Regulation and Function of GATA Transcription Factors in Adrenocortical Tumors and Granulosa Cells

Helka Parviainen

Clinical Graduate School in Pediatrics and Gynecology
and
Pediatric Graduate School

Children’s Hospital
University of Helsinki
Finland

ACADEMIC DISSERTATION

To be publicly discussed with permission of the Medical Faculty, University of Helsinki, in the Niilo Hallman auditorium of the Children’s Hospital, on September 9th 2011, at 12 noon

Helsinki 2011
Supervisor

Professor Markku Heikinheimo, M.D., Ph.D.
Children’s Hospital, University of Helsinki
Helsinki, Finland

Reviewers

Docent Jarmo Jääskeläinen, M.D., Ph.D.
Department of Pediatrics, Kuopio University Hospital
Kuopio, Finland

and

Docent Camilla Schalin-Jäntti, M.D., Ph.D.
Department of Endocrinology, Helsinki University Central Hospital
Helsinki, Finland

Official opponent

Professor Heikki Ruskoaho, M.D., Ph.D.
Department of Biomedicine, University of Oulu
Oulu, Finland

ISBN 978-952-10-7138-6 (PDF)
http://ethesis.helsinki.fi

Helsinki University Print 2011
To my family
# Table of Contents

**ABSTRACT** 6

**ORIGINAL PUBLICATIONS** 8

**ABBREVIATIONS** 9

**INTRODUCTION** 10

**REVIEW OF THE LITERATURE** 11

1 DEVELOPMENT AND FUNCTION OF THE ADRENAL GLAND 11
1.1 FETAL PERIOD 11
1.2 POSTNATAL PERIOD 13

2 GATA FAMILY OF TRANSCRIPTION FACTORS 15
2.1 STRUCTURE 15
2.2 TISSUE-SPECIFIC EXPRESSION AND KNOCKOUT PHENOTYPES 16
2.3 GATA FACTORS IN THE DERIVATIVES OF ADRENOGONADAL PRIMORDIUM 19

3 ADRENOCORTICAL TUMORS 21
3.1 HUMAN ADRENOCORTICAL TUMORS 21
3.2 MOUSE MODELS 23

4 MOLECULAR PATHOPHYSIOLOGY OF ADRENOCORTICAL TUMORIGENESIS 26
4.1 SUBCAPSULAR STEM/PROGENITOR CELLS 26
4.2 WNT/β-CATENIN SIGNALING 27
4.3 TGF-β/SMAD SIGNALING 28
4.4 CAMP SIGNALING 28
4.5 MOLECULAR MECHANISMS IN HUMAN ADRENOCORTICAL TUMORS 29

**AIMS OF THE STUDY** 32

**MATERIALS AND METHODS** 33

1 PATIENTS AND CLINICAL DATA (I, IV) 33
2 EXPERIMENTAL ANIMALS, SERUM SAMPLES AND TREATMENTS (II, III) 33
3 TISSUE SAMPLES 33
3.1 MOUSE (II, III) 33
3.2 HUMAN (I, IV) 34
4 CELL CULTURES AND TRANSIENT TRANSFECTIONS (I, III) 34
5 mRNA EXPRESSION 35
5.1 IN SITU HYBRIDIZATION (II, III) 35
TABLE OF CONTENTS

5.2 Nuclear extracts and electrophoretic mobility shift assays (EMSAs) (I) 35
5.3 Reverse-transcriptase PCR (RT-PCR) (III, IV) 36
5.4 RNase protection (II, III) 36
6 PROTEIN EXPRESSION 36
6.1 Immunohistochemistry (I–IV) 36
6.2 Immunocytochemistry (I) 37
6.3 Protein co-immunoprecipitation (I) 38
6.4 Western blotting (I) 38
7 STATISTICAL ANALYSIS (I, III, IV) 38

RESULTS AND DISCUSSION 39

1 MOLECULAR INTERACTIONS OF GATA-4/6 (I) 39
1.1 GATA-4/6 is required for inhibin-α promoter activation by TGF-β 39
1.2 Functional and physical interaction of GATA-4 and Smad3 41
1.3 SF-1 cooperates with GATA-4 and Smad3 41
2 LH-DEPENDENT NEOPLASIA OF THE MOUSE ADRENAL CORTEX (II, III) 43
2.1 Gonadal characteristics in neoplastic cells 43
2.2 GATA-4 and LHR in the adult adrenal cortex 46
2.3 Mechanisms of subcapsular stem/progenitor cells 49
3 HUMAN ADRENOCORTICAL TUMORS AND REGULATORY PATHWAYS (I–IV) 50
3.1 Human adrenocortical tumors 50
3.2 Signaling cascades in adrenocortical tumors 53

CONCLUSIONS AND FUTURE PERSPECTIVES 55

CLINICAL SIGNIFICANCE 56

PUBLICATIONS NOT INCLUDED IN THIS THESIS 57

ACKNOWLEDGEMENTS 58

REFERENCES 60
Abstract

Transcription factors play a key role in tumor development, in which dysfunction of genes regulating tissue growth and differentiation is a central phenomenon. The GATA family of transcription factors consists of six members that bind to a consensus DNA sequence (A/T)GATA(A/G) in gene promoters and enhancers. The two GATA factors expressed in the adrenal cortex are GATA-4 and GATA-6. In both mice and humans, GATA-4 can be detected only during the fetal period, whereas GATA-6 expression is abundant both throughout development and in the adult. It is already established that GATA factors are important in both normal development and tumorigenesis of several endocrine organs, and expression of GATA-4 and GATA-6 is detected in adrenocortical tumors. The aim of this study was to elucidate the function of these factors in adrenocortical tumor growth.

In embryonal development, the adrenocortical cells arise and differentiate from a common pool with gonadal steroidogenic cells, the urogenital ridge. As the adult adrenal cortex undergoes constant renewal, it is hypothesized that undifferentiated adrenocortical progenitor cells reside adjacent to the adrenal capsule and give rise to daughter cells that differentiate and migrate centripetally. A diverse array of hormones controls the differentiation, growth and survival of steroidogenic cells in the adrenal gland and the gonads. Factors such as luteinizing hormone and inhibins, traditionally associated with gonadal steroidogenic cells, can also influence the function of adrenocortical cells in physiological and pathophysiological states.

Certain inbred strains of mice develop subcapsular adrenocortical tumors in response to gonadectomy. In this study, we found that these tumors express GATA-4, normally absent from the adult adrenal cortex, while GATA-6 expression is downregulated. Gonadal markers such as luteinizing hormone receptor, anti-Müllerian hormone and P450c17 are also expressed in the neoplastic cells, and the tumors produce gonadal hormones. The tumor cells have lost the expression of melanocortin-2 receptor and the CYP enzymes necessary for the synthesis of corticosterone and aldosterone. By way of xenograft studies utilizing NU/J nude mice, we confirmed that chronic gonadotropin elevation is sufficient to induce adrenocortical tumorigenesis in susceptible inbred strains. Collectively, these studies suggest that subcapsular adrenocortical progenitor cells can, under certain conditions, adopt a gonadal fate.

We studied the molecular mechanisms involved in gene regulation in endocrine cells in order to elucidate the role of GATA factors in endocrine tissues. Ovarian granulosa cells express both GATA-4 and GATA-6, and the TGF-β signaling pathway is active in these cells. Inhibin-α is both a target gene for, and an atypical or antagonistic
member of the TGF-β growth factor superfamily. In this study, we show that GATA-4 is required for TGF-β-mediated inhibin-α promoter activation in granulosa cells, and that GATA-4 physically interacts with Smad3, a TGF-β downstream protein.

Apart from the regulation of steroidogenesis and other events in normal tissues, TGF-β signaling is implicated in tumors of multiple organs, including the adrenal cortex. Another signaling pathway found often to be aberrantly active in adrenocortical tumors is the Wnt pathway. As both of these pathways regulate the expression of inhibin-α, a transcriptional target for GATA-4 and GATA-6, we wanted to investigate whether GATA factors are associated with the components of these signaling cascades in human adrenocortical tumors. We found that the expression of Wnt co-receptors LRP5 and LRP6, Smad3, GATA-6 and SF-1 was diminished in adrenocortical carcinomas with poor outcome. All of these factors drive inhibin-α expression, and their expression in adrenocortical tumors correlated with that of inhibin-α. The results support a tumor suppressor role previously suggested for inhibin-α in the mouse adrenal cortex, and offer putative pathways associated with adrenocortical tumor aggressiveness. Unraveling the role of GATA factors and associated molecules in human and mouse adrenocortical tumors could ultimately contribute to the development of diagnostic tools and future therapies for these diseases.
Original Publications


In addition, some unpublished data are presented.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACA</td>
<td>adrenocortical adenoma</td>
</tr>
<tr>
<td>ACC</td>
<td>adrenocortical carcinoma</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>AMH</td>
<td>anti-Müllerian hormone</td>
</tr>
<tr>
<td>CREB</td>
<td>cAMP response element binding protein</td>
</tr>
<tr>
<td>CRH</td>
<td>corticotropin-releasing hormone</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>DAX-1</td>
<td>dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1</td>
</tr>
<tr>
<td>DBA</td>
<td>dilute brown strain of mice</td>
</tr>
<tr>
<td>DHEA</td>
<td>dehydroepiandrosterone</td>
</tr>
<tr>
<td>DN</td>
<td>dominant negative</td>
</tr>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>FOG</td>
<td>friend of GATA</td>
</tr>
<tr>
<td>GnRH</td>
<td>gonadotropin-releasing hormone</td>
</tr>
<tr>
<td>hCG</td>
<td>human chorionic gonadotropin</td>
</tr>
<tr>
<td>HU</td>
<td>Hounsfield unit</td>
</tr>
<tr>
<td>LH</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>LHR</td>
<td>luteinizing hormone receptor</td>
</tr>
<tr>
<td>LRP</td>
<td>low density lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>MAP</td>
<td>mitogen-activated protein</td>
</tr>
<tr>
<td>MC2R</td>
<td>melanocortin-2 receptor</td>
</tr>
<tr>
<td>MEN1</td>
<td>multiple endocrine neoplasia, type 1</td>
</tr>
<tr>
<td>P450c17</td>
<td>cytochrome P450 17α-hydroxylase/17,20-lyase</td>
</tr>
<tr>
<td>PCOS</td>
<td>polycystic ovary syndrome</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PPNAD</td>
<td>primary pigmented nodular adrenocortical disease</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SF-1</td>
<td>steroidogenic factor-1</td>
</tr>
<tr>
<td>sFRP1</td>
<td>secreted frizzled-related protein-1</td>
</tr>
<tr>
<td>StAR</td>
<td>steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>SULT2A1</td>
<td>dehydroepiandrosteronesulfotransferase</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>Wnt</td>
<td>wingless-related mouse mammary tumor virus integration site</td>
</tr>
<tr>
<td>wt</td>
<td>wild type</td>
</tr>
<tr>
<td>WT-1</td>
<td>Wilm’s tumor-1</td>
</tr>
<tr>
<td>zF</td>
<td>zona fasciculata</td>
</tr>
<tr>
<td>zG</td>
<td>zona glomerulosa</td>
</tr>
<tr>
<td>zR</td>
<td>zona reticularis</td>
</tr>
</tbody>
</table>
Introduction

The adrenal gland is a major steroid-secreting organ and part of the hypothalamic-pituitary-adrenal axis. In mammalian embryogenesis, the cells destined to form the adrenal cortex arise from a pool of mesodermal progenitors, termed the urogenital ridge, that also gives rise to the steroidogenic cells of the gonads. The adrenocortical progenitors then associate with neural crest derivatives that will give rise to the adrenal medulla.

Transcription factors are proteins that regulate gene expression in cells by binding to specific DNA sequences – promoters and enhancers of the genes. In mammalian development, differentiation and growth of the organs is regulated by a complex set of genes. Transcription factors also play a key role in tumor development, in which dysfunction of genes regulating tissue growth and differentiation is one of the central phenomena. This study focuses on one group of transcription factors, the GATA factors, and on their significance in tumors of the adrenal cortex.

Of the six GATA factors, GATA-4 and GATA-6 are expressed in the mouse and human adrenal cortex. GATA-4 expression is confined to the fetal cortex, while GATA-6 is expressed in both fetal and adult adrenal glands. In vitro, GATA-4 regulates inhibin-α and steroidogenic factor-1, which are important for normal adrenal function. GATA-6 probably has roles in the regulation of steroidogenesis and in the development and differentiation of adrenocortical cells. Disruption of either the Gata4 or Gata6 gene in mice results in early embryo lethality which, while underscoring their importance in embryonal development, has hampered investigations of these factors in the adrenal cortex and other endocrine tissues.

GATA factors have already been found to be important in both normal development and tumor growth of several endocrine organs. In granulosa cell tumors and adrenocortical carcinoma, high GATA-4 expression has been associated with aggressive behavior. We investigated the function of these factors and their cofactors in granulosa cell signaling and in tumors of the human and mouse adrenal cortex, to elucidate their role in the molecular mechanisms of tumor growth.
Review of the Literature

1 Development and function of the adrenal gland

1.1 Fetal period

In embryonal development, the adrenal cortices are formed from mesoderm-derived cells in the urogenital ridge. The cells of the adrenal medulla migrate from the ectodermal neural crest. In addition to the adrenal cortices, the urogenital ridge gives rise to kidneys and the stromal cells of the gonads. As a result of this common embryonal origin, adult gonads may sometimes contain patches of adrenocortical cells, and in turn, adrenocortical cells are in some circumstances able to adopt gonadal characteristics.

By embryonal day 9.5 in mice, a subset of urogenital cells termed the adrenogonadal primordium can be distinguished by expression of the transcription factor steroidogenic factor-1 (SF-1) (Hanley, et al. 1999; Ikeda, et al. 1994). A separate adrenocortical primordium is distinguishable by embryonal day 11.5 (Hatano, et al. 1996). In addition to SF-1, other early regulators of adrenogonadal development are Wilm’s tumor-1 (WT1), wingless-related mouse mammary tumor virus integration site 4 (Wnt4) and dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1 (DAX-1), also expressed in adrenogonadal primordium. Each of these factors is critical to proper differentiation of adrenocortical and gonadal stromal cells (Bardoni, et al. 1994; Keegan and Hammer 2002; Kreidberg, et al. 1993; Luo, et al. 1994; Vainio, et al. 1999; Yu, et al. 1998) (figure 1).

Early development of the adrenal gland is very similar in humans and mice. By the 4th week of human gestation, the adrenogonadal primordium is evident, and by 8th week of gestation the caudal cells destined to form the adrenal gland have migrated to the cranial end of the mesonephros, forming the adrenocortical primordium (Mesiano and Jaffe 1997). At this stage, the primordium comprises an inner fetal zone and a thin, outer definitive zone. Shortly thereafter the medullary cells migrate from the neural crest into the primordium and the adrenal becomes encapsulated (Mesiano and Jaffe 1997).
Figure 1.
Development of the urogenital ridge derivatives and a simplified model of developmental regulation as studied in genetically engineered mice. Mutations in the key regulatory genes $WT1$, $SF1$ and $Wnt4$, expressed in the urogenital ridge or adrenogonadal primordium, disrupt both adrenal and gonadal development (Keegan and Hammer 2002; Kreidberg et al. 1993; Luo et al. 1994; Vainio et al. 1999). Mutations in melanocortin-2 receptor (MC2R, adrenocorticotropic hormone receptor) or luteinizing hormone receptor (LHR), expressed in cells differentiated to adrenal or gonadal directions, respectively, have effects mostly on postnatal development (Chida, et al. 2007; Lei, et al. 2001; Zhang, et al. 2001).
1.2 Postnatal period

After birth, the adrenal cortex undergoes marked changes. In humans, the fetal zone regresses and the definitive zone expands, forming the zona glomerulosa (zG) and the zona fasciculata (zF) (Mesiano and Jaffe 1997). The adult zonal pattern is established during 10–20 years of age (Sucheston and Cannon 1968); the adult adrenal cortex consists of the outermost zG, the middle zF and the inner zona reticularis (zR). In mouse adrenal cortex, in contrast, zonation is already complete by birth. In addition to the zG and the zF, the postnatal adrenal cortex consists of a layer termed the X-zone, which is derived from the recently recognized mouse fetal zone (Zubair, et al. 2006). The X-zone, which has been suggested to participate in progesterone catabolism (Hershkovitz, et al. 2007), regresses during puberty in males and during the first pregnancy in females. In the mouse adrenal cortex, no zR is seen, and consequently its function also differs from that of humans (table 1).

<table>
<thead>
<tr>
<th>Human</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zona glomerulosa</td>
<td>Zona glomerulosa</td>
</tr>
<tr>
<td>Zona fasciculata</td>
<td>Zona fasciculata</td>
</tr>
<tr>
<td>Zona reticularis</td>
<td>X-zone / fetal zone</td>
</tr>
<tr>
<td>Fetal zone</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.** Comparative anatomy and physiology of human and mouse adrenal cortex. Modified from (Bielinska, et al. 2006). Abbreviation: P450c17, cytochrome P450 17α-hydroxylase/17,20-lyase

The adult adrenal cortex undergoes constant renewal. The adrenocortical cells are believed to be derived from subcapsular stem/progenitor cells and to migrate centripetally, in turn adopting characteristics specific for each zone (Kim and Hammer 2007; Schulte, et al. 2007). The human adrenal cortex secretes various steroid hormones (figure 2), mainly aldosterone (zG), cortisol (zF), and dehydroepiandrosterone (DHEA) and other androgens (zR). The most prominent difference between mouse adrenal steroid production, in contrast to humans and most other rodents, is the lack of cytochrome P450 17α-hydroxylase/17,20-lyase (P450c17) in the adult mouse adrenal cortex (Keeney, et al. 1995). Thus, no
androgens are produced in the mouse adrenal, and the main glucocorticoid is corticosterone instead of cortisol.

Figure 2.
Steroid hormone biosynthetic pathways. The steroidogenic capacity in a given cell depends on the enzymes available. Abbreviations: P450scc, P450 side-chain cleavage; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; DOC, deoxycorticosterone; DHEA, dehydroepiandrosterone; DHEA-S, dehydroepiandrosterone sulfate; P450aldo, aldosterone synthase; P45019a1, aromatase; P450c11, cytochrome P450 11β-hydroxylase; P450c17, cytochrome P450 17α-hydroxylase/17,20-lyase; P450c21, cytochrome P450 21-hydroxylase.

The adrenal cortex functions as part of the hypothalamic-pituitary-adrenal axis (see figure 5 in 2.3.2). The hypothalamus secretes corticotropin-releasing hormone (CRH), which promotes the secretion of adrenocorticotropic hormone (ACTH), the pituitary tropic hormone responsible for the secretion of cortisol and androgens from the adrenal cortex (Mesiano and Jaffe 1997). The adrenocortical corticosteroids in turn inhibit excess CRH production from the hypothalamus, creating a negative feedback loop. The regulation of aldosterone secretion is not dependent on ACTH. The chromaffin cells of the adrenal medulla, derived from neural crest cells, are the source of the stress hormones adrenaline and noradrenaline.
2 GATA family of transcription factors

Transcription factors are regulator proteins critical for cellular diversity. They bind to specific sites on the promoter region of a gene, thus regulating gene expression in a given cell. The GATA family of zinc finger transcription factors is named after a (A/T)GATA(A/G) binding site on a gene’s promoter (Tremblay and Viger 2003a). The family consists of six members, GATA-1 through GATA-6 (table 2). GATA-1, GATA-2 and GATA-3 are primarily associated with hematopoiesis, while GATA-4, GATA-5 and GATA-6 are expressed in various endocrine and other mesoderm-derived tissues. Null mutations of Gata genes, except for Gata5, are embryonic lethal, underscoring their pivotal role in early development.

2.1 Structure

The structure of the GATA factors is based on two zinc fingers binding DNA and cofactors. Situated next to the C-terminal zinc finger is a nuclear localization signal, which together with the zinc fingers constitutes the DNA binding and protein-protein interaction domain, and additional transcriptional activation domains are located N-terminally (Molkentin 2000; Morrisey, et al. 1997). The C-terminal zinc finger and the adjacent nuclear localization signal are conserved through species (Lowry and Atchley 2000). In addition, among the GATA family members, the zinc fingers share considerable homology (figure 3), which could be expected to lead to functional redundancy. However, in vivo the roles of the GATA factors often seem to be relatively distinct and non-redundant, and the presence of many other factors controlling tissue-specific transcription is documented in many tissues (Viger, et al. 2008).
Figure 3.
Structure and homology of the GATA proteins. The light gray area represents the zinc finger (ZnF) domain and the dark gray area represents the nuclear localization sequence. This region binds DNA and is involved in protein interactions with cofactors and other transcriptional partners. The percentage homology in the zinc finger domain is indicated. Abbreviations: ZnF, zinc finger; N, N-terminus; C, C-terminus. Modified from (Viger et al. 2008).

2.2 Tissue-specific expression and knockout phenotypes

GATA factors are expressed in a wide variety of tissues (table 2). Null mutations of the Gata genes, excluding Gata5, each arrest development at an early stage, which has been a challenge for research into the function of GATA factors in adult tissues.

GATA factors can be divided in two subgroups based on their expression pattern, the hematopoietic group and the cardiac group (figure 3 and table 2). GATA-1, GATA-2 and GATA-3 are primarily expressed in hematopoietic cells and are essential to the differentiation and proliferation of the hematopoietic lineage (Weiss and Orkin 1995). GATA-4, GATA-5 and GATA-6 are, in contrast, expressed in a variety of mesoderm- and endoderm-derived tissues, including the heart, gut, gonads and adrenal gland (Molkentin 2000). In particular, the role of GATA-4 and GATA-6 in the heart has been extensively studied (Pikkarainen, et al. 2004). Mice lacking GATA-4 or GATA-6 die in utero by E10.5 as a result of severe defects in development; Gata4 null mice have defects in ventral morphogenesis and heart tube formation, while in Gata6 null mice, endoderm differentiation is blocked (Kuo, et al. 1997; Molkentin, et al. 1997; Morrisey, et al. 1998). The Gata5 null mutation is the only one of the GATA factor
mutations resulting in a viable mouse (Molkentin, et al. 2000). However, a recent report suggests functional redundancy for GATA-4 and GATA-5 in the heart, as double mutant mice, Gata5 null and heterozygous for Gata4, die in utero as a result of cardiovascular defects (Singh, et al. 2010).

The friend of GATA (FOG) proteins FOG-1 and FOG-2 are specific co-regulators of GATA factors. They contain zinc fingers by which they bind and interact with GATA factors, either by enhancing or antagonizing (Cantor and Orkin 2005). Null mutation of Fog1 results in a failure in hematopoiesis, and mice null for Fog2 have severe cardiac defects; both knockout phenotypes are embryonic lethal (Svensson, et al. 2000b; Tevosian, et al. 2000; Tsang, et al. 1998). FOG-1 is expressed in hematopoietic cells, as expected from the null mutation phenotype, and also in other tissues such as testis and gut (Cantor and Orkin 2005; Jacobsen, et al. 2005). FOG-2 is expressed in the heart, where it interacts with GATA-4: if the N-terminal zinc finger of GATA-4 is mutated to prohibit the interaction, a mouse phenotype similar to the Fog2 null mouse develops (Crispino et al., 2001). This underlines the importance of GATA:FOG interactions. FOG-2 is also expressed in the gonads (Viger et al. 2008), and in addition to heart defects, gonadal dysgenesis has been detected in a human mutation of the Fog2 gene (Finelli, et al. 2007).
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA-5</td>
<td>Heart, genitourinary tract</td>
<td>Female genitourinary abnormalities (Molkentin et al. 2000)</td>
<td>Not detected</td>
</tr>
<tr>
<td>GATA-6</td>
<td>Adrenal gland, gonads, heart</td>
<td>Lethal (E7.5), failure in gastrulation (Morrisey et al. 1998)</td>
<td>Not detected</td>
</tr>
<tr>
<td>FOG-1</td>
<td>Hematopoietic lineage, testis</td>
<td>Lethal (E11.5), failure in hematopoiesis (Tsang et al. 1998)</td>
<td>Not detected (see GATA-1)</td>
</tr>
<tr>
<td>FOG-2</td>
<td>Gonads, heart, brain</td>
<td>Lethal (E12.5), defects in heart development (Svensson et al. 2000b; Tevosian et al. 2000)</td>
<td>Congenital heart defects and gonadal dysgenesis (loss-of-function mutation) (Finelli et al. 2007)</td>
</tr>
</tbody>
</table>

**Table 2.** GATA and FOG factor expression, knockout phenotypes and mutations detected in humans. Abbreviations: FOG, friend of GATA; CNS, central nervous system; HDR, hypoparathyroidism-deafness-renal dysplasia
2.3 GATA factors in the derivatives of adrenogonadal primordium

Within the Gata family, GATA-4 and GATA-6 are specifically involved in development and differentiation of the steroidogenic tissues of the adrenals and gonads. The adrenal cortex in both humans and mice expresses GATA-4 and GATA-6. GATA-6 is abundantly expressed throughout development and adulthood (Kiiveri, et al. 2002; Nemer and Nemer 2003). During human fetal development, GATA-6 expression is seen both in the fetal and definitive zones. In adults, GATA-6 is expressed in the zR and the zF. In mice, GATA-6 is also expressed both in the fetal and adult adrenal cortex. GATA-4, in contrast, is expressed only in fetal adrenals, both in humans and mice (Kiiveri et al. 2002). The Gata4 expression pattern in the fetal mouse adrenal resembles that of P450c17 (Keeney et al. 1995), and although there is no direct proof, it can be speculated that GATA-4 enhances expression of P450c17 in the fetal adrenal.

GATA-4 does not seem to be essential for early adrenocortical development, as studied in mice chimeric for Gata4-/- and wild-type cells (Kiiveri et al. 2002; Tevosian, et al. 2002), but it is a cofactor for the developmentally critical protein SF-1 (Tremblay and Viger 1999). In vitro, it also upregulates the expression of several proteins involved in the function of the adrenal gland, such as inhibin-α, P450c17, and steroidogenic acute regulatory protein (StAR) (Tremblay and Viger 2003a). GATA-6 also regulates several steroidogenic enzymes in transactivation experiments. By acting in synergy with SF-1, it upregulates the expression of P450c17, P450scC, StAR and dehydroepiandrosteronesulfotransferase (SULT2A1), which are proteins necessary for adrenal steroid biosynthesis (Jimenez, et al. 2003; Tremblay and Viger 2003a).

Although the gonads share the same developmental origin with the adrenals, the GATA factor expression profile is different. As early as in the urogenital ridge, GATA-4 is expressed in the cells destined to form gonadal somatic cells, and the expression persists throughout development. In adults, GATA-4 is present in several gonadal cell lineages, including male Leydig cells and female granulosa and theca cells (Viger et al. 2008). GATA-6 is also expressed in gonadal somatic tissue during development and adulthood (Heikinheimo, et al. 1994; Ketola, et al. 1999). Several of the GATA target genes are shared between the adrenals and gonads, including SF-1, StAR and inhibin-α. Specifically gonadal regulators are the GATA-4 target genes anti-Müllerian hormone and aromatase. GATA-6 has also been proposed to transactivate several of these regulators of gonadal development and function (Tremblay and Viger 2003a).

GATA-6 protein expression has been found to be significantly diminished in most human adrenocortical adenomas and carcinomas. However, supporting its proposed
3 Adrenocortical tumors

3.1 Human adrenocortical tumors

Incidentalomas are adrenal masses found by incident when a radiologic examination, e.g. computed tomography (CT) of the abdomen, is performed for an indication other than evaluation of the adrenals. As ultrasonography, CT and magnetic resonance imaging are increasingly being utilized in medical diagnostics, the occurrence of incidentalomas has increased: the probability of finding an adrenal mass in an abdominal CT examination is now about 7% in a patient over 70 years of age (Kloos, et al. 1995; Young 2007).

The majority of incidentalomas are benign adrenocortical adenomas. The true incidence is unknown, but according to autopsy studies, adrenocortical adenomas affect approximately 5% of the population (Brunt and Moley 2001). Most often they do not require treatment, being clinically nonhypersecreting and asymptomatic. If, however, the tumor is functional, i.e. hormone-secreting, or if its radiological features are suspicious as regards malignancy, it is surgically removed (Bertherat, et al. 2002). The CT imaging features that associate with adenomas are small size (< 3 cm diameter), sharp margins and low density (< 10 Hounsfield units (HU)) (Brant and Helms 2006). Approximately 30% of adenomas have a low fat content, resulting in a density of 10–30 HU. The washout of intravenously administered contrast material is measured in these cases; benign lesions typically demonstrate more than 50% washout (Brant and Helms 2006).

The hormones secreted by adrenocortical adenomas are usually the same as those that are produced by the normally functioning cortex. Hormonally active tumors are divided according to the major hormone secreted: Cushing’s (glucocorticoids) which are the most common, Conn’s (aldosterone), and the rare virilizing (androgens) or feminizing (estrogen) adenomas (Bertherat et al. 2002; DeLellis, et al. 2004). Most of the androgen- and estrogen-secreting adrenocortical tumors are carcinomas.

Other than an adrenocortical adenoma, an incidentaloma can be a pheochromocytoma originating from adrenal medullary cells, a metastasis, or primary adrenocortical carcinoma, among other things. Adrenocortical carcinoma is a rare disease, with an incidence of approximately 1 case per million people per year, but its prognosis is poor and it often presents with metastases (Barlaskar and Hammer 2007; Volante, et al. 2008). About 80% of these carcinomas are functional, most commonly secreting glucocorticoids (45%), glucocorticoids plus androgens (45%), or
androgens (10%). Primarily aldosterone-secreting adrenocortical carcinomas are very rare, representing less than 1% of all the cases.

The histopathological grading of adrenocortical tumors is often carried out according to the criteria of Weiss (DeLellis et al. 2004; Weiss 1984; Weiss, et al. 1989) (table 3). Of the 9 criteria, the presence of three or more highly correlates with subsequent malignant behavior. Generally, scores 0–2 are considered clearly benign and 4–9 clearly malignant; score 3 has been called the “grey zone” (Tissier 2010), as although originally considered malignant by Weiss (Weiss et al. 1989), there have been several reports of score-3 tumors that did not recur (Tissier 2010). On the other hand, that is not proof that these tumors were indeed benign. Weiss score-3 tumors could be described as having malignant potential. Other systems for distinguishing between benign and malignant adrenocortical tumors have also been developed, including nonhistologic criteria such as tumor mass and clinical features (Hough, et al. 1979; van Slooten, et al. 1985). A modification of the Weiss system, adding more weight to the mitotic rate and the cytoplasmic characteristics, has been proposed (Aubert, et al. 2002), and some investigators believe that the criteria for malignancy differ for pediatric tumors (Ribeiro, et al. 1990).

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>High nuclear grade; Fuhrman criteria</td>
</tr>
<tr>
<td>2.</td>
<td>&gt;5 mitoses per 50 high-power fields</td>
</tr>
<tr>
<td>3.</td>
<td>Atypical mitotic figures</td>
</tr>
<tr>
<td>4.</td>
<td>&lt;25% of tumor cells are clear cells</td>
</tr>
<tr>
<td>5.</td>
<td>Diffuse architecture (&gt;33% of tumor)</td>
</tr>
<tr>
<td>6.</td>
<td>Necrosis</td>
</tr>
<tr>
<td>7.</td>
<td>Venous invasion</td>
</tr>
<tr>
<td>8.</td>
<td>Sinusoidal invasion</td>
</tr>
<tr>
<td>9.</td>
<td>Capsular invasion</td>
</tr>
</tbody>
</table>

**Table 3.** Histopathologic criteria proposed by Weiss (Weiss 1984; Weiss et al. 1989) for distinguishing benign from malignant adrenocortical neoplasms. The presence of three or more criteria highly correlates with subsequent malignant behavior.

It has been reported that postmenopausal women (Fidler 1977), and men with acquired testicular atrophy (Romberger and Wong 1989) develop subcapsular adrenocortical neoplasms that histologically resemble ovarian tissue. It has been
speculated that elevated luteinizing hormone (LH) levels or decreased sex steroid levels might account for the development of these tumors (Bielinska et al. 2006). Interestingly, a similar phenomenon has been observed in mice and will be discussed in the next chapter.

### 3.2 Mouse models

The close developmental relationship between the adrenal gland and the gonads can become manifest in pathological conditions. In certain instances, factors traditionally associated with gonadal steroidogenesis may influence the differentiation, proliferation and function of adrenocortical progenitor cells.

As early as in the 1940s it was observed that after gonadectomy, mice develop subcapsular neoplasia in their adrenals (Fekete, et al. 1941). However, this phenomenon is evident only in certain inbred mouse strains, such as DBA/2J, C3H and CE/J, of which the latter develops carcinomas, while other strains such as C57BL/6J and FVB/N are resistant to gonadectomy-induced neoplasia (Bielinska, et al. 2009). The susceptibility was later linked to a polymorphism in the SF-1 gene (Frigeri, et al. 2002). Additionally, linkage analysis of crosses between a susceptible and a non-susceptible strain (DBA/2J and C57BL/6J) showed that tumorigenesis following gonadectomy is a dominant trait and that a major locus for tumorigenesis is on chromosome 8 (Bernichtein, et al. 2008). One of the candidate genes in this region is proposed to be that for secreted frizzled-related protein-1 (sFRP1), which is a tumor suppressor inhibiting the Wnt/β-catenin signaling pathway (Bernichtein et al. 2008).

The adrenocortical neoplasms that develop in susceptible strains arise subcapsularly and invade the deeper layers of the cortex, displacing normal adrenocortical tissue. Two different populations of cells make up the neoplasms: small, basophilic cells termed A cells and large, lipid-laden cells termed B cells (Fekete et al. 1941; Woolley, et al. 1952) (figure 4).
Genetically modified mouse models of adrenocortical tumors have also been developed. Mice lacking the inhibin-α gene (Inha -/-) develop gonadal tumors but, when gonadectomized, adrenocortical tumors with gonadal characteristics arise (Matzuk, et al. 1994; Matzuk, et al. 1992). Transgenic mice expressing simian virus T-antigen driven by the inhibin-α promoter (Inha/Tag) similarly initially develop gonadal tumors and, post-gonadectomy, adrenocortical tumors (Kananen, et al. 1996; Kananen, et al. 1995). In the adrenals of gonadectomized Inha/Tag mice, type A- and type B-like cells can be observed near the adrenal capsule (Rahman, et al. 2004), although the actual tumors are situated at a juxtamedullar position in the X zone. It has been proposed that the origin of these tumors could also be the subcapsular progenitor cells (Looyenga and Hammer 2006).

Chronic elevation of circulating gonadotropin concentrations is a prerequisite for tumor formation in all of these mouse models, as studied by either surgical, pharmacological or mutational abrogation of the gonadotropin effects (Huseby and Bittner 1951; Kumar, et al. 1995; Rahman, et al. 1998; Rilianawati, et al. 1998). LH is a dimeric glycoprotein hormone, secreted in a pulsatile manner from the pituitary in response to a surge of gonadotropin-releasing hormone (GnRH). LH receptors
(LHRs), present on gonadal steroidogenic cells and, at a very low level, in normal human adrenal cortex (Couzinet, et al. 2001; Pabon, et al. 1996), bind the LH protein on the cell surface. Via several signaling pathways, including the cAMP/protein kinase A (PKA) pathway, the mitogen-activated protein (MAP) kinase pathway and the phosphatidylinositol-3-kinase pathway, LHRs transmit effects on cell growth, differentiation and steroid production by activation of transcription factors inducing expression of steroidogenic enzyme genes (Huhtaniemi, et al. 2002; Tremblay and Viger 2003a). Under normal conditions, the sex steroids secreted signal back to the hypothalamus, inhibiting GnRH production; a similar negative feedback loop as in the hypothalamic-pituitary-adrenal axis. After gonadectomy, however, the negative feedback is absent, allowing continuously elevated levels of LH and other gonadotropins. Figure 5 summarizes the hypothalamic-pituitary-adrenal/gonadal axes in intact and gonadectomized mice.

**Figure 5.**
The hypothalamic-pituitary-adrenal/gonadal axis in intact (left) and gonadectomized (right) mice. In intact mice, the steroids produced by the adrenals and gonads send negative feedback to the hypothalamus and the pituitary, while in gonadectomized mice the negative feedback from the gonads is absent. Abbreviations: CRH, corticotropin-releasing hormone; GnRH, gonadotropin-releasing hormone; ACTH, adrenocorticotropic hormone; LH, luteinizing hormone. Modified from (Bielinska et al. 2006).
4 Molecular pathophysiology of adrenocortical tumorigenesis

Signaling pathways involved in normal embryonal development are often aberrantly active in neoplastic processes. Adrenocortical tumors are no exception, as many of the growth and differentiation factors important for adrenal development are implicated in tumors of the adrenal cortex.

4.1 Subcapsular stem/progenitor cells

The adrenal cortex constantly renews itself by turnover of the steroidogenic cells. It is theorized that multipotential stem/progenitor cells reside in the subcapsular region of the cortex and give rise to daughter cells that differentiate, migrate centripetally, and in turn adopt the characteristics of each functional zone (Bielinska et al. 2006; Kim and Hammer 2007; Schulte et al. 2007). The molecular characteristics of the progenitor cells that are derived from the quiescent subcapsular stem cells include expression of SF-1 and DAX-1 (figure 6). DAX-1 acts as a repressor of steroidogenic gene expression, which limits the steroidogenic capacity of the subcapsular progenitors. In response to ACTH, Dax1 is downregulated and the cells differentiate into corticoid-producing cells that express GATA-6. The binding of ACTH to its G-protein-coupled receptor, melanocortin-2 receptor (MC2R), drives differentiation of adrenocortical progenitors into corticoid-producing cells. It is hypothesized that the subcapsular stem/progenitor cells are principal targets of neoplastic transformation in the adrenal cortex (Bielinska et al. 2006; Doghman, et al. 2007; Kim and Hammer 2007).
Figure 6.
Differentiation of multipotential subcapsular adrenocortical progenitor cells into corticoid-producing cells. Dax-1 is downregulated in response to ACTH, and the cells differentiate into MC2R- and GATA-6-expressing steroidogenic cells.

4.2 Wnt/β-catenin signaling

The Wnt signaling pathway regulates many developmental processes, such as cell-fate determination, cell proliferation and adhesion (Prunier, et al. 2004). Molecules of the Wnt family bind to cell surface frizzled receptors, which bind the low density lipoprotein receptor-related protein (LRP) co-receptors and initiate a signaling cascade. The canonical Wnt signal is transmitted via the cytoplasmic mediator β-catenin, which is translocated to the nucleus and can there enhance transcription of a specific gene, together with cofactors such as SF-1 on inhibin-α activation in steroidogenic cells (Gummow, et al. 2003). Wnts can also act through non-canonical, β-catenin independent pathways, the jun N-terminal kinase pathway and the calcium pathway (Rao and Kuhl 2010). In the mouse adrenal cortex, Wnt4 is expressed in the zG and, as shown in knockout studies, contributes to its function (Heikkila, et al. 2002). Wnt4 null mice have decreased expression of P450aldo in the adrenal cortex and in contrast, ectopic expression of adrenocortical P450c21 in gonadal tissue (Heikkila et al. 2002; Jeays-Ward, et al. 2004). Wnt4 may thus play a role in cell migration or in the sorting of adrenocortical and gonadal cells in early development. Another member of the Wnt family expressed in the adrenal cortex is Wnt2b, which is found in the subcapsular progenitor cells (Lin, et al. 2001).
4.3 TGF-β/Smad signaling

Transforming growth factor beta (TGF-β) signaling is mediated through a receptor complex comprising type I and type II serine/threonine kinase receptors. The ligand binds to the type II receptor, which activates the complex and transmits the signal by phosphorylating the intracellular Smad proteins. These ubiquitous proteins then become translocated to the nucleus and interact with cell-specific transcription factors and other regulators to drive expression of target genes (Shi and Massague 2003) (figure 7). The cell-specific cofactors are essential for the functional diversity of TGF-β signaling, as only two types of Smad responses are known: the bone morphogenetic protein subfamily activates Smad1, Smad5 and Smad8, whereas the TGF-β subfamily activates Smad2 and Smad3. Other Smad proteins participating in signal transmission are Smad4, a coactivator, and co-inhibitors Smad6 and Smad7.

Activin and inhibin are dimeric glycoproteins that belong to the TGF-β superfamily. They function as endocrine, paracrine and autocrine regulators of pituitary and gonadal function, and also influence the adrenal gland (Beuschlein, et al. 2004; Drummond, et al. 2004; Risbridger, et al. 2001). The inhibin-activin system involves three genes, Inha, Inhba and Inhbb, that respectively encode monomeric protein subunits α, βA and βB. These subunits form homo- and heterodimers: inhibins, in which the α-subunit is present (inhibin A and inhibin B), and activins, which are formed by the β-subunits alone (activin A, activin B, or activin AB).

There is no known specific receptor for inhibins, so they are considered to antagonize activin by preventing its binding to the TGF-β receptor (Stenvers and Findlay 2010). Activin receptors are expressed in mouse adrenal gland, and activins have been shown to inhibit adrenocortical cell growth and enhance apoptosis in the X zone in mice (Beuschlein, et al. 2003). Although the phenotype of Inha -/- mice suggests that the α subunit of inhibin is a tumor suppressor, the absence of inhibin-α is not required for tumor formation. In contrast, strong inhibin-α expression has been detected in at least ferret and human adrenocortical tumors (Arola, et al. 2000; Peterson, et al. 2004; Vanttinen, et al. 2003).

4.4 cAMP signaling

Activation of G-protein-coupled receptors on the surface of endocrine cells has various effects on cell growth, differentiation and steroid production. The ACTH receptor MC2R and the LH receptor are examples of G-protein-coupled receptors, mediating their signals in part through the well characterized cAMP/PKA pathway. The cAMP response element binding protein (CREB) translocates to the nucleus to enhance the expression of steroidogenic enzyme genes. Because many of the
cAMP-responsive endocrine genes lack cAMP response element (CRE) sites, the response requires other (cell-specific) transcription factors such as GATA-4, GATA-6 and SF-1 (Parker and Schimmer 1997; Tremblay and Viger 2003a). Dysregulation of cAMP/PKA signaling is manifest in many of the human mutations associated with adrenocortical neoplasia.

Figure 7. A simplified schematic illustration of the canonical Wnt/β-catenin signaling pathway, the TGF-β/Smad pathway and the cAMP/PKA pathway from the cell surface to the nucleus. Signals are transmitted via intracellular mediators to the nucleus, where the mediator proteins bind to gene promoter regions together with cell-specific transcription factors. As a result, transcription of a given gene, such as inhibin-α, is enhanced or inhibited. See text for details.

4.5 Molecular mechanisms in human adrenocortical tumors

The pathogenesis of adrenocortical tumors in humans remains mostly unresolved. Certain genetic conditions predispose individuals to adrenocortical tumors (table 4),
and although each of them is rare, they have given valuable insight into the mechanisms of adrenocortical neoplastic growth.

*Carney complex* is a multiple neoplasia syndrome featuring a variety of mucocutaneous pigmented lesions and cardiac, cutaneous, neural and endocrine tumors (Carney, et al. 1986). A mutation in the *PRKAR1A* gene causes increased PKA activity by affecting its regulatory subunit (Kirschner, et al. 2000), and its adrenocortical manifestation is primary pigmented nodular adrenocortical disease (PPNAD). Another syndrome in which the cAMP/PKA pathway is dysregulated is *McCune-Albright syndrome*. It is a pediatric sporadic syndrome defined by the triad of polyostotic fibrous dysplasia, café-au-lait skin lesions and sexual precocity. Patients may develop nodular hyperplasia or adenomas of the adrenal cortex, and hypersecretion of hormones may also occur. McCune-Albright syndrome is caused by activating mutations on Gsα, a heterotrimeric G protein α-subunit (Lumbroso, et al. 2002). *Li-Fraumeni syndrome* is caused by an inactivating germline mutation in tumor suppressor TP53 and it leads to familial susceptibility to cancer (Malkin 1993; Malkin, et al. 1990). Affected individuals may also develop adrenocortical carcinoma, although rarely. *Multiple endocrine neoplasia, type 1* (MEN1) is an autosomal dominant syndrome in which the *MEN1* tumor suppressor gene is heterozygously mutated. MEN1 is characterized by predisposition to pituitary, parathyroid, endocrine pancreas, and adrenocortical tumors (Lemos and Thakker 2008). The adrenocortical lesions are usually adenomas. Individuals with *Beckwith-Wiedemann syndrome*, linked to the 11p15 chromosomal locus which includes the growth regulatory gene *P57kip2*, IGF2 and H19, exhibit macrosomia, visceromegaly and various tumors, including adrenocortical tumors (Hatada and Mukai 2000; Hatada, et al. 1996; Ping, et al. 1989).

The molecular bases of sporadic adrenocortical tumors are somewhat similar to the mutations in the above syndromes. Mutation in the *TP53* gene is also implicated in sporadic adrenocortical cancer, and it is overall the most frequently mutated gene in human cancers (Hollstein, et al. 1991). Mutations in *TP53* conserved exons 5–8 have been found in 20–27% of adrenocortical carcinomas and a small proportion of sporadic adrenocortical adenomas (Ohgaki, et al. 1993; Reincke, et al. 1994). Insulin-like growth factor (IGF) system mutations at the 11p15 locus, such as the *P57kip2* mutation in Beckwith-Wiedemann syndrome, are documented in sporadic adrenocortical carcinoma (Soon, et al. 2008). Gsα has been reported as being occasionally mutated in sporadic adenomas, but not carcinomas (Fragoso, et al. 2003; Yoshimoto, et al. 1993).
<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Gene (locus)</th>
<th>Mutation</th>
<th>Manifestation of tumor syndrome</th>
<th>Prevalence of ACTs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Li-Fraumeni syndrome</td>
<td>TP53, hCHK2, 1q23</td>
<td>Inactivating mutations in tumor suppressor TP53</td>
<td>Soft tissue sarcoma, osteosarcoma, breast cancer, brain tumor, leukemia, ACC</td>
<td>ACC, 3–4%</td>
</tr>
<tr>
<td>Carney complex</td>
<td>PRKAR1A, 1q24</td>
<td>Increased PKA activity caused by mutations in its regulatory subunit</td>
<td>Cardiac, endocrine, cutaneous, and neural myxomatous tumors, and pigmented lesions of the skin and mucosa</td>
<td>PPNAD, 90–100%</td>
</tr>
<tr>
<td>McCune-Albright syndrome</td>
<td>GNAS, 20q13.2</td>
<td>Activating mutations in a G protein subunit Gsα</td>
<td>Sporadic pediatric genetic disease: polyostotic fibrous dysplasia, café-au-lait spots, and endocrine dysfunction</td>
<td>ACA, rare</td>
</tr>
<tr>
<td>Beckwith-Wiedemann syndrome</td>
<td>IGF2, H19, CDKN1C, KCNQ1</td>
<td>Mutations in the 11p15 locus</td>
<td>Exomphalos, macroglossia, gigantism, ACC, nephroblastoma, hepatoblastoma, rhabdomyosarcoma</td>
<td>ACC, 5%</td>
</tr>
<tr>
<td>Multiple endocrine neoplasia 1</td>
<td>MEN1, 11q13</td>
<td>Heterozygous inactivating mutation in tumor suppressor MEN1</td>
<td>Parathyroid, pancreatic islet cell, anterior pituitary and adrenocortical tumors</td>
<td>ACA, 55%; ACC, rare</td>
</tr>
</tbody>
</table>

Table 4. Syndromes that can be associated with adrenocortical neoplasia. Modified from (Soon et al. 2008). Abbreviations: MEN1, multiple endocrine neoplasia-1; PKA, protein kinase A; ACA, adrenocortical adenoma; ACC, adrenocortical carcinoma; PPNAD, primary pigmented nodular adrenocortical disease.
**Aims of the Study**

This study was undertaken to elucidate the function and mechanisms of GATA factors in tumors of the adrenal cortex and in granulosa cells.

The specific aims of the present study were:

1. To investigate the cooperation of GATA-4 with TGF-β signaling in regulation of the inhibin-α gene in granulosa cells.

2. To investigate the expression of GATA-4, GATA-6, LHR and steroidogenic markers in the gonadectomy-induced adrenocortical tumors of certain inbred strains of mice, and to assess the relative importance of the lack of gonadal hormones vs. the increase in gonadotropin levels in relation to the neoplastic phenomenon.

3. To study the expression of components of the TGF-β and Wnt signaling cascades, compared with GATA-4/6, SF-1 and inhibin-α in human adrenocortical tumors.
Materials and Methods

1 Patients and clinical data (I, IV)

The Helsinki University Central Hospital Ethics Committee approved the use of human tissue samples and collection of clinical data from the patient registry, the Finnish Population Registry and Statistics Finland. The ovarian tissue samples were from women under 35 years of age operated upon because of cervical cancer, and the adrenocortical samples were from patients operated upon because of adrenocortical tumors.

2 Experimental animals, serum samples and treatments (II, III)

All animal work was carried out in the USA, in accordance with NIH Guidelines for the Care and Use of Laboratory Animals, and was approved by the institutional animal studies committee.

Weanling FVB/N mice were obtained from the Department of Pediatrics, CHRC Genetically Altered Mouse Core. Weanling DBA/2J mice, C57BL/6J mice and mice homozygous for the Foxn1nu mutation in an NU/J background were purchased from The Jackson Laboratory (Bar Harbor, ME). At 3–4 wk of age, the mice were anesthetized and subjected to gonadectomy or sham surgery. Immediately after operations, nude NU/J mice were injected sc in each flank with 0.2 ml of hCG-CHO cells (see below) or control cell suspension.

At specified times the mice were killed by CO₂ inhalation, blood samples were collected and adrenals were harvested. Serum LH concentrations were measured using an immunofluorometric assay (Wallac Oy, Turku, Finland), as described previously (Haavisto, et al. 1993). Serum levels of hCG and testosterone were measured using commercial assay kits (Diagnostic Products Corp., Los Angeles, CA). Serum estradiol levels were determined by RIA (Spectria, Orion Diagnostica, Espoo, Finland). Corticosterone levels were also measured by RIA (IDS, Boldon, UK).

3 Tissue samples

3.1 Mouse (II, III)

Harvested tissue was fixed overnight in 4% paraformaldehyde, embedded in paraffin, and sectioned for histochemical staining, immunohistochemistry or in situ hybridization. Alternatively, isolated adrenals were frozen in OCT compound (Tissue-Tek, Torrance, CA) and sectioned.
Materials and Methods

3.2 Human (I, IV)
Tissue samples were fixed overnight in formalin or Bouin’s fixative, embedded in paraffin, and sectioned for histochemical staining or immunohistochemistry. In some instances, small amounts of adrenocortical tumor tissue were additionally frozen for RNA analysis.

4 Cell cultures and transient transfections (I, III)
Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂. COS-7 cells were cultured in DMEM supplemented with fetal bovine serum (FBS; 10%), penicillin (100 U/ml) and streptomycin (100 U/ml). KK-1 cells were originally established from an ovarian tumor of an Inha/Tag transgenic mouse and cultured in DMEM supplemented with glucose (4.5 g/l), L-glutamine (0.365 g/l), 20 mM Hepes, penicillin (100 U/ml) and streptomycin (100 U/ml). Chinese hamster ovary (CHO) cells stably expressing a biologically active single chain hCG variant (hCG-CHO cells) were prepared as described elsewhere (Ben-Menahem, et al. 2001; Sugahara, et al. 1995). hCG-CHO cells and control CHO cells were maintained in Ham’s F-12 medium supplemented with fetal bovine serum (5%), G418 antibiotic solution (125 µg/ml), penicillin (100 µg/ml) and streptomycin (100 µg/ml) and glutamine (2 mM).

In cases of stimulation with TGF-β, the cells were treated with 0, 10, 20 or 50 ng TGF-β₁/ml in FBS. COS-7 cells were transfected with FuGene6 (Roche) and KK-1 cells with Lipofectamine 2000 (Invitrogen) according to the manufacturers’ instructions. The expression of all transfected plasmids was verified by Western blotting. In all experiments, pCMVβ-galactosidase (Clontech) was cotransfected to monitor transfection efficiency. When protein-expressing plasmids were transfected, an equal amount of corresponding empty plasmids was transfected to the other wells. Cells were lysed after transfection, and a luciferase assay normalized to β-galactosidase activity was performed as previously described (Martelin, et al. 2000).

The dominant negative GATA-4 mutant plasmid pMT2-GATA-4-DN2 (DN2) was generated by mutating the carboxy-terminal zinc-finger in the sequence coding for GATA-4 protein, using a Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The following primers were designed to change cysteines into serines in both positions of the zinc-finger: wild-type nucleotides 1437–1466, 5’-GAGCCTGTATGTAATGCCTGCCTAC-3’; mutant nucleotides 1437–1466, 5’GAGCCTGTATCTAATGCCTGCCTAC-3’. The changes were confirmed by sequencing, and the integrity of the mRNAs and proteins was confirmed by Northern and Western blotting respectively, as described by (Arceci, et al. 1993). The other plasmids employed have been previously described: pMT2-GATA-4 (wild-type GATA-4) and pMT2-GATA-4-DN1 (DN1) (Arceci et al. 1993), pGL2 –679 bp-inhibin-α–luciferase (Tremblay and Viger 2001), Flag-pcDEF3-Smad2 and Flag-pcDEF3-
MATERIALS AND METHODS

Smad4 (Kawabata, et al. 1998), FlagpcDNA3-Smad3 (Nakao, et al. 1997), and pMT2-FOG-1 (Tsang et al. 1997) and pCS2+-FOG-2 (Tevosian, et al. 1999). In the wild-type and mutant –180 bp-inhibin-α–luciferase plasmids, we replaced the original pTKGH reporter vector (Ketola et al. 1999) with pGL2 by subcloning to the HindIII sites and sequencing to verify orientation.

5 mRNA expression

5.1 In situ hybridization (II, III)
Frozen or paraffin-embedded tissue sections were subjected to in situ hybridization. The sections were deparaffinized, permeabilized, rehydrated, and then incubated in a final volume of 80 µl with 106 cpm 33P-labeled antisense riboprobe, prepared using [33P]UTP (3000 Ci/mmol; ICN Biomedicals) and the linearized plasmid templates described before: GATA-4 (Narita et al. 1997); GATA-6 (Narita et al. 1997); 11β-hydroxylase (Domalik, et al. 1991); P450 aldosterone synthase (Domalik et al. 1991); inhibin-α (Esch, et al. 1987); StAR (Caron, et al. 1997); LHR (Kero, et al. 2000); SF-1 (Sadovsky, et al. 1995); MC2R (Beuschlein et al. 2003); steroid 21-hydroxylase (Milstone, et al. 1992); and anti-Müllerian hormone (AMH) (Mishina, et al. 1996).
Partial-length cDNAs for mouse P450scc and P450c17 were generated via RT-PCR of mRNA isolated from the Leydig cell tumor line mLTC-1 (Rebois 1982). Previously described primers (Arensburg, et al. 1999) were used to generate a PCR product spanning nucleotides 110–455 of mouse P450scc. A forward primer of 5'-CCAGATGGTGACTCTAGGCCTCTTGTC and a reverse primer of 5'-GGTCTATGGTAGTCAGTATGC were used to generate a PCR product spanning nucleotides 331–631 of mouse P450c17 (Youngblood, et al. 1991). PCR reaction mixtures were processed for 30 cycles. The resultant PCR products were ligated into the T-A cloning site of pCRII (Invitrogen, Carlsbad, CA).

5.2 Nuclear extracts and electrophoretic mobility shift assays (EMSAs) (I)
To prepare nuclear extracts, cells were harvested in ice-cold PBS. After lysis in a buffer containing protease inhibitor cocktail P8340 (Sigma-Aldrich), 10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl and 1 mM DTT, the cells were left on ice for 15 min and then vortexed for 10 s. After centrifugation for 5 s at 14 000 rpm, proteins from the nuclear pellet were extracted for 20 min on ice in a buffer containing protease inhibitor cocktail, 20 mM HEPES, 25% (vol./vol.) glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 420 mM NaCl and 1 mM DTT. Nuclear debris was removed by centrifugation for 5 min at 4°C at 14 000 rpm. Protein concentrations were measured using Bio-Rad DC protein assay (Bio-Rad Laboratories). Oligonucleotide probes for the desired area were end-labeled with γ-32P-ATP, using T4 polynucleotide kinase, and annealed
to dimers. In binding reactions, 10 µg of nuclear protein were incubated with 10 000–30 000 c.p.m. of the labeled probe for 5 min on ice in 10 mM Hepes (pH 7.8), 1 mM EDTA, 5 mM MgCl₂, 5% glycerol, 1 mM DTT and 1 µg poly-dIdC. The total reaction volume was 20 µl. The samples were loaded on a 5% acrylamide gel in 0.5x TBE, run at 200 V for 1.75–2 h at room temperature, and visualized by autoradiography.

5.3 Reverse-transcriptase PCR (RT-PCR) (III, IV)
Total RNA was isolated from frozen tissues by ultracentrifugation through a cesium chloride gradient; purified RNA (1 µg) was reverse-transcribed and 2 µl of the reaction mixture was used for each PCR reaction. Agarose gel electrophoresis (2%) demonstrated a band of expected size for each of the primer pairs. The following primers were used: β-catenin, 5'-GTTCGTGACATGATGAC-3' and 5'-CGATAGCTAGGATCATCCTG -3'; LRP5, 5'-GCAAGAAGCTGTACTGGACG-3' and 5'-TGTTGACAGGATGATGAG-3'; LRP6, 5'-GATTATCCAGAGACATGCGAG-3' and 5'-TCCATGACCACCTTCTCA-3'; Smad2, 5'-GCTCCGTGGCATGACAC-3' and 5'-TCTCTTGGCCAGGATGCTT-3'; Smad3, 5'-TGCTGTTGACTGGATGAC-3' and 5'-CTCCTGGGAAGGTGCTT-3'; aromatase, 5'-ACAGCATTGTAGTCCCTCT-3' and 5'-CATCTTTGCTATTGTCCTC-3'; hypoxanthine-guanine phosphoribosyl transferase (HPRT), 5'-GTGGTGAAAGGACTT-3' and 5'-CACAGGACTAGACCTCTG-3'.

5.4 RNase protection (II,III)
The assays were performed with a commercially available kit (Ambion, Austin, TX) and 5 µg total RNA. 32P-labeled antisense riboprobes were prepared by in vitro transcription in the presence of [32P]CTP (800 Ci/mmol; ICN Biomedicals, Irvine, CA) using linearized plasmid templates and RNA polymerases. Protected fragments were separated by PAGE and quantified using phosphorimage analysis, normalizing to the expression of β-actin. Plasmid templates have been described previously: GATA-4 (Narita et al. 1997); GATA-6 (Narita et al. 1997); and LHR (Kero et al. 2000). Preparation of the P450c17 template is described above (5.1).

6 Protein expression
6.1 Immunohistochemistry (I–IV)
Paraffin-embedded tissue sections were deparaffinized and rehydrated. They were treated by boiling with citric acid (10 mM) and in some instances were also treated with H₂O₂ to quench endogenous peroxidase. After incubation with blocking buffer, the sections were incubated with primary antibodies (see table 5) for 1 h at 37 °C. Detection was performed using Vectastain ABC kits (Vector Laboratories, Burlingame, CA) and diaminobenzidine stain (Sigma-Aldrich Co., St. Louis, MO)
according to the manufacturers' instructions. The sections were counterstained with hematoxylin.

Staining results were evaluated by light microscopy. Human adrenocortical tumor samples were scored from 0 (benign) to 9 (highly malignant) according to the criteria of Weiss (Weiss 1984; Weiss et al. 1989) by an experienced pathologist (J.A.). Immunohistochemistry results were assessed by light microscopy and the percentage of positive tumor cells was scored from 0% to 100%, rounded to the nearest 10%.

<table>
<thead>
<tr>
<th>antibody</th>
<th>company</th>
<th>cat #</th>
<th>dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>Santa Cruz Biotechnology, Inc.</td>
<td>sc-6886</td>
<td>1:200</td>
</tr>
<tr>
<td>aromatase</td>
<td>Santa Cruz Biotechnology, Inc.</td>
<td>sc-14245</td>
<td>1:200</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Zymed Laboratories</td>
<td>18-0226</td>
<td>1:500</td>
</tr>
<tr>
<td>β-catenin</td>
<td>Cell Signaling Technology, Inc.</td>
<td>9562</td>
<td>1:1000</td>
</tr>
<tr>
<td>ERα</td>
<td>Dako</td>
<td></td>
<td>1:500</td>
</tr>
<tr>
<td>FOG-2</td>
<td>Santa Cruz Biotechnology, Inc.</td>
<td>sc-9364</td>
<td>1:100</td>
</tr>
<tr>
<td>GATA-4</td>
<td>Santa Cruz Biotechnology, Inc.</td>
<td>sc-1237</td>
<td>1:200</td>
</tr>
<tr>
<td>GATA-6</td>
<td>Santa Cruz Biotechnology, Inc.</td>
<td>sc-9055</td>
<td>1:500, 1:50</td>
</tr>
<tr>
<td>inhibin-α</td>
<td>AbB Serotec</td>
<td>MCA951S</td>
<td>1:20, 1:25</td>
</tr>
<tr>
<td>LHR</td>
<td>American Type Culture Collection</td>
<td>CRL-2685</td>
<td></td>
</tr>
<tr>
<td>LRP5</td>
<td>Abcam</td>
<td>38311</td>
<td>1:200</td>
</tr>
<tr>
<td>LRP6</td>
<td>Cell Signaling Technology, Inc.</td>
<td>2560S</td>
<td>1:200</td>
</tr>
<tr>
<td>PCNA</td>
<td>Santa Cruz Biotechnology, Inc.</td>
<td>sc-1797</td>
<td>1:800</td>
</tr>
<tr>
<td>SF-1</td>
<td>Affinity Bioreagents, Inc.</td>
<td>PAI-800</td>
<td>1:1000</td>
</tr>
<tr>
<td>SF-1</td>
<td>Upstate Biotechnology</td>
<td>06-431</td>
<td>1:200</td>
</tr>
<tr>
<td>Smad2</td>
<td>Zymed Laboratories</td>
<td>51-1300</td>
<td>1:100</td>
</tr>
<tr>
<td>Smad3</td>
<td>Zymed Laboratories</td>
<td>51-1500</td>
<td>1:500</td>
</tr>
</tbody>
</table>

Table 5. Antibodies utilized in immunohistochemistry, immunocytochemistry, protein co-immunoprecipitation and Western blotting.

### 6.2 Immunocytochemistry (I)

Cells were grown to form a monolayer and fixed with 4% paraformaldehyde. The staining procedure was the same as for the tissue sections.
6.3 Protein co-immunoprecipitation (I)
Cells were transfected to express equal amounts of the proteins under study. Aliquots (100 µg) of cell nuclear extracts were immunoprecipitated by end-over-end mixing overnight at 4 °C with specific antibodies in PBS supplemented with NP40 (1%), 1 mM EDTA and protease inhibitor cocktail. The protein complexes were collected by adding protein G-Sepharose for 1 h, followed by five washes with the buffer, and subjected to SDS–PAGE. Non-specific antibodies were employed in control experiments.

6.4 Western blotting (I)
Cell nuclear proteins or precipitated proteins were separated by means of 7.5% SDS-PAGE and transferred to Immobilon-P membrane. Non-specific antibody binding was blocked with 5% skimmed milk in 0.1% Tween-TBS buffer. The membrane was incubated with the desired primary antibody for 1 h at room temperature, followed by secondary antibody, and visualized by using an Enhanced Chemiluminescence Plus Kit. For controls, the membranes were subsequently stripped and reprobed.

7 Statistical analysis (I, III, IV)
Cell culture results are presented as mean ± standard error of the mean of three independent experiments. The statistical significance of hormone measurement data was determined by using Student’s t test. In analysis of immunohistochemistry scoring results, differences between two groups were tested by using Wilcoxon’s (nonparametric) test, and correlations between continuous variables by using Spearman’s (nonparametric) rho. The analyses were performed using JMP 7.0 software. A p value <0.05 was considered significant.
Results and Discussion

1 Molecular interactions of GATA-4/6 (I)

GATA-4 is an activator of many genes regulating endocrine function, including LHR, aromatase, anti-Müllerian hormone, inhibin-βB and inhibin-α (Feng, et al. 2000; Ketola et al. 1999; Rahman et al. 2004; Tremblay and Viger 1999, 2001). GATA-6 has also been proposed to transactivate several of these regulators, albeit more weakly (Tremblay and Viger 2003a). Gonadotropins signal in endocrine cells through the cAMP-PKA pathway, which leads to phosphorylation of GATA-4, increasing its transactivation of target genes (Tremblay and Viger 2003a, b). Additionally, GATA factors are known to cooperate with TGF-β signaling in other organ systems (Blokzijl, et al. 2002; Brown, et al. 2004).

We wanted to investigate the molecular interactions of GATA-4 in endocrine cell signaling. Our hypothesis was that GATA-4 cooperates with the TGF-β/Smad signaling cascade to regulate endocrine genes. We chose a cell line termed KK-1, derived from an ovarian tumor from a transgenic Inha/Tag mouse (Kananen et al. 1995). This cell line expresses factors present in normal ovarian granulosa cells, such as inhibin-α, GATA-4 and SF-1. We also utilized another cell line, COS-7 epithelial cells, which does not express these endocrine factors but responds to a TGF-β/Smad3-type signal (Dennler, et al. 1998; Mazerbourg, et al. 2004). To study the effects on gene transcription, we utilized the inhibin-α promoter as an endocrine target.

1.1 GATA-4/6 is required for inhibin-α promoter activation by TGF-β

We found that in transient transfections TGF-β stimulation activated the inhibin-α promoter only in the presence of GATA-4. Exogenous TGF-β administered to the KK-1 cells upregulated GATA-4 protein levels and increased the transcription of the inhibin-α promoter coupled to a luciferase reporter. In COS-7 cells, forced expression of GATA-4 was needed to activate the inhibin-α promoter (figure 8A), and a similar effect, although weaker, was observed with forced expression of GATA-6 (Parviainen et al., unpublished data). Overexpressing FOG-1 or FOG-2, in contrast, resulted in abolishment of the promoter activation in both cell lines (shown in COS-7 cells in figure 8A). As FOG proteins are known to be both coactivators and repressors of GATA factors (Svensson, et al. 2000a; Tsang, et al. 1997), the findings support our hypothesis that GATA proteins are activating factors in this setting.
In KK-1 cells, dominant negative (DN) GATA-4 variants or mutation of GATA-binding sites in the promoter could attenuate TGF-β responsiveness. These results are summarized in (figure 8B, C), and suggest that GATA-4 is required for TGF-β-mediated *inhibin-α* activation.
Figure 8. Transient transfections of COS-7 and KK-1 cells. Luciferase activity, representing *inhibin-α* promoter activation, is presented as fold activation relative to control. A: COS-7 cells were transfected with the *inhibin-α*-luciferase construct, in the presence of GATA-4, FOG-1 and FOG-2 expression plasmids or the corresponding empty plasmids, and with or without TGF-β. B: KK-1 cells were transfected with the *inhibin-α*-luciferase construct in the presence of dominant negative (DN) GATA-4 expression plasmids or the corresponding empty plasmids, and with or without TGF-β. DN1 lacked the N-terminal end of GATA-4 protein, and DN2 had the cysteines mutated on the C-terminal zinc-finger. C: KK-1 cells were transiently transfected with either a wild-type or a mutant *inhibin-α*-luciferase construct, and with or without TGF-β. In mut1, the 5’ GATA site was mutated by replacing nucleotide G with C; in mut2, the 3’ GATA-site; and in mut1 and 2, both. D, E: COS-7 cells were transiently transfected with the *inhibin-α*-luciferase construct, in the presence of GATA-4, Smad2 and Smad3 expression plasmids or the corresponding empty plasmids, and with or without TGF-β.

1.2 Functional and physical interaction of GATA-4 and Smad3

Smad proteins are the ubiquitous intracellular transmitters of the TGF-β signal (Shi and Massague 2003). Because the TGF-β-type signal is mediated via Smad2 and Smad3, we chose to investigate whether these two proteins cooperate with GATA-4. In transfections of COS-7 cells, GATA-4 synergism was specific to Smad3; with Smad2 administration, no enhanced transcription of *inhibin-α* was observed (figure 8D, E). Functional GATA-4:Smad3 synergism was also evident in KK-1 cells. A Smad3 null mutation has been observed to downregulate *inhibin-α* expression in the ovary (Tomic, et al. 2004), which supports our finding that Smad2 and Smad3 behave differently in this environment.

We also introduced mutant GATA-4 constructs together with Smad3 in COS-7 cells and found that only administration of the DN construct that had the C-terminal zinc finger abolished resulted in failure to activate the *inhibin-α* promoter. Also in cardiac cells, GATA-4 and the TGF-β superfamily signal transmitters Smad1 and 4 cooperate to activate the *Nkx2.5* promoter, and the interaction seems to require the C-terminal region of GATA-4 (Brown et al. 2004). Further, we were able to demonstrate (by means of immunoprecipitation experiments) endogenous physical Smad3:GATA-4 interaction in KK-1 cells, and the same phenomenon was detected in transfected COS-7 cells (figure 9A, B).

1.3 SF-1 cooperates with GATA-4 and Smad3

*Steroidogenic factor-1* (SF-1) is a GATA target gene (Tremblay and Viger 2001) and a key regulator of steroidogenic enzyme expression and the development of
mammalian adrenals, testes and ovaries (reviewed in Parker and Schimmer 1997). SF-1 has been shown to have significant synergism with GATA-4 in activating multiple gonadal gene promoters, including those of *aromatase*, *anti-Müllerian hormone* and *inhibin-a* (Tremblay and Viger 1999, 2001). We introduced SF-1 to the synergistic setting of GATA-4:Smad3, to improve the picture of these key players in the endocrine organs. In COS-7 cells, with no endogenous SF-1 expression, SF-1 activated the *inhibin-a* promoter similarly to GATA-4, and acted synergistically with GATA-4 and with the GATA-4:Smad3 combination. In KK-1 cells, SF-1 similarly showed synergism with GATA-4 and Smad3, and the combination of the three players strongly enhanced TGF-β stimulation of the promoter, up to 11-fold (figure 9C) (Parviainen et al., unpublished data).

![Figure 9](image.png)

**Figure 9.** A: COS-7 cells were transfected with GATA-4 and Smad3, and nuclear protein extracts were immunoprecipitated (IP) with anti-Smad3, following immunoblotting (IB) with GATA-4 and Smad3 antibodies. B: Nuclear protein extracts of KK-1 cells were immunoprecipitated with anti-Smad3, followed by immunoblotting with GATA-4 and Smad3 antibodies. C: KK-1 cells were transiently transfected with the *inhibin-a*-luciferase construct, in the presence and absence of GATA-4, SF-1 and Smad3 expression plasmids and TGF-β. Luciferase fold activity is presented relative to the controls.

Considering the other gonadal GATA targets, such as *anti-Müllerian hormone* (Tremblay and Viger 1999) and *LHR* (Rahman et al. 2004), these results altogether...
suggest that GATA-4 is a link between endocrine and paracrine factors in endocrine cell gene regulation. The role of Smads in the transactivation of gonadal promoters has, however, only recently begun to evolve. By studying the GATA-4:Smad3 link further, we found that SF-1, both a target gene and an interacting partner for GATA factors in endocrine cells, is able to further enhance the GATA-4:Smad3 synergism. Thus, the regulation of inhibin-α expression may be orchestrated by a transcriptional module including GATA-4, Smad3 and SF-1.

2 LH-dependent neoplasia of the mouse adrenal cortex (II, III)

2.1 Gonadal characteristics in neoplastic cells

We wanted to examine the expression profile of adrenocortical tumors in mice susceptible to gonadectomy-induced adrenocortical neoplasia. When gonadectomized, mice of the susceptible DBA/2J strain developed typical adrenocortical neoplasia consisting of A and B cells, as previously described (Fekete et al. 1941; Woolley et al. 1952). FVB/N mice, a non-susceptible strain, did not develop these changes. In the adrenals of DBA/2J mice, small A cells began to accumulate in the subcapsular region as early as a few weeks after gonadectomy. The A cells then migrated centripetally between the cell columns of the zF, and eventually disrupted the morphology of the zG and zF. By a few months after gonadectomy, large, lipid-laden B cells could be observed in between the A cells.

The expression profiles of GATA-6 and GATA-4 in these tumors were of interest, since the former is abundantly expressed in normal adrenocortical cells and the latter is not normally detected postnatally. Immunohistochemical staining of the adrenals of gonadectomized DBA/2J mice, harvested 4 months after gonadectomy, showed that the normal GATA-6 nuclear expression was downregulated in the tumor area, whereas strong GATA-4 nuclear expression could be seen in tumor cells (figure 10). The small, spindle-shaped A cells and the large, lipid-laden B cells shared these characteristics.
RESULTS AND DISCUSSION

Figure 10.
GATA-4 immunohistochemical staining of a neoplastic region in the adrenal cortex of a gonadectomized DBA/2J mouse. Both A and B cells have strong GATA-4 nuclear expression (brown stain), whereas the nuclei of adjacent normal adrenocortical cells remain negative (blue). Yellow arrows: A cells, red arrows: B cell. Modified from (II).

We then further investigated the expression profile of the tumors, utilizing in situ hybridization to localize mRNA expression in the adrenal glands. As expected, A and B cells expressed GATA-4 mRNA. In addition, B cells expressed p450c17, a steroidogenic enzyme required for the synthesis of sex steroids, and normally expressed in the mouse adrenal cortex only during the fetal period. LH receptor (LHR) and inhibin-α were also expressed in B cells; inhibin-α also in A cells to a lesser extent. In contrast, A cells did not express p450c17 or LHR, and the expression pattern also significantly differed from that of normal adrenocortical cells, as A cells did not express SF-1, StAR or P450scc mRNA. This implies limited steroidogenic capacity in A cells, whereas B cells express the markers needed for sex steroid production, resembling the expression pattern in gonadal steroidogenic cells.

To further characterize the contribution of gonadectomy and, on the other hand, gonadotropin elevation to the induction and progression of adrenocortical neoplasia, we created a mouse model employing female NU/J nude mice. The NU/J strain is an inbred strain carrying a loss-of-function mutation in the Foxn1 gene (Foxn1nu) that is associated with athymia (Balciunaite, et al. 2002). After gonadectomy, the NU/J mice developed adrenocortical neoplasia, morphologically comparable to the changes seen in DBA/2J mice. Also similarly to the phenotype of gonadectomized DBA/2J mice, normal adrenocortical markers such as MC2R and 21-hydroxylase were absent from the neoplastic area, while ectopic expression of p450c17 and AMH receptor mRNA could be localized in B cells in in situ hybridization analysis. Ectopic immunohistochemical expression of GATA-4 in A and B cells was also similarly evident, as was the expression of LHR, inhibin-α and several other markers in B cells (figure 11).
**RESULTS AND DISCUSSION**

**Figure 11.**
Immunohistochemical expression of steroidogenic differentiation markers in the adrenal cortex of gonadectomized NU/J mice. GATA-4 expression is seen both in A and B cells (black arrows). SF-1 is expressed in normal adrenocortical cells and B cells, but is absent from A cells. LHR, AMH, inhibin-α, Smad3, aromatase and β-catenin expression is seen in B cells.

Taking advantage of the ability of NU/J mice to accept xenografts, we next assessed whether the adrenocortical tumorigenesis phenomenon is dependent directly on elevation of gonadotropin concentrations or on other changes induced by gonadectomy, e.g. lack of gonadal hormones. We utilized subcutaneous injections of cells secreting a single-chain variant of hCG known to bind to LH receptor (Sugahara et al. 1995). We found that non-gonadectomized mice with the hCG-secreting xenografts developed adrenocortical neoplasia morphologically very similar to the gonadectomized mice, and the expression patterns of GATA-4, inhibin-α and FOG-2 were also similar. The expression of the sex steroidogenic markers LHR, P450c17 and aromatase was, however, attenuated in the adrenals of hCG-treated mice compared with gonadectomized mice, as studied by RNase protection assays and RT-PCR.
RESULTS AND DISCUSSION

Our results suggest that chronic elevation of gonadotropin concentrations is the principal signal responsible for adrenocortical tumorigenesis in susceptible strains of mice, but that it does not explain all the phenotypic changes, as the sex steroidogenic capacity of the hCG-treated mice was reduced. This might be a result of the effects of hCG on the ovaries, stimulating production of estrogen and testosterone. In addition, LH signaling could be decreased through down-regulation of LHR as a result of elevated hCG levels (Ascoli, et al. 2002; Wang and Menon 2005).

2.2 GATA-4 and LHR in the adult adrenal cortex

Early investigations on gonadectomized mice revealed that the developing adrenocortical neoplasia morphologically resembled gonadal stroma and was able to induce hormonal changes in mice normally achieved by ovarian steroid production (Fekete et al. 1941). Our studies suggest that the neoplastic cells not only are transformed to express gonad-specific genes, but also lose expression of characteristic adrenocortical genes. The ectopic expression of GATA-4, normally not expressed in the adult adrenal cortex, but abundantly in the gonads, has emerged as a hallmark of these tumors. In addition to the inbred mouse strains, the genetically modified models Inha/Tag and Inha/- also exhibit GATA-4 expression in adrenocortical tumors (Looyenga and Hammer 2006; Rahman et al. 2004). Many of the other features of the inbred models are also similar (table 6).
RESULTS AND DISCUSSION

<table>
<thead>
<tr>
<th></th>
<th>Normal cortex</th>
<th>DBA</th>
<th>NU/J nude</th>
<th>Inhα/Tag</th>
<th>Inh-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA-4</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>GATA-6</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>LHR</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inhibin-α</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>AMH</td>
<td>-</td>
<td>n/a</td>
<td>+</td>
<td>n/a</td>
<td>+</td>
</tr>
<tr>
<td>P450c17</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>n/a</td>
<td>+</td>
</tr>
<tr>
<td>aromatase</td>
<td>-</td>
<td>n/a</td>
<td>+</td>
<td>n/a</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 6. Gene expression in adrenocortical neoplasms of gonadectomized DBA/2J, NU/J nude, Inhα/Tag and Inh-/- mice, and in normal adult mouse adrenal cortex. Modified from (Parviainen, et al. 2007). Abbreviations: LHR, luteinizing hormone receptor; AMH, anti-Müllerian hormone; P450c17, cytochrome P450 17α-hydroxylase/17,20-lyase; n/a, unknown.

Studying *Inha-/-* mice, two distinct cell types have been identified in the neoplastic area, one resembling theca cells and the other granulosa cells (Looyenga and Hammer 2006), corresponding to the A and B cells, respectively. Also similarly to our results, these neoplastic cells do not express typical adrenocortical genes such as *Mc2r*, *Cyp11b1* or *Cyp21*. Type A cells, having a limited steroidogenic capacity, resemble postmenopausal ovarian stromal cells (Jabara, et al. 2003). The steroidogenic B cells resemble follicular theca cells found in patients with polycystic ovary syndrome (PCOS) (Kaaijk, et al. 2000), a condition characterized by elevated LH levels in the circulation.

For LH to have a direct effect on cells, its receptor must be present. LHR is normally expressed in gonadal cells in adults, and very low levels of LHR have been detected in normal human adrenal cortex (Couzinet et al. 2001; Pabon et al. 1996). In fetal mice, however, considerable LHR expression has also been detected in the adrenal cortex; a developmental expression pattern resembling those of GATA-4 and P450c17 (Apaja, et al. 2005; Kiiveri et al. 2002; Rahman et al. 2004). The mechanism by which LHR expression is upregulated in neoplastic adrenocortical tissue is unknown, but it is conceivable that a subset of adrenocortical stem/progenitor cells expresses functional LHR and proliferates in response to the upregulated LH levels. Expression of GATA-4 is one of the earliest events in the neoplastic process and positive and reciprocal feed-forward amplification seems to exist between GATA-4 and LHR expression (Rahman et al. 2004). Furthermore, a
recent study has demonstrated that GATA-4 binds to LH β-subunit receptor and activates it in pituitary cells (Lo, et al. 2011). Whether a similar mechanism is active in the LH-dependent adrenocortical tumors is an interesting target for future investigation.

Supporting the central role of GATA-4 in the expression of steroidogenic genes in the tumors, GATA-4 has been found to be sufficient to induce expression of steroidogenic markers in adrenocortical Y1 cells (Looyenga and Hammer 2006). We have examined the influence of exogenous GATA-4 protein in the adult mouse adrenal cortex of a non-susceptible strain (FVB/N) in the absence of LH stimulation (Parviainen et al., unpublished data). GATA-4 expression was driven in the mouse adrenal cortex by a p450c21 promoter (21OH/Gata4 mouse), which is expressed in differentiated mouse adrenocortical cells (Morley, et al. 1996). Preliminary results indicate that these mice develop subcapsular patches of GATA-4-positive cells in the adrenal cortex, resembling the A cells in the tumors of the inbred susceptible strains. GATA-6 expression is diminished and LHR expression upregulated in the neoplastic area (figure 12). Steroidogenic B cells do not seem to be present, in contrast to the other mouse models described above; this might be due to the introduction of GATA-4 to the already differentiated adrenocortical cells. These results, however, highlight the role of GATA-4 in the neoplastic process.

Figure 12.
Expression of GATA-4 is upregulated and that of GATA-6 and SF-1 downregulated in the spindle-shaped neoplastic cells of 21OH/Gata4 mice, compared to the adjacent normal adrenocortical cells. Ectopic LHR expression is seen in the neoplastic area.

The hypothesis that mouse adrenocortical tumors arise from improperly specified adrenocortical stem/progenitor cells is supported by analogous human conditions. Several cases of thecal metaplasia of the adrenal have been reported in post-menopausal women and in men with acquired testicular atrophy – both conditions characterized by chronically elevated levels of gonadotropins (Fidler 1977; Romberger and Wong 1989). The rare ACTH-independent Cushing syndrome in
pregnant women is also interesting as regards LH; in this condition, the adrenals respond to LH/hCG stimulation (Kasperlik-Zaluska, et al. 2000; Wy, et al. 2002).

2.3 Mechanisms of subcapsular stem/progenitor cells

The stem/progenitor cell theory in LH-dependent adrenocortical tumorigenesis is summarized in figure 13: in response to LH (instead of ACTH), the SF-1-positive subcapsular progenitors downregulate Dax1 and upregulate GATA-4, subsequently driving expression of *P450c17* and other genes involved in sex steroidogenesis.

![Figure 13.](image)

In response to elevated LH levels, the multipotential adrenocortical subcapsular progenitor cell differentiates into a gonadal-like cell. Modified from (Bielinska et al. 2009).

It is interesting to note that SF-1, β-catenin and Smad3 are all expressed in the type B cells of NU/J mice. SF-1 has been shown to synergize with β-catenin in inducing the *inhibin-α* promoter in adrenocortical cells (Gummow et al. 2003), while GATA-4 can also activate the SF-1 promoter and cooperate with SF-1 at the protein level (Tremblay and Viger 2003a). Upregulation of Smad3 together with GATA-4 has also been observed in *Inh-/-* adrenocortical tumors (Looyenga, et al. 2004), and we have shown in ovarian granulosa cells that GATA-4:Smad3 interaction is a mediator of TGF-β signaling in the regulation of *inhibin-α* (I). All in all, the coexpression of GATA-4, SF-1, β-catenin and Smad3 in gonadectomy-induced adrenocortical tumors would
suggest potential involvement of TGF-β and Wnt signaling cascades in the mechanisms of phenotypic switching from adrenocortical to gonadal-type cells.

3 Human adrenocortical tumors and regulatory pathways (I–IV)

The mouse models of adrenocortical tumorigenesis are indeed models, and it is of interest to investigate whether similar factors and mechanisms are present in human adrenocortical tumors. As mouse and human adrenal glands are functionally different, it could be presumed that the factors implicated in human adrenocortical tumor growth are not completely similar either. For instance, the human tumors do not generally depend on elevated levels of circulating gonadotropins – with the exceptions of thecal metaplasia in post-menopausal women (Fidler 1977) and in men with acquired testicular atrophy (Romberger and Wong 1989), and the ACTH-independent Cushing syndrome in pregnant women, in which the adrenals respond to LH/hCG stimulation (Kasperlik-Zaluska et al. 2000; Wy et al. 2002). The mouse models have, however, provided information that can be utilized in the search for the mechanisms responsible for the growth of human tumors.

3.1 Human adrenocortical tumors

Next, we wanted to study whether the transcription factors and other regulators implicated in mouse studies are expressed in human adrenocortical tumors, and investigate the possible correlations of these factors in benign vs. malignant phenotype or other features of the tumors. We utilized a series of 31 human adrenocortical tumors, of which 13 were carcinomas.

Inhibin-α seems to be a tumor suppressor in the mouse, as Inha−/− mice develop gonadal tumors and, when gonadectomized, adrenocortical tumors (Kananen et al. 1996; Kananen et al. 1995). In human adrenocortical tumors, in contrast, no significant difference in inhibin-α expression between benign and malignant tumors has been found (Arola et al. 2000). Inhibin-α is nonetheless an interesting target for further research in adrenocortical tumors, as it is regulated by many of the factors expressed in the mouse tumor models, such as GATA-4 and 6, SF-1, β-catenin and Smad3 (I–III and Gummow et al. 2003; Looyenga et al. 2004; Tremblay and Viger 2003a). β-catenin is the canonical intracellular mediator of the Wnt pathway, which is aberrantly active in various neoplastic processes, including human adrenocortical tumors (Tissier, et al. 2005; Willert and Jones 2006). The TGF-β pathway, in which Smad3 is a mediator, is also implicated in many malignant processes, either as a promoter or as an attenuator of tumor growth (Massague, et al. 2000).
RESULTS AND DISCUSSION

We chose to study expression of the TGF-β pathway components Smad3 and Smad2 and the Wnt pathway components β-catenin and the co-receptors LRP5 and LRP6, in correlation with inhibin-α, GATA-6 and SF-1 expression. Our previous results with the same tumor series indicated that GATA-6 and SF-1 are predominantly expressed in benign tumors (Kiiveri et al. 2005), and we now wanted to further dissect the role of these transcription factors in the tumors.

The Wnt pathway co-receptors LRP5 and LRP6 were predominantly expressed in benign tumors, although some expression was also detected in carcinomas. We divided the tumors by their Weiss scores into two groups, Weiss scores 0–3 and 4–9, and the expression of both receptors was significantly higher in the Weiss score 0–3 group. In contrast, in β-catenin staining, no difference could be detected between the groups. This finding differs from previous results in a French tumor series, in which β-catenin expression was higher in carcinomas (Gaujoux, et al.; Tissier et al. 2005).

The expression profiles of Smad2 and Smad3 were clearly different, as Smad3 expression was highest in tumors with a low Weiss score and Smad2 expression, in contrast, highest in the most malignant tumors. In addition, Smad3 expression correlated with that of inhibin-α, whereas expression of Smad2 did not, which is interesting considering our previous results concerning GATA-4/6 interaction in inhibin-α activation, which is specific to Smad3 (I). Of note, inhibin-α expression in our material also correlated with the Wnt pathway components LRP5, LRP6 and β-catenin, and with SF-1. In the future, it would be interesting to investigate further whether or not the in vitro cooperation of SF-1 and β-catenin on inhibin-α activation (Gummow et al. 2003) is also present in human adrenocortical tumors. The expression of GATA-6 also correlated with that of inhibin-α, possibly relating to the ability of GATA-6 to transactivate the inhibin-α promoter (Tremblay and Viger 2001).

Finally, both GATA-6 and SF-1 expression in carcinomas, although generally lower than in adenomas (Kiiveri et al. 2005), was also significantly more downregulated in those carcinomas that proved to be lethal. The results obtained concerning adrenocortical carcinomas are presented in figure 14. Considering the proposed tumor suppressor role of inhibin-α in the adrenal cortex (Looyenga et al. 2004), it could be hypothesized that in the most aggressive carcinomas, with poor outcome, inhibin-α transcription is attenuated as the intracellular and nuclear mediators Smad3, GATA-6 and SF-1 are lost or diminished.
RESULTS AND DISCUSSION

Figure 14. Expression of the TGF-β signaling mediators Smad2 and Smad3 in adrenocortical carcinoma differed in that Smad2 expression was upregulated and Smad3 downregulated as compared to adenomas. Expression of both LRP5 and LRP6 was reduced in carcinomas. SF-1 and GATA-6 expression was downregulated in the most aggressive carcinomas. Abbreviations: DOD, died of disease; NDOD, not dead of disease.

Aberrant GATA-4 expression has been detected in a subset of human adrenocortical tumors (Bassett et al. 2005; Kiiveri et al. 2005; Kiiveri et al. 1999), and high GATA-4 expression has been associated with aggressive behavior (Barbosa et al. 2004). In our tumor material, GATA-4 mRNA expression could be detected in a subset of the tumors by RT-PCR, but no significant correlation was detected with the TGF-β cascade components or other factors studied (Kiiveri et al., unpublished data). We also assessed LHR mRNA expression in the tumors, which was evident in approximately half of the adenomas and in a minority of the carcinomas for which frozen tissue was available, but it also did not correlate with the other factors studied. These findings contrast with those in mouse models of adrenocortical tumorigenesis, in which GATA-4 and LHR expression are hallmarks of neoplastic growth (Bielinska et al. 2006). Although there obviously are differences between human adrenocortical tumors and the mouse models studied, some phenomena (such as diminished expression of GATA-6) are present in both.

Adding to the tumor studies, we also studied the expression of the same factors in Wnt4 knockout mice (Parviainen et al., unpublished data). Wnt4 null mice have an adrenal phenotype of decreased expression of P450aldo and ectopic expression of P450c21 in the gonads, which suggests a role for Wnt4 in cell migration from the
RESULTS AND DISCUSSION

urogenital ridge (Heikkila et al. 2002). We studied (by way of immunohistochemistry) the expression of Wnt pathway components and factors implicated in mouse and human adrenocortical tumorigenesis in Wnt4-/ mice. Since the mice do not survive postnatally as a result of kidney failure (Vainio et al. 1999), only fetal samples could be studied. Smad3 expression was slightly upregulated in the adrenals of knockout mice, while the expression levels of Smad2 and other factors such as inhibin-α, β-catenin, LRP5/6 and GATA-4 were not significantly altered. These preliminary data suggest that the TGF-β/Smad3 pathway is upregulated in the adrenal cortex when Wnt signaling is absent, but additional studies are needed.

3.2 Signaling cascades in adrenocortical tumors

Presumably, there are several signaling cascades concomitantly active in both normal steroid-producing cells and adrenocortical tumor cells in humans and mice. We have studied the function of GATA factors that can affect gene expression in the cells both by direct transcriptional activation and by interacting with other regulators present. Our results suggest interactions of GATA-4 and GATA-6 with the TGF-β and Wnt signaling pathways.

The putative intracellular cascades involving the GATA factors are summarized in figure 15. Signals are transmitted through cell surface receptors for TGF-β (RI and RII complex) and Wnt (complex of frizzled receptor and LRP co-receptor), and the G-protein-coupled receptors for ACTH (MC2R) or LH (LHR), depending on the cell type. GATA-4 cooperates with Smad3 to upregulate inhibin-α transcription in the nucleus, and GATA-6 is also likely to be able to participate in the transcriptional module. SF-1 and β-catenin cooperate to induce the same promoter (Gummow et al. 2003), while GATA-4 and GATA-6 are able to upregulate SF-1 transcription (Tremblay and Viger 2001), and GATA-4 has been shown to interact directly with SF-1 in endocrine promoter transactivation (Tremblay and Viger 1999). The ACTH or LH signal is transmitted through the cAMP-PKA pathway, which, among its other effects, induces GATA-4 and GATA-6 expression (Tremblay and Viger 2003a, b), increasing the transactivation of, for example, inhibin-α. Other as yet unidentified factors are likely to be present in the promoter transactivation complex.
Figure 15.
A hypothetical model of the participation of GATA-4 and GATA-6 in gene transcription in steroid-producing cells. See text for details.
Conclusions and Future Perspectives

1. GATA-4 and Smad3 physically cooperate to induce inhibin-α in granulosa cells and are likely to participate in a transcriptional module orchestrating inhibin-α activation in endocrine tissues.

2. GATA-4 expression is dramatically upregulated and GATA-6 downregulated in gonadectomy-induced adrenocortical tumors of inbred mice. Ectopic expression of GATA-4 and LHR in these tumors is associated with changes in cell proliferation, differentiation and steroid production.

3. Chronic elevation of circulating gonadotropin concentrations, caused by either gonadectomy or hCG administration, induces the neoplastic process in the adrenal cortex of susceptible inbred mice. A population of cells in the subcapsular region undergoes differentiation along a gonadal rather than an adrenal route, leading to sex steroid production by the tumors.

4. The signaling cascade components and transcription factors driving inhibin-α expression are reduced in ACC with poor outcome, suggesting a role for inhibin-α as a tumor suppressor. The results suggest pathways associated with adrenocortical tumor aggressiveness.

Research on adrenocortical tumors has in recent years led to the identification of dysregulated gene mutations and signaling pathways, by means of gene expression profiling of tumor samples and analysis of human genetic disorders that predispose individuals to adrenocortical tumors. However, the molecular events behind the frequent benign tumors and the rare event of malignant transformation in the adrenal cortex remain unknown. The availability of inbred and genetically engineered mouse models has enabled research on adrenocortical stem/progenitor cells that may elucidate the genetic and epigenetic events behind both tumor types. Specifically, it would be of interest to further dissect the role of GATA-4 in adrenocortical tumors by introducing ectopic GATA-4 expression in mouse subcapsular progenitor cells. Another approach is investigation of the adrenal phenotype in intact and gonadectomized Gata4 haploinsufficient mice; a report is already in preparation.
Clinical Significance

Based on current knowledge it can be assumed that GATA factors and their cofactors are important regulators of the development and function of the adrenal gland, and that abnormalities in their expression or in the genes coding for them may lead to disease and tumors in the adrenal cortex. Abnormalities in transcription have been linked to developmental diseases and tumorigenesis in many organ systems, but such events have until recently been poorly understood in the adrenal cortex.

Analyses of heritable and spontaneous types of human adrenocortical tumors have revealed alterations in either cell surface receptors or their downstream effectors, and mouse models have enabled research on the mechanisms behind neoplastic transformation and tumor growth. Better understanding of the basic molecular mechanisms controlling organ development and leading to tumor formation is needed to ultimately improve diagnostics and to develop new treatment strategies for benign and malignant adrenocortical tumors.
Publications Not Included in this Thesis


Acknowledgements

This study was carried out at the Research Laboratory of the Children’s Hospital, University of Helsinki. I wish to express my gratitude to those who provided me with such excellent research facilities: Docent Jari Petäjä, Director of the Department of Gynecology and Pediatrics, Professors Mikael Knip and Christer Holmberg, who both have filled the Chairmanship of the Children’s Hospital, and Docent Eero Jokinen, Head of the Department of Pediatrics. I also warmly thank the former Head of the Research Laboratory, Professor Erkki Savilahti.

I had the privilege to join the Clinical Graduate School in Pediatrics and Gynecology and the Pediatric Graduate School, which both provided me with financial support and valuable scientific education, and I wish to thank the Heads of the graduate schools, Professors Jorma Paavonen, Markku Heikinheiro and Mikael Knip. The Finnish Medical Society Duodecim, the Finnish Cultural Association, the Emil Aaltonen Foundation, the Maud Kuistila Foundation, the Helsinki University Research Funds and the Helsinki University Central Hospital Research Funds are also acknowledged for financial support.

I owe my deepest gratitude to my supervisor, Professor Markku Heikinheiro, for introducing me to the world of science. Your continuous support, encouragement and insight have been invaluable during these years. I also greatly value your more official supportive roles as Head of the Institute of Clinical Medicine, Head of the Clinical Graduate School in Pediatrics and Gynecology and former Head of the Pediatric Graduate School, making it possible for me and many other young investigators to concentrate on research.

I want to thank the official referees, Docents Jarmo Jääskeläinen and Camilla Schalin-Jäntti for the time they devoted to improve my thesis and for helpful discussions by phone and in person. I also sincerely thank the members of my thesis follow-up group, Docent Päivi Tapanainen and Professor Marikki Laiho, for giving valuable advice and encouragement during the ups and downs of the project.

I have had the opportunity to work with great colleagues. This thesis results from years of teamwork, and I thank you all: Professors Irving Boime, Ilpo T. Huhtaniemi, Juhani Leppäluoto, Louis J. Muglia, Seppo Vainio and David B. Wilson, Docents Johanna Arola, Caj Haglund, Nafis Rahman and Olli Ritvos, and Doctors Mikko Anttonen, Malgorzata Bielinska, Elena Genova, Sanne Kiiveri, Antti Kyrölähti and Renata Prunskaitė-Hyyryläinen. Dave, thank you for sharing your knowledge on
GATA and the adrenal, and for the opportunity to visit your laboratory in WUSTL in 2005 and 2008. Johanna, thank you for introducing me to the world of pathology. Taru Jokinen, Ritva Löfman, Elina Aspiala, Eija Heiliö and Susan B. Porter-Tinge are thanked for expert technical assistance. Dr. Nick Bolton is thanked for editing the language of this thesis, and Dr. Markku Kallio for the help with statistics.

I thank the past and present members of our wonderful research group FOGs: Ilkka Ketola, Sanne Kiiveri, Mikko Anttonen, Susanna Mannisto, Noora Andersson, Hanna Haveri, Jonna Salonen, Antti Kyrönlähti, Reeta Pökkylä, Anniina Färkkilä, Marjut Kauppinen, Tea Soini, Sanna Vattulainen, Jenni Elo, Riika Vähätalo, Maarit Rämö and Maija Tamminen. I especially want to mention the “senior FOGs” Sanne, Mikko, Ilkka, Susa and Noora for sharing their knowledge and experience; Hanna, Jonna, Antti, Marjut and Anniina for the many discussions; and Reeta for your “peer support” and friendship. Docent Leila Unkila-Kallio has always been supportive and encouraging. All the research groups in the program have participated in making the lab a nice place to work. There are many people to thank; Arvi-Matti Kuusniemi, Saija Näse-Stålhammar, Sauli Toikka, Sari Linden and Tuike Helmiö to name a few.

My dear friends from med school, Eeva, Eliisa, Kanerva, Marianna, Ninnu, Sara and Ulla, thank you for being there for ten years and sharing many unforgettable moments. Thank you for your friendship, dear TYK friends Outi, Enni, Ilari, Ilmari, Suvi, Hanna, Janiika and Markus.

I thank my Mom and my mother-in-law Pirkko for sharing their time with Sisu. The help has been indispensable and made the completion of this thesis possible. Mom and Dad, thank you for always believing in me. I also thank Henna and Panu for being there.

Finally, I thank my dear family. Perttu, thank you for your love and support. Our dear son Sisu, and his little sister soon to be born, constantly remind me of what really is important in life.

Espoo, August 2011

Helka Parviainen
References

Apaja PM, Aatsinki JT, Rajaniemi HJ & Petaja-Repo UE 2005 Expression of the mature luteinizing hormone receptor in rodent urogenital and adrenal tissues is developmentally regulated at a posttranslational level. *Endocrinology* **146** 3224-3232.


Brant WE & Helms CA 2006 Fundamentals of Diagnostic Radiology.


REFERENCES


DeLellis RA, Lloyd RV, Heitz PU & Eng C 2004 WHO Classification of Tumours: Pathology and Genetics of Tumours of Endocrine Organs.


Huseby RA & Bittner JJ 1951 Differences in adrenal responsiveness to postcastrational alteration as evidenced by transplanted adrenal tissue. *Cancer Res* **11** 954-961.


REFERENCES


Woolley GW, Dickie MM & Little CC 1952 Adrenal tumors and other pathological changes in reciprocal crosses in mice. I. Strain DBA x strain CE and the reciprocal. *Cancer Res* **12** 142-152.


