.05$.*

Altogether, our data indicate that GATA4 acts as an anti-apoptotic factor in GCT cells, and that GATA4 and SMAD3 exhibit distinct effects on cell survival and apoptosis compared to wt FOXL2. Furthermore, these factors do not modulate the deacreased ability of mutated FOXL2 to induce apoptosis, suggesting that the disturbance of the delicately balanced regulation of cell survival and apoptosis due to the C134W mutation is likely to contribute to GCT pathogenesis (hypothetical model is presented in figure 18). The regulation of granulosa cell growth and apoptosis is complex and includes numerous para- and autocrine factors, as well as transcription factors that have to co-operate precisely. In this study we chose to explore the effects of only three of these factors on GCT cell viability and apoptosis. Therefore it is plausible that several other factors are also involved in the complex molecular events leading to the malignant transformation of granulosa cells.

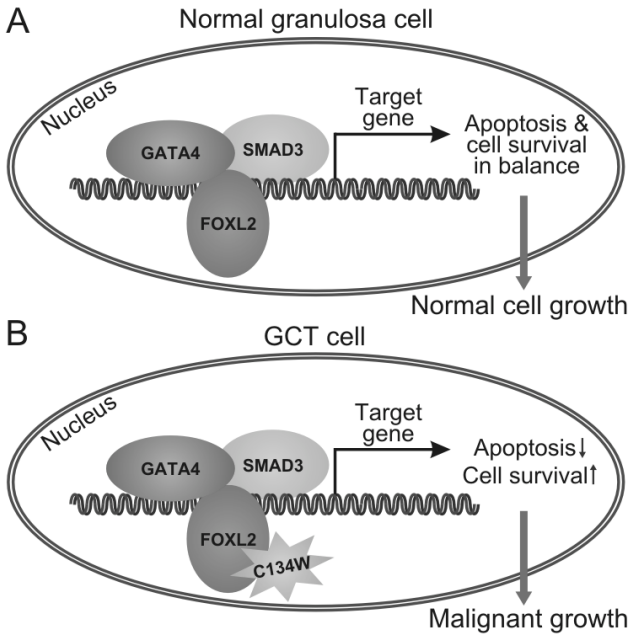

Figure 18 *Hypothetical model of the actions of C134W mutated FOXL2 in GCT pathogenesis. C134W mutation in FOXL2 gene gives GCT cells a growth advantage. A) Normal granulosa cell growth is modulated by interaction and co-operation between wt FOXL2, GATA4, and SMAD3. B) C134W mutation in FOXL2 disrupts this balance leading to malignant cell growth.*

3. TRAIL and anti-VEGF treatment inhibit growth in GCTs (III, IV)

Although TRAIL ligand and its receptors are known to be expressed in various tissues (117), TRAIL has been most intensively studied in cancer cells due to its ability to induce apoptosis in malignant cells without affecting the healthy cells (242). The mechanism by which TRAIL induces apoptosis only in cancerous cells has been under debate, and it is

still largely unknown. Decoy receptor expression in normal cells but not in cancerous cells is thought to be one possible explanation (243, 244). Owing to its unique potential to selectively kill malignant cells, TRAIL has become a promising target for cancer therapy. Multiple preclinical and clinical studies have been carried out to assess the usefulness and safety of recombinant human TRAIL (rhTRAIL) in treatment of several cancer types, including metastatic colorectal cancer, prostate cancer, pancreatic cancer, and ovarian cancer (245).

Another potential target for cancer therapy is anti-VEGF treatment with either VEGF targeting antagonists or VEGFR blocking agents. One of the most promising anti-VEGF drugs is bevacizumab, a humanized monoclonal antibody against VEGF (186, 187). Interestingly, in addition to endothelial cells, various cancer cells, including GCT cells (183), express VEGF and its receptor VEGFR-2, but the role of VEGF signaling in GCT cell growth has not been assessed before.

3.1 GCTs express functional TRAIL receptors

TRAIL pathway components are expressed in normal granulosa cells (121), but their expression in GCT cells have not been extensively studied before. Furthermore, we now assessed the functionality of TRAIL pathway in freshly isolated primary GCT cells.

The expression of functional TRAIL receptors on the cell surface is a prerequisite for TRAIL-indused apoptosis in tumor cells. To study the protein expression of TRAIL receptors DR4 and DR5 in GCTs, the tumor tissue microarray of 93 GCT samples was subjected to immunohistochemical staining. Tumors were classified in three groups (low, intermediate, high) based on the staining intensity. Majority of GCTs exhibited strong or intermediate immunostaining for both DR4 and DR5 (Figure 19A and B, Table 5). Interestingly, 11/12 of recurrent GCTs showed strong or intermediate DR4 expression, and 12/12 tumors expressed DR5 at high or intermediate level. In epithelial ovarian cancer patients and breast cancer patients with invasive ductal carcinoma, DR4 expression levels have been shown to correlate with tumor grade (246, 247). No such correlation was found in GCT patients, and neither did DR4 and DR5 expression patterns correlate with the other clinicopathological parameters studied (clinical stage, tumor size, nuclear atypia, mitotic index, and recurrence tendency).

Table 5 *The expression of TRAIL receptors in GCT tissue microarray*

	DR4	DR5
Low/negative	8 (9%)	7 (8%)
Intermediate	64 (73%)	65 (75%)
Strong	16 (18%)	15 (17%)
Total	88	87

Next, we characterized the functional TRAIL receptor expression in six primary GCT cell cultures (two primary and four recurrent tumors). Immunocytochemistry staining and reverse transcription-PCR analysis of DR4 and DR5 showed that all the six primary GCT cell cultures expressed both of the receptors.

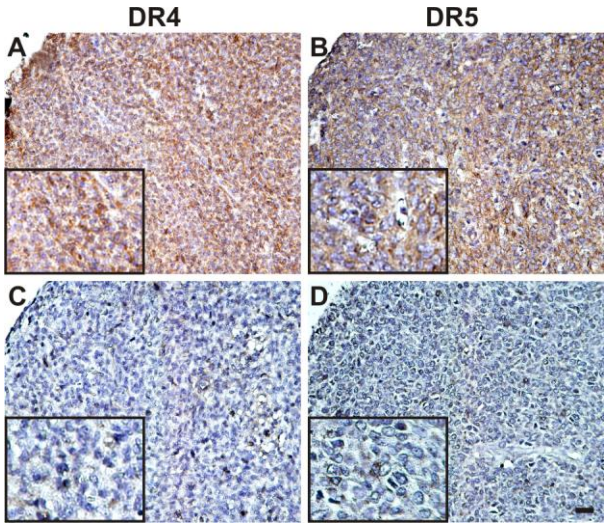

Figure 19 *TRAIL receptors are expressed in adult GCTs. Representative immunostaining images of high/intermediate expression (A and B) and low/negative expression (C and D) tumors. Scale bar = 50 µm.*

3.2 TRAIL pathway is active in GCT cells

RhTRAIL induces apoptosis in GCT derived KGN cell line (121, 248). Now we tested the ability of TRAIL to indude apoptosis in freshly isolated primary GCT cells. Cultured primary cells were stimulated with increasing doses of rhTRAIL for 24h, after which apoptosis was measured by western blotting and DAPI staining. In all six primary GCT cell cultures studied, TRAIL dose dependently activated apoptosis (Figure 20A and B).

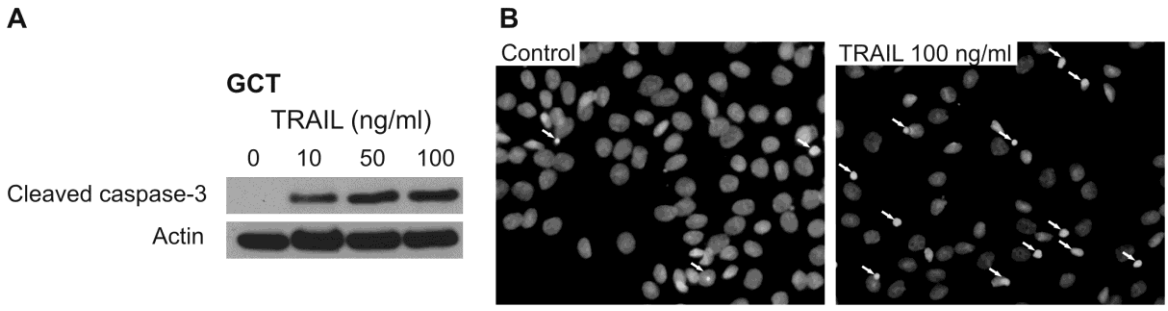

Figure 20 *TRAIL induces apoptosis in primary GCT cells. A) Western blotting showing the activated caspase 3 after TRAIL treatment. B) DAPI staining of primary GCT cells after TRAIL administration. Arrows indicate apoptotic cells.*

Together these findings demonstrate a functional TRAIL pathway in GCT cells similarly to other ovarian cancer types (249), and set a preclinical basis for therapeutic use of TRAIL in treatment of GCT patients. In addition to rhTRAIL, another promising tool for TRAIL pathway activation are agonistic monoclonal antibodies towards functional TRAIL receptors. Several of these antibodies have been tested, and they have shown promising safety and efficacy in diverse preclinical cancer models as well as in clinical studies (250). Numerous preclinical studies have revealed that combination treatments of rhTRAIL and various chemotherapeutics (e.g. DNA damaging agents) or agents that target other points in the apoptosis pathway (e.g. BCL2 antagonists) lead to synergistic apoptotic activity (250). This synergism is thought to result from the combinatorial stress that sensitises the cell and triggers apoptosis more efficiently than single stress factor. In the future, combination of sensitizing treatment and the stimulation of TRAIL-dependent extrinsic apoptotic pathway could be used in the treatment of GCT patients.

3.3 Serum VEGF is elevated in GCT patients

Cancer patients often present with elevated levels of serum VEGF (176-178). To assess whether this is the case also in GCT patients we measured the circulating VEGF from the serum of 54 GCT patients. Mean serum VEGF was significantly higher in patients with non-operated GCT when compared with disease-free patiens (Figure 21A). Furthermore, in paired analyses of an individual patient's with-disease and disease-free samples, tumor removal significantly decreased serum VEGF (Figure 21B). No significant difference was seen when the serum VEGF levels of patients with large (<10 cm in diameter) tumors were compared with those of small (>10 cm in diameter) tumors. Furthermore, serum VEGF levels did not correlate with any of the clinicopathological parameters studied (patient age at sample retrieval, the type of treatment, haemoglobin levels, haematocrit, leucocyte, or platelet count). Since most of the GCTs in our study were diagnosed at early stage, the serum VEGF levels were only moderately increased in the GCT patients, and thus are of limited value as tumor marker in GCT patients.

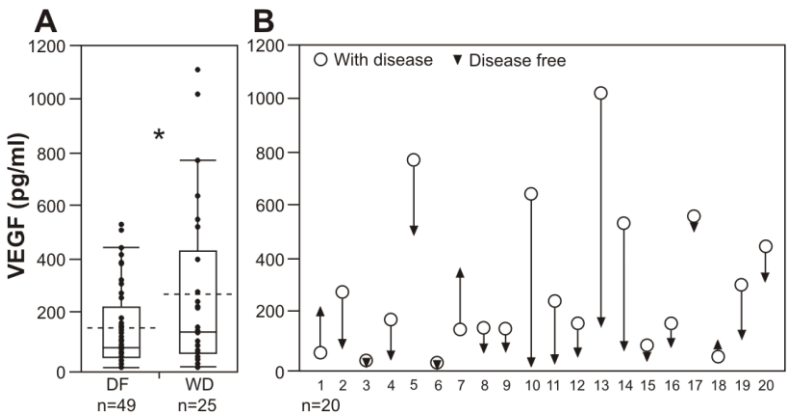

Figure 21 *Sirculating VEGF is elevated in GCT patients. A) Serum VEGF was measured from 54 GCT patients. B) The paired analyses of serum VEGF in 20 GCT patients. Abbreviations: DF, disease free; WD, with disease. *P<0.05.*

To explore the origin of circulating VEGF in GCT patients, we analysed the VEGF production from primary GCT cell cultures. All 14 primary cell cultures studied secreted significant amounts of soluble VEGF into the supernatant, suggesting that the source of the serum VEGF are the tumor cells. This is in line with the finding that serum VEGF levels decrease after tumor removal.

3.4 Human GCTs express phosphorylated VEGFR-2

Various tumor cells, including GCTs, express VEGFR-2, the functional receptor of VEGF (180, 183). Furthermore, studies in ovarian (182) and various other cancer cells (180) have revealed an autocrine VEGF/VEGFR-2 signaling loop that promotes growth and survival of the tumor cells via phosphorylation and activation of VEGFR-2. In this study we analysed the expression of phosphorylated VEGFR-2 (pVEGFR-2) in our GCT tissue microarray of 93 GCT samples by immunohistochemisty. Tumors were classified in three groups (high, low, negative) based on the staining intensity, and the localisation of antigen was further categorized as cytoplasmic or nuclear.

Most of the tumors (73/89) showed positive staining for pVEGFR-2, and 21 of them were highly positive. Interestingly, 95% of pVEGFR-2 positive tumors exhibited nuclear staining. This is in agreement with other studies in endothelial cells showing the nuclear localization of VEGFR-2 upon activation (251, 252). Nuclear localization of activated VEGFR-2 mediates distinct responses on gene expression depending on the VEGF isoform, phosphorylated tyrosine residues of the intracellular domain of receptor, and cell type (253, 254). No differences were found in pVEGFR-2 expression between primary and recurrent GCTs, and the pVEGFR-2 expression pattern did not correlate with any of the clinicopathological parameters studied (tumor size, subtupe, nuclear atypia, and mitotic index). Interestingly, high pVEGFR-2 expression positively correlated with the

expression of VEGF and VEGFR-2 (expression patterns previously published in (183)), suggesting an autocrine VEGF/VEGFR-2 signaling loop in GCT cells.

3.5 BVZ inhibits GCT cell growth by inducing apoptosis

To further study the possible growth-promoting role of VEGF in GCT cells, we blocked the tumor produced VEGF with BVZ, a humanized monoclonal antibody that inhibits VEGF. KGN cells were treated with increasing doses of BVZ and analysed for viable cell count and apoptosis. BVZ was able to induce apoptosis (Figure 22A and B), and it significantly decreased the viable cell number (Figure 22C) with highest concentration used. Similar results were obtained from primary GCT cells; BVZ induced apoptosis in all the six (4 primary and 2 recurrent tumors) primary cell cultures studied. Furthermore, BVZ treatment reduced the expression of pVEGFR-2 in KGN cells.

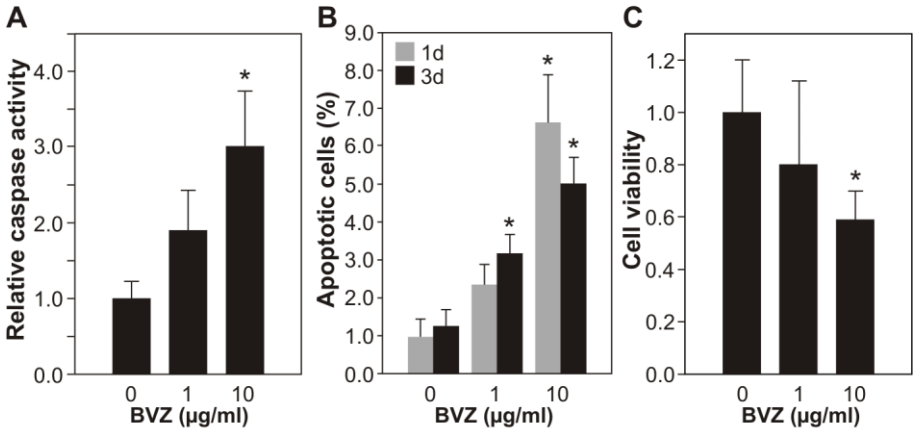

Figure 22 *BVZ inhibits GCT cell growth and activates apoptosis. KGN cells were treated with increasing doses of BVZ. After 1-3 d treatment apoptosis was measured using caspase 3/7 assay (A) and DAPI staining (B). C) Viable cell count was analysed using MTT assay. *, significant difference compared with BVZ 0 µg/ml. P<0.05.*

Our findings demonstrate an active VEGF signaling pathway in GCT cells, and that BVZ inhibits GCT cell growth by inhibiting this pathway. Thus, these data further prove the existence of survival-promoting VEGF/VEGFR-2 autoloop in GCT cells. Furthermore, our results set a molecular basis for the clinical use of BVZ in the treatment of GCT patients. BVZ is already widely used in the treatment of multiple cancer types, including epithelial ovarian carcinoma (186, 187). Moreover, small retrospective clinical studies have shown that BVZ is also active in treatment of recurrent GCTs (188-190). However, BVZ treatment has some severe side effects, including gastrointestinal perforations (255). Therefore other VEGF/VEGFR-2 targeting drugs, such as a monoclonal antibody against VEGFR-2, ramucirumab, that inhibits VEGF binding to its receptor, and has shown survival benefits in patients with advanced gastric adenocarcinoma, hepatocellular

carcinoma, and other cancer types (256-258), may provide safer and better-tolerated target for treatment of recurrent GCTs.

4. GATA4 protects GCT cells from TRAIL-induced apoptosis (III)

As discussed above, transcription factor GATA4 acts as an anti-apopotic factor in human GCT cells. Furthermore, during folliculogenesis in normal ovary GATA4 downregulation precedes the physiological apoptosis of ovulating follicles (96). To test whether TRAIL-treatment affects the endogenous GATA4 levels, KGN cells were treated with increasing doses of rhTRAIL and GATA4 protein levels were determined by western blotting. Our results showed that the endogenous GATA4 levels did not change after TRAIL administration indicating that the apoptosis-inducing effect of TRAIL does not require GATA4 downregulation.

Next, to assess whether GATA4 modulate the extrinsic apoptosis pathway, we overexpressed and silenced GATA4 in KGN cells using either adenoviral GATA4 construct or lentiviral vectors expressing two different shGATA4. We also disrupted GATA4 function using dominant negative GATA4 adenoviral construct. Transfected cells were treated with increasing doses of rhTRAIL, after which apoptosis was measured. Overexpression of GATA4 effectively protected KGN cells from TRAIL-induced apoptosis (Figure 23A), while silencing GATA4 significantly enhanced it (Figure 23B). Furthermore, disrupting GATA4 function with dominant negative mutant GATA4 also sensitized the cells to TRAIL-induced apoptosis (Figure 23C).

These findings further strengthen the anti-apoptotic role of GATA4 in GCTs. Similarly to our findings, in murine heart GATA4 has shown to protect cardiomyocytes from doxorubicin-induced apoptosis (149). The mechanism by which GATA4 protects GCT cells from TRAIL-induced apoptosis still remains unknown, but it is likely to exert its effects via intrinsic apoptotic pathway, since it is known that GATA4 regulates the intrinsic pathway by regulating the expression of anti-apoptotic BCL2 in cardiomyocytes and GCT cells (148, 150). Other parameters that sensitise tumor cells to TRAIL induced apoptosis include mutations in proapoptotic *BAX* and *Caspase 8* genes, and altered expression of certain anti- and proapoptotic factors (250). Since GATA4 protein levels are known to be elevated in more aggressive tumors (147), one may speculate that in these tumors elevated GATA4 levels protect the tumor cells from exogenous apoptosis-inducing factors such as TRAIL. Furthermore, the evaluation of GATA4 expression levels of individual GCT patients could be used as a predictive marker of TRAIL treatment efficacy in clinic in the future.

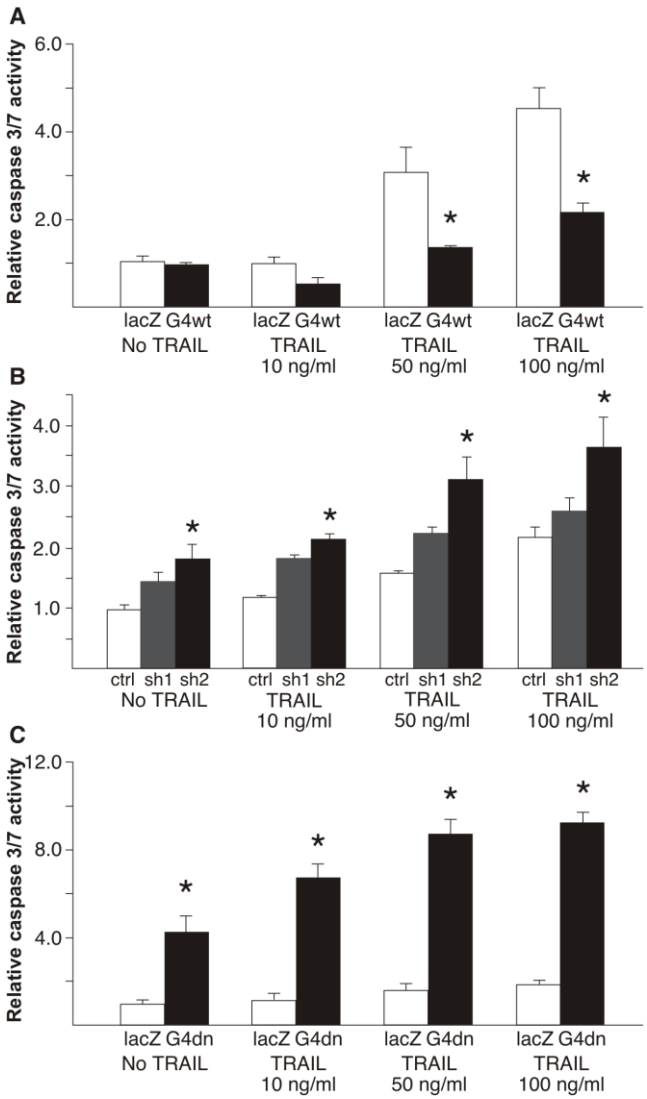

Figure 23 *GATA4 protects GCT cells from TRAIL induced apoptosis. KGN cells were transfected with A) adenoviral GATA4 construct (G4wt), B) lentiviral shGATA4 constructs (sh1 and sh2) A), or C) adenoviral dominant negative GATA4 construct (G4dn). Transfected cells were treated with rhTRAIL for 6 h and caspase 3/7 activity was measured. All caspase activities are presented relative to control transfection as the mean ±SEM of three independent experiments performed in triplicate. *P<0.05.*

All in all, our data increase the knowledge of regulation of the GCT cell apoptosis. We show that transcription factor GATA4 acts as an anti-apoptotic factor in these cells by protecting them from FOXL2- and TRAIL-induced apoptosis. Furthermore, disrupting GATA4 function promotes GCT cell apoptosis. Our findings also indicate the existence of prosurvival VEGF/VEGFR-2 autoloop in GCT cells, and that blocking this pathway by BVZ leads to GCT cell apoptosis. A schematic depiction of these findings is presented in Figure 24.

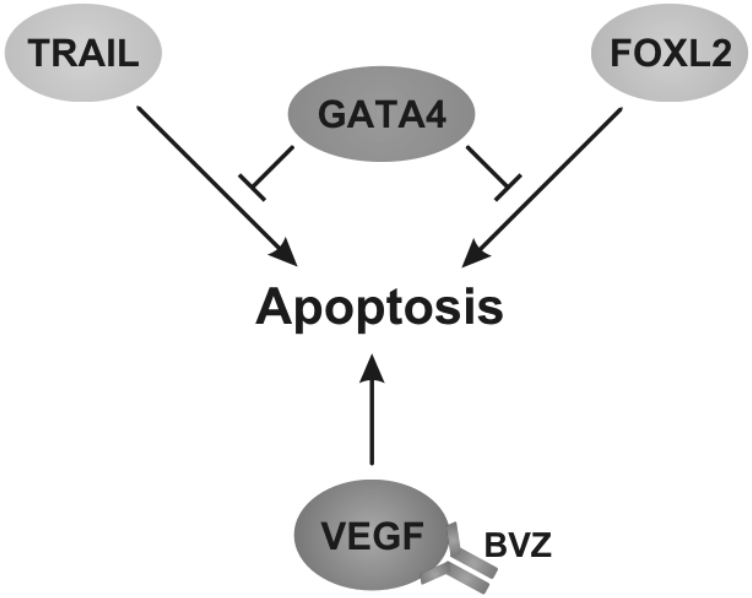

Figure 24 *Summary of the regulation of GCT cell apoptosis.*

Conclusions and future prospects

The main steroidogenic organs, adrenal cortex and gonads, originate from the common progenitor, and share partly the same molecular machinery, including transcription factors and signaling pathways that regulate their cell differentiation, hormone production, growth, and cell death. The studies presented herein focus on the transcriptional regulation of adrenocortical cell differentiation and GCT cell survival, as well as finding new potential targets for GCT treatment.

1) Transcription factor GATA6 is important for the proper development and differentiation of murine adrenal cortex. Conditional deletion of *Gata6* gene from *Sf1*-positive adrenocortical cells results in a complex adrenal phenotype including a thin and cytomegalic adrenal cortex, blunted aldosterone production, lack of X-zone, and increased subcapsular cell hyperplasia. All in all, this study demonstrates that GATA6 regulates the balance between progenitor cell prolifearation and differentiation in the adrenal cortex.

In the present study we describe the phenotype of GATA6 cKO mouse, but further studies are needed to elucidate the mechanisms behind this pleiotropic phenotype, e.g. whether the key signaling pathways, including pathways implicated in stem cell function are disrupted in *Gata6* cKO adrenals.

2) Transcription factors GATA4, FOXL2, and SMAD3 interact and co-operatively modulate GCT cell viability and apoptosis. Our study strengthens the concept of the anti-apoptotic role of GATA4 in GCTs by showing that disrupting its function significantly increases apoptosis in these cells, and that GATA4 protects GCT cells from FOXL2-induced apoptosis. Furthermore, GATA4 and SMAD3 demonstrate distinct effects compared to wild type FOXL2 in the regulation of cell survival, whereas they do not modulate the reduced ability of mutated FOXL2 to induce GCT cell apoptosis. Taken together, these findings suggest that C134W mutation in *FOXL2* gene destabilises the balanced control of GCT cell growth and apoptosis leading to malignant transformation.

This study demonstrates the previously unknown interaction of GATA4, FOXL2, and SMAD3. However, it does not unveil the exact molecular mechanisms by which C134W-mutated FOXL2 causes the GCT formation. Further studies are therefore needed to better understand the functional consequences of this mutation.

3) TRAIL and BVZ induce apoptosis in GCT cells. Human GCTs express functional TRAIL receptors, and TRAIL pathway is active in primary GCT cell cultures leading to apoptosis. Furthermore, GATA4 protects GCT cells from TRAIL-induced apoptosis. GCTs also express activated VEGF receptor VEGFR-2, and serum VEGF levels are elevated in GCT patients. Moreover, blocking the autocrine VEGF/VEGFR-2 pathway with BVZ results in GCT cell apoptosis. These findings set a preclinical basis for targeting these two pathways in the treatment of GCTs.

The treatment of recurrent GCTs is challenging due to lack of biologically targeted treatment modalities. Our study provides two potential targets for new treatment options. However, several issues have to be considered before their clinical use. In addition to malignant cells, TRAIL receptors are also expressed in normal granulosa cells, and TRAIL has been suggested to involve in the regulation of follicular atresia. Therefore thorough evaluation of the effects of TRAIL on normal granulosa cells needs to be done.

BVZ has shown to have severe side effects. Thus, other VEGF/VEGFR2 pathway inhibitors, such as VEGFR-2 blockers, may serve better-tolerated options for GCT treatment. However, the efficacy and safety of these drugs must be assessed in preclinical and clinical studies before their clinical use.

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