OVARIAN DEVELOPMENT, FUNCTION, AND GRANULOSA CELL TUMORIGENESIS:

Role of GATA Transcription Factors and Anti-Müllerian Hormone

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ACADEMIC DISSERTATION

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To my family

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ABSTRACT

Mammalian gonadogenesis is a complex orchestration of genes and signaling cascades, leading to differentiation of the gonadal primordium into either an ovary or a testis. The Y-chromosomal Sry drives testis differentiation, and ovarian development requires a means of disabling the testis pathway. Anti-Müllerian hormone (AMH) is a testicular product that induces regression of the Müllerian ducts in males. Postnatally, ovarian folliculogenesis is regulated by endocrine, paracrine, and autocrine mechanisms that pprepare the oocyte for fertilization. In addition to follicle-stimulating hormone (FSH), AMH and oocyte-derived ligands of the transforming growth factor β (TGF-β) superfamily influence follicle growth, involving balanced proliferation and apoptosis of the granulosa cells. Intracellularly, the regulatory cascades employ a number of secondary messengers as well as transcription factors to drive cell-specific gene expression.

In Finland, the fourth most common malignancy among women is ovarian cancer, with sex cord-derived granulosa cell tumors (GCTs) accounting for 5% of all ovarian cancer. GCTs are steroidogenically active and may present with abdominal pain, menstrual cycle disorders, and secondary findings due to hyperestrogenism. Granulosa cell tumorigenesis is thought to arise from misregulation of granulosa cell proliferation. With the help of animal models, some molecular defects have been elucidated, but the exact mechanisms underlying human GCT pathogenesis are poorly known.

The GATA transcription factors are suggested to play a role in gonadogenesis and folliculogenesis in mice. Each of the six GATA factors possess two zinc fingers and regulate cell-specific gene expression in various organs. Together with steroidogenic factor 1 (SF-1), GATA-4 and GATA-6 activate several endocrine target genes, such as *AMH*, *inhibin-α*, and *aromatase*, the proper expression of which is essential for ovarian function. Furthermore, GATA-4 is known to be an intracellular effector in the FSH pathway. The specificity of gene regulation by GATA factors is achieved by cofactors acting in concert with them. Accordingly, SF-1, CREB-binding protein (CBP), as well as FOG(friend of GATA)-1 and FOG-2 are able to modulate gene transactivation by a given GATA protein.

This thesis aimed at clarifying the impact of GATA-4, GATA-6, and FOG-2 on the development and function of the mammalian ovary and on GCT pathogenesis by profiling mRNA and protein expression in the normal mouse and human ovary and in the GCTs. In the fetal mouse, GATA-4 and FOG-2 were readily expressed in the bipotential urogenital ridge and in tovarian somatic cells. Also in the fetal human ovary, both GATA-4 and FOG-2 were expressed in somatic cells, in which GATA-4 may have an anti-apoptotic function. Given the role of GATA target *AMH* in the fetal testis, transfection assays with the *AMH* promoter were performed, and the results indicated that FOG-2 participates in inhibiting *AMH* activation by GATA-4 in the fetal ovary.

In the postnatal human ovary, GATA-4 and FOG-2 expression was initiated in the granulosa cells of primary follicles, along with activation of granulosa cell proliferation. At later follicle stages, expression of GATA-4, GATA-6, and FOG-2 was intermediate or high in granulosa and theca cells. Based on the expression profiles for these factors, their function and regulation in the granulosa cells were studied

with the use of *in vitro* cell cultures. In cultured human granulosa-luteal cells, which resemble the luteal gland, GATA-6 was upregulated by gonadotropins, supporting the role of GATA-6 in the luteal glands, which also express FOG-2. Furthermore, the growth factor TGF-β was able to upregulate GATA-4 in mouse granulosa cells. GATA-4 was found to be essential for the activation of *inhibin-α* by TGF-β.

Finally, the impact of GATA-4, GATA-6, FOG-2, and AMH on GCT pathogenesis was analyzed by comparing their expression levels in tumor tissue with markers of clinical behavior in a series of 80 GCT patients. Most of the GCTs exhibited intermediate or high expression of GATA-4, GATA-6, and FOG-2. However, AMH expression was more often downregulated, which was associated with an increase in tumor size, indicating that AMH may be a growth suppressor for GCT. Moreover, high and thus normal sustained GATA-4 expression was associated with more aggressive clinical behavior of GCTs, supporting a role for GATA-4 in granulosa cell proliferation.

In conclusion, the expression profiling and functional analysis coupled with results of previous studies indicate that transcription factor GATA-4 is a key component in the granulosa cell function. GATA-6, on the other hand, is involved in regulating steroidogenic function in gonads as well as in adrenals, whereas FOG-2 has a putative impact on the fetal ovary with regard to *AMH* inhibition. Lastly, the results indicate yet another functional role for AMH in human cancer. Since transcription factors are nuclear mediators for several ovarian signaling pathways, proper knowledge of their function in regulating development as well as tumorigenesis will give us insight into how to control and modify these cascades. This information may lead to improved diagnostics and enable new treatment strategies to be developed.

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, referred to in the text by their Roman numerals:

I) **Anttonen M**, Ketola I, Parviainen H, Pusa A-K, and Heikinheimo M: FOG-2 and GATA-4 are coexpressed in the mouse ovary and can modulate Müllerian-inhibiting substance expression. Biol Reprod 2003 Apr;68(4):1333-40.

II) Vaskivuo TE, **Anttonen M**, Herva R, Billig H, Dorland M, Te Velde ER, Stenbäck F, Heikinheimo M, and Tapanainen JS: Survival of human ovarian follicles from fetal to adult life: apoptosis, apoptosis-related proteins, and transcription factor GATA-4. J Clin Endocr Metab 2001 Jul;86(7):3421-9.

III) Laitinen MPE*, **Anttonen M***, Ketola I, Wilson DB, Ritvos O, Butzow R, and Heikinheimo M: Transcription factors GATA-4 and GATA-6 and a GATA family cofactor, FOG-2, are expressed in human ovary and sex cord-derived ovarian tumors. J Clin Endocr Metab 2000 Sep;85(9):3476-83.

IV) **Anttonen M**, Unkila-Kallio L, Leminen A, Butzow R, and Heikinheimo M: Expression profiles of GATA-4, GATA-6, SF-1, and anti-Müllerian hormone reveal a subgroup of granulosa cell tumors with aggressive behavior. Submitted.

In addition, some unpublished data are presented.

* These authors contributed equally to this work.

ABBREVIATIONS

AMH	Anti-Müllerian hormone (also known as MIS, Müllerian inhibiting substance)
Bcl-2	B cell leukemia-2
BMP15	Bone-morphogenic protein 15 (also known as GDF9B, see below)
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein
C/EBP	CCAAT enhancer binding protein
cDNA	Complementary deoxynucleic acid
CRE	cAMP-responsive element
CREB	cAMP-responsive element binding protein
DAX-1	Dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome, gene 1
E	Embryonal day
ERK	Extracellular signal-regulated kinase
FIGO	International Federation of Gynecology and Obstetrics
FOG	Friend of GATA
FSH	Follicle-stimulating hormone
FSHR	Follicle-stimulating hormone receptor
GCT	Granulosa cell tumor
GDF9	Growth and differentiating factor 9
hGL	Human granulosa-luteal cell
hCG	Human chorionic gonadotropin
IVF	*In vitro* fertilization
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
MAPK	Mitogen-activated protein kinase
MI	Mitotic index
mRNA	Messenger RNA
PKA	Protein kinase A
SF-1	Steroidogenic factor 1
Sox9	A SRY-box containing gene 9
Sry	Sex-determining region of the Y chromosome
StAR	Steroidogenic acute regulatory protein
TGF-β	Transforming growth factor β
Wnt	Wingless-related mouse mammary tumor virus (MMTV) integration site

INTRODUCTION

Gene regulation is a fundamental process enabling a fertilized egg to differentiate and grow into an individual mammal. Majority of the molecular mechanisms have been evolutionally conserved from invertebrates to vertebrates. All of the information required for development and functioning of an individual is located in the DNA. In eukaryotic cells, histone proteins pack the DNA into chromatins, which may be in an inactive or active state. The state of the chromatin depends on its condensation; when the chromatin is condensed (heterochromatin), gene transcription is in an inactive state. However, during early embryogenesis, all genes are potentially able to be transcribed to mRNA.

Transcriptional control is paramount in gene regulation, which involves proteins known as transcription factors. In general, a given gene has promoter and enhancer element(s), the DNA sequences of which are bound by transcription factors to activate expression of the gene in a temporal and spatial manner. Transcription factors make up roughly 10% of the genes in the genome and can be combined in families. The zinc finger transcription factors possess at least one finger-like formation that mediates DNA binding. Interestingly, the nuclear receptors for steroid hormones, such as androgens, estrogens, and cortisol, are zinc finger transcription factors that are activated by the given hormone.

Transcription factors play key roles in the temporal and spatial activation or silencing of genes during embryonic development and during adaptive responses in differentiated cells of a grown individual. The different transcription factors form transcriptional modules to drive cell-specific gene expression. While all the cells contain the same genetic information, the complex and specific network of transcription factors determines which genes remain active and which are silenced. The basic principle is that genes are not expressed unless transcription factors activate their promoters and enhancers; likewise, genes are silenced through inhibition of transcription factors' function on the gene promoters, followed by inactivation of the promoters by methylation. Given that the methylated state of a given gene passes through cell division, a number of genes are permanently inactivated in a differentiated line of cells.

An organ is comprised of a number of differentiated cell lines that provide its functional characteristics. The mammalian ovary possesses haploid germ cells, i.e. the oocytes, as well as cells of diploid somatic lineage, i.e. granulosa, theca, interstitial, and epithelial cells. Ovarian function has evolved as a specific interplay of these differentiated cells to prepare the oocytes for ovulation and fertilization, enabling them to develop into a new individual. Transcription factors play a key part in the interplay between endocrine, paracrine, and autocrine regulators in the ovary, as they orchestrate cell-specific gene expression and respond to extracellular signals to e.g. promote adequate proliferation and steroidogenesis. During recent years, the zinc finger transcription factor GATA-4 has evolved into one of the intracellular effectors in the gonadotropin pathway, the proper function of which is crucial for normal folliculogenesis. The roles of the six GATA transcription factors have been delineated in various organs, including the heart, lung, testis, and adrenals. Little is, however, known about their temporal and spatial expression and function in the mammalian ovary.

Impaired function of the transcriptional regulation machinery has direct implications for human disease. For example, abrogated *GATA1* function causes problems in hematopoietic cells, haploinsufficiency of the *GATA3* gene causes a hypoparathyroidism, deafness and renal dysplasia syndrome, and haploinsufficiency of the *GATA4* gene is associated with congenital heart disease. Dysfunctional transcription machinery is also involved in tumorigenesis and cancer. Many cancer cells have lost significant parts of the differentiated phenotype of normal human cells and escaped cell cycle control. Cancer cells often have striking similarities with the undifferentiated embryonic cells. Considering that transcription factors may be targeted by pharmacological treatment modalities in cancer, improved understanding of their normal expression profiles and function will enable the development of novel and more efficient treatment strategies for cancer.

This study was conducted to gain insight into the role of transcription factors GATA-4 and GATA-6 and their cofactor FOG-2 in the development and function of the mouse and human ovary and in human ovarian malignancies.

REVIEW OF THE LITERATURE

1 GATA family of transcription factors

1.1 Structure and mode of action

Transcription factors are proteins that bind to specific DNA sequences in the promoters and enhancers of genes and form specific transcriptional modules for a given gene to drive its expression. The GATA family consists of six zinc-finger transcription factors that commonly recognize and bind a consensus DNA sequence, (A/T)GATA(A/G), known as the GATA motif, in gene promoters and enhancers [1,2]. The six vertebrate GATA proteins (GATA-1 to GATA-6) contain a homologous DNA-binding domain, comprising of two distinctive zinc fingers, Cys-X-X-Cys-X_{17-18}-Cys-X-X-Cys (X represents any amino acid). GATA proteins also contain a basic domain with the nuclear localization sequence adjacent to the C-terminal finger and two separate transcriptional activation domains in the N-terminal part of the protein [1,3]. Further, the C-terminal zinc finger coupled with the basic domain is necessary and sufficient for DNA binding, and is able to bind a GATA-like sequence (AGATCTT, GATT, or GATC) in addition to the consensus GATA motif (Figure 1) [1,3,4]. The C-terminal zinc finger and the basic domains have been evolutionally highly conserved among species; in fact, the vertebrate GATA genes seem to have one or two common ancestors [5].

GATA transcription factors act in concert with a number of cofactors, coactivators, or corepressors to regulate gene expression in a given cell (Figure 1). For example, GATA-4 interacts with Nkx2.5 and MEF2 proteins to drive cardiac gene expression [1,6], and with C/EBPβ and steroidogenic factor 1 (SF-1) proteins to drive endocrine gene expression [7]. GATA-4 is also able to recruit and bind large transcriptional coactivator proteins, such as CBP (CREB-binding protein), which possess epigenetic tools to convert the chromatin to an active state [8,9]. In addition, GATA transcription factors themselves may act to open the chromatin to facilitate gene expression [10]. Finally, interactions with related GATA cofactors FOG-1 and FOG-2 may fine-tune the gene activation. In initial studies on cardiac cells, FOG-2 was found to

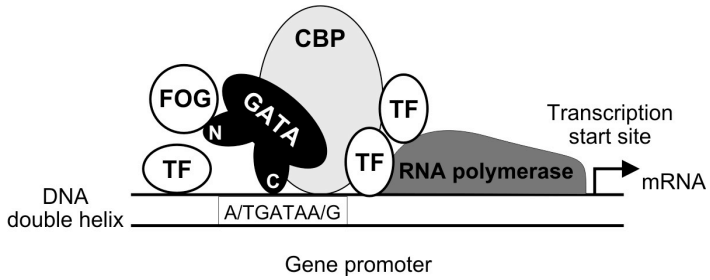

Figure 1. *Simplified presentation of mode of action of GATA transcription factors on a gene promoter.* The given GATA protein binds the GATA motif in the DNA predominantly by its C-terminal zinc finger. The cofactor FOG-1 or FOG-2 binds the N-terminal zinc finger, whereas CBP, a large coactivating transcription factor, binds the C-terminal finger. The transcriptional machinery that activates RNA polymerase to transcribe mRNA requires a number of additional transcription factors (TF).

enhance or suppress the promoter transactivation by GATA factors *in vitro* [11-14]. Interestingly, the multi-zinc finger FOG proteins bind the N-terminal zinc finger of GATA proteins, in contrast to Nkx2.5, CBP, and p300, which bind the C-terminal zinc finger (Figure 1) [1].

1.2 Tissue-specific expression and function during development

Based on similarities in amino acid sequence and expression pattern, the six vertebrate GATA factors have been divided into two subgroups. GATA-1, GATA-2, and GATA-3 are predominantly expressed and functional in the hematopoietic cell lineage, whereas GATA-4, GATA-5, and GATA-6 belong to the endoderm-, and mesoderm-derived cell lineage found in the heart, blood vessels, lung, gut, adrenals, and gonads (Table 1). The expression patterns nicely correlate with the evolution of the six GATA factors, assuming that they had two ancestors or, alternatively, one that subsequently gave rise to the two GATA lineages by duplication of the DNA sequence [5]. With regard to embryonic functions, the phenotypes of various *Gata*-null mice show that GATA-6 is required for the earliest phases of endoderm differentiation, while GATA-4 is essential or lateral-ventral folding throughout the embryo (Table 1). However, as null mice for all *Gata* and *Fog* genes (except for *Gata5*) die in embryonic stage, these models do not allow analysis of the impact of these genes on later developmental stages and adult animals. Nevertheless, studies on expression patterns followed by functional analysis have delineated a great number of processes involving these transcription factors.

GATA-4 and GATA-6 play distinctive roles in the development and function of the heart and adrenals. Fetal and postnatal cardiac cells express GATA-4 and GATA-6 [6], suggesting functions for both of these factors in this cell lineage. Indeed, a number of cardiac-specific genes possess GATA motifs in their promoters and are activated in concert by GATA-4 and GATA-6 [6,26]. With an extracellular stress stimulus, such as mechanical stretching of the ventricular walls, GATA-4 activity is enhanced through

Table 1. Expression profiles and phenotypes of mice null for *Gata* or *Fog* genes.

Gene/Protein	Examples of expression	Mouse -/- phenotype (reference)
GATA-1	Hematopoietic cells, testis	Lethal by E11 due to failure in hematopoiesis [15]
GATA-2	Hematopoietic cells, embryonic brain, endothelial cells, fetal germ cells in ovary	Lethal by E11 due to failure in hematopoiesis [16]
GATA-3	T lymphocytes, placenta, kidney, adrenal medulla, central nervous system (CNS)	Lethal by E12 due to failure in hematopoiesis and CNS [17,18]
GATA-4	Gonads, fetal adrenal, heart, pancreas, intestine, primitive endoderm	Lethal by E10 due to failure in ventral morphogenesis [19,20]
GATA-5	Heart, lung, spleen, intestine, primitive endoderm	Malformations in vagina, urethra, and clitoris in females [21]
GATA-6	Gonads, adrenal, heart, lung, liver, pancreas, intestine, primitive endoderm	Lethal by E7 due to block in endoderm differentiation [22]
FOG-1	Hematopoietic cells, testis, liver	Lethal by E12 due to failure in hematopoiesis (like GATA-1) [23]
FOG-2	Gonads, heart, central nervous system	Lethal by E15 due to failure in heart development [24,25]

activation of the mitogen-activated protein kinase (MAPK) pathway [27-29]. The activated GATA-4 may thus promote the development of cardiac hypertrophy, a disease characterized by increased size of cardiac cells and compromised cardiac function [27-29]. The GATA cofactor FOG-2 is also required for cardiac development; *Fog2*-null mice die of congestive heart failure *in utero*, with atresia of the tricuspid valve, among other malformations [24,25]. Moreover, human mutations in the *GATA4* gene have been associated with cardiac malformations, including atrial and ventricular septal defects (ASD and VSD, respectively) [30].

In the adrenals, GATA-4 and GATA-6 are expressed in specific patterns during development. Fetal adrenocortical cells in mice and humans express GATA-4 and GATA-6, whereas postnatally only GATA-6 is expressed [31]. These findings suggest that GATA-4 might support the proliferation of less differentiated cells in fetal life, while GATA-6 has roles in differentiation and high steroidogenic activity in adult adrenals. Consistent with this speculation, GATA-4 is upregulated in adrenal tumors in mice [32,33]. In addition to the adrenals and the heart, murine gonads express GATA-4 and GATA-6 [34-36]. The putative roles of these factors in the gonads, especially in the ovary, will be presented and discussed here.

2 Embryonic development of the female reproductive tract

2.1 Bipotential gonad and sex ducts

The capability of the gonadal primordium to differentiate into two gonads is a unique situation in embryology [37]. Prior to differentiation, a common precursor, the urogenital ridge, appears in the intermediary mesoderm of the embryo, later giving rise to the gonads and adrenals [37,38]. The mammalian germ cells migrate from the extraembryonic mesoderm of the yolk sac to the urogenital ridge. Germ cell arrival subsequently induces proliferation of the somatic epithelial cells of the urogenital ridge and development of the bipotential gonadal primordium (Figure 2) [37,38]. Besides the undifferentiated gonad, an XY or XX embryo also possesses the anlagen for the rest of the male and female reproductive tract, the Wolfian and Müllerian ducts, respectively (Figure 2). These sex ducts form within the mesonephros in a process requiring proper function of a number of genes, such as *Wnt4* and *Wnt7a* [39,40]. The development of the Wolfian duct derivates, i.e. the epididymides, *vas deferens*, and seminal vesicles, in males and the Müllerian duct derivates, i.e. the fallopian tubes, uterus, and upper vagina, in females is dependent on anti-Müllerian hormone and testosterone produced by the fetal testis and estrogen produced by the ovary.

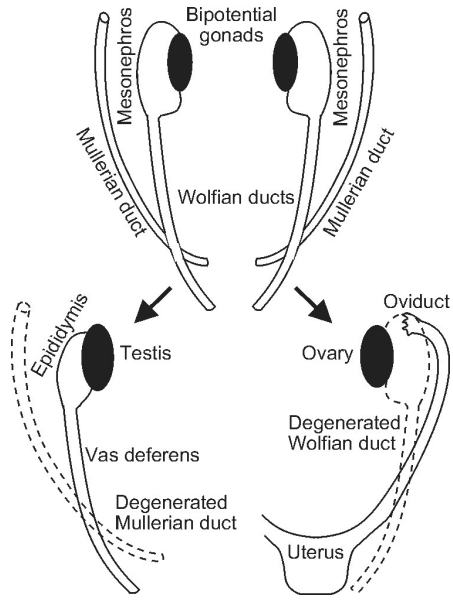

Figure 2. *Schematic presentation of the fetal gonads, the sex ducts, and their sexual differentiation*. The sex ducts, Wolfian and Müllerian, and the bipotential gonads arise from the mesonephros. The Y chromosome driven signaling cascade differentiates the gonad into the testis and promotes the development of the Wolfian derivates and the regression of the Müllerian ducts in males. In female, the ovary and the Müllerian duct derivates develop, while the Wolfian ducts regress. Adapted from [37].

2.2 Regulation of sexual differentiation

The differentiation of the mammalian ovary is commonly explained as a passive event occurring in the absence of Y chromosome. Presence of the Y chromosome, by contrast, triggers a cascade that differentiates the gonadal primordium into the testis and induces testicular gene expression in somatic cells. In the XX gonad or ovary, most of these genes are either not expressed or downregulated immediately beyond the undifferentiated bipotential stage [41]. Expression patterns of the essential genes in this process in the mouse are depicted in Figure 3; the function of these genes is discussed here based on the data obtained from mice, unless otherwise indicated. The initial switch in the testis cascade is the wave-like expression of Sry (sex-determining region of the Y chromosome) at E10.5-E12.5 in the somatic cells of the indifferent gonad [42]. The role of Sry in mice was verified by forcing Sry expression in the somatic cells of

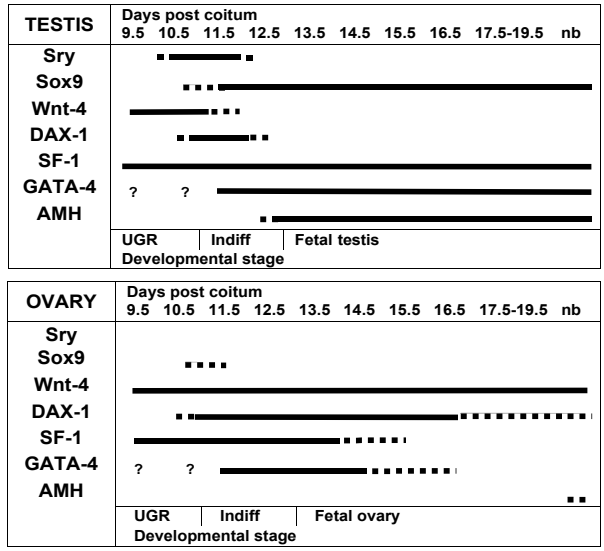

Figure 3. *Expression profiles of essential genes during gonadogenesis and sex determination.* Gene expression in testicular and ovarian somatic cells presented at the fetal (nb, new born) developmental stages (UGR, urogenital ridge; indiff, indifferent, i.e. the bipotential gonad). Sox9, SF-1, and GATA-4 directly activate the *AMH* promoter, whereas DAX-1 may modulate this action. Wnt4 is an extracellular signaling molecule, that works upstream of DAX-1. GATA-4 expression in the early developmental stages is unknown.

XX gonads, which then resulted in a testis [43]. While the *Sry* gene was identified and characterized more than a decade ago [44,45], its molecular function is still poorly understood. Rather than directly activating gene expression, Sry may remodel the chromatin, thus facilitating specific gene expression [46]. Most probably, Sry contributes to the activation of Sox9 expression, which in turn activates downstream genes in the testis cascade [47,48]. Although both Sry and the Sry-related Sox9 possess an high mobility group box DNA-binding domain, only Sox9 seems to function as a transcription factor. Similarly to Sry, the introduction of Sox9 expression in XX gonads gives rise to a testis [49].

Anti-Müllerian hormone (AMH)

The differentiating testis produces two hormones essential for male reproductive development: testosterone and anti-Müllerian hormone. The model of two testicular hormones causing sexual differentiation of the reproductive tract anlagen was first suggested by Dr. Jost in the 1940s [50,51]. His pioneering experiments in which a testis or a ovary was removed and then grafted onto male or female rabbits indicated that two testicular hormones were needed to enable male differentiation of the sex ducts. Years later, the specific inhibitory hormone for the Müllerian duct was purified and its cDNA isolated [52-54]. This important hormone has two equally common names: anti-Müllerian hormone (AMH) and Müllerian inhibiting substance (MIS); the name AMH is used in the present work, except in Study I. Experiments on mice, including null mutation and overexpression of the gene, have verified that AMH induces regression of the Müllerian duct in males, and represses Leydig cell proliferation and steroidogenesis in the testis [55-57]. Likewise, the absence of AMH in the female embryo allows the proper development of Müllerian duct derivates, i.e. the oviduct, uterus, and upper vagina. AMH is a homodimeric glycoprotein that belongs to the transforming growth factor β (TGF-β) superfamily, the members of which are involved at various points of growth and differentiation [58,59].

As one might expect, AMH is expressed in a sexually dimorphic pattern in the gonads (Figure 3). In the testis, AMH is upregulated in the developing Sertoli cells immediately after the onset of testicular differentiation, whereas in the ovary AMH is only expressed in postnatal granulosa cells [60,61]. Given the AMH function, its fetal expression is under strict regulation and involves several factors [41,58,62]. Expression patterns of these genes in fetal gonads are, once again, dimorphic, accompanying AMH expression (Figure 3). Sox9, SF-1, and GATA-4 induce *AMH* by binding directly on the promoter [63,64], whereas DAX-1 more likely acts as a modulator of the SF-1 action [65]. The *AMH* action of Sox9 in fetal testis was elucidated by an engineered mutation in the Sox9 binding site of the *AMH* promoter. The mutation resulted in impaired AMH expression, testis formation, and Müllerian duct regression in XY mice [66]. Moreover, forced Sox9 expression in an XX mouse led to abnormal *AMH* induction and subsequent sex reversal of the female reproductive tract [49].

Steroidogenic factor 1 and GATA-4

Steroidogenic factor 1 (SF-1) is widely involved in endocrine function in the pituitary gland, adrenals, and gonads [67]. SF-1 is an orphan nuclear receptor, acts as a transcription factor, and is readily expressed in the indifferent urogenital ridge at E9.0, prior to Sry, Sox9, and AMH [67,68]. Later, SF-1 expression is downregulated in the fetal ovary, but sustained in the testis (Figure 3). Null mutation of SF-1 results in gonadal and adrenal agenesis due to deterioration of the urogenital ridge and adrenal primordium by E12.5 through apoptosis [69]. These mice die within eight days after birth because of adrenocortical insufficiency. Subsequent studies have detailed that *AMH* upregulation in the testis is intimately associated with SF-1 [68,70]. Mutation of the SF-1 binding site in the *AMH* promoter results in significantly reduced levels of AMH in male mice *in vivo* [66]. In these mice, AMH was, however, expressed in sufficient amounts to promote Müllerian regression; this did not occur when the Sox9 site was mutated. Interestingly, SF-1 interacts directly with Sox9 [63], suggesting that the resulting synergism on the *AMH* promoter is essential to achieve high levels of AMH in the fetal testis.

SF-1 has also been shown to physically interact with GATA-4 in order to synergistically activate *AMH* [35,64,71]. Although not verified *in vivo*, the proper GATA-4 action, the endogenous GATA-4:SF-1 synergism, and the corresponding binding sites on the *AMH* promoter are essential for full *AMH* activity in cultured rat Sertoli cells [71,72]. Furthermore, the promoter of the SF-1 encoding gene, *FTZF1,* is activated by GATA-4 [73]. The GATA-4 protein directly binds its target sequence on the *FTZF1* and *AMH* promoters, and in the initial study [35] the GATA-4 expression pattern was found to be dimorphic in fetal gonads, resembling that of the other "testis genes" (Figure 3). This very likely benefits the activation of AMH expression by GATA-4 in the fetal testis, as part of the transcriptional orchestration for AMH. Proper function and interaction of GATA-4 and FOG-2 are required for normal male differentiation and for testicular gene expression, including expression of Sry, Sox9, and AMH [74].

2.3 Passive versus active signaling in the fetal ovary

The old theory or dogma on passive differentiation of the gonadal primordium into the ovary in the absence of Y chromosome and testis-producing factors is losing its strength, as active signaling in the differentiating ovary has been demonstrated. In addition to the lack of AMH, the fetal ovary is devoid of androgen production. In

males, testosterone is required for the development of Wolfian duct derivates epididymides, *vas deferens*, and seminal vesicles (Figure 2). Leydig cell-produced testosterone also acts paracrinally in the testis and is required for the testes to descend. In females, ectopic or abnormal levels of testosterone production by the gonads or adrenals lead to masculinization of the ovary and external genitalia, and thus the pseudohermaphroditism most commonly seen in congenital adrenal hyperplasia [75]. The ability to suppress fetal testosterone production is therefore essential for normal female development. Female development also requires proper estrogen function, given that mice null for estrogen receptors undergo postnatal sex reversal and abnormally express Sox9 and AMH [76]. Light has been shed on the mechanisms of "female signaling" via mouse models and findings in human disorders.

Wnt4 and DAX-1

Wnt4, a member of the wingless-related MMTV integration site family of locally acting secreted factors, has been determined to be essential for female reproductive development through studies on *Wnt4*-deficient mice [40]. In its absence, the Müllerian duct fails to develop, while the Wolfian duct continues to develop. Even more importantly, the mice have masculinized ovaries with Leydig-like cells producing androgens and expressing the necessary steroidogenic enzymes [40]. In addition, macroscopically, the *Wnt4*-deficient ovary is round and resembles the testis. Thus, Wnt4 appears to be required to suppress Leydig cell development, testosterone production, and the resulting preservation of the Wolfian ducts by testosterone. External genitalia were not, however, virilized or masculinized in *Wnt4*-deficient mice. Subsequent studies have shown that Wnt4 is upstream of DAX-1 [77,78], a regulator able to repress SF-1-mediated promoter activation [65]. Not surprisingly, Wnt4 and DAX-1 are expressed in the bipotential stage, together with SF-1, but are downregulated in the testis at the onset of its differentiation (Figure 3). The picture recently became more complex, with results showing that in *Wnt4*-deficient mice the Sertoli cell differentiation in the fetal testis is compromised at least for a short while [79]. Interestingly, however, both the XY and XX gonads of *Wnt4* deficient mice have increased numbers of steroidogenic cells [40,79].

DAX-1 is suggested to be an "ovarian-determining" gene, given the human phenotype of genetically XY females who possess an extra copy of this X chromosomal gene [80]. Moreover, overly high levels of DAX-1 in mice, achieved by introduction of an extra *DAX1*, are able to antagonize the Sry action and retard testis differentiation [81], leading to the conclusion that DAX-1:SF-1 dimer may repress testis genes such as Sox9. Likewise, Sry in the testis may prevent the DAX-1:SF-1 function [81]. In another mouse model, however, null mutation for *DAX1* did not affect ovarian development or female fertility, instead abrogating postnatal spermatogenesis [82]. Thus, rather than being "ovarian-determining" genes, Wnt-4 and DAX-1 appear to be "anti-testis" or "anti-male" factors in the ovarian cascade. Moreover, a given gene may act differently in humans and mice. Interestingly, DAX-1 is able to abrogate the SF-1:GATA-4 synergism on the *AMH* promoter [83]. Whether GATA-4 has a role in the Wnt4 pathway remains unknown; the role of GATA factors in the development of the female reproductive tract also has not been addressed.

2.4 Human reproductive tract development disorders

Given the elaborate molecular mechanisms underlying the differentiation of the gonads and the sex ducts into male or female

reproductive organs, the process may sometimes be disrupted. Aberrations in the genes involved may cause complex developmental disorders, i.e. syndromes with gonadal dysgenesis, total or partial sex reversal of the reproductive tract, and/or dysgenesis in the organs derived from the sex ducts (Table 2). In general, most of the syndrome phenotypes have similarities with the corresponding genetic aberrations in the engineered mouse.

Hitherto, three cases with mutations in the gene encoding SF-1 (*FTZF1)* have been demonstrated as the cause of 46,XY sex reversal with gonadal dysgenesis and adrenal insufficiency [85-88]. Two of these cases present with retained Müllerian duct derivates, indicating insufficient AMH production in fetal life; two of the mutations are heterozygous. Initially, the mutant SF-1 protein (G35E; heterozygous) was found to have no dominant negative effect in *in vitro* gene expression assays, indicating that haploinsufficiency in *FTZF1* causes the clinically severe phenotype [86,89]. However, subsequent *in vitro* analysis revealed that the mutated SF-1 protein (G35E) may act as a dominant negative competitor to the synergistic AMH activation by GATA-4 and SF-1 [90]. These findings suggest that proper GATA-4:SF-1 synergism is essential for human gonadal and adrenal development.

The two sex chromosomes and the genes they possess clearly have a special role in human gonadal differentiation. First of all, SRY has a key role in determining testis development, whereas duplication of the X chromosomal *DAX1* has been identified as the cause of the dosage-sensitive sex reversal syndrome [80]. Somewhat similarly to mice, XY individuals with an extra *DAX1,* develop as females, despite the presence of SRY [80,81]. On the other hand, mutations in *DAX1* are associated with adrenal hypoplasia congenita, which is an X-linked syndrome

Table 2. Selected genes, the aberrations of which cause defects in human reproductive tract development (adapted from tables in [75,84]).

Gene	**Location**	**Protein and function**	**Phenotype** (**M**=mutation, **D**=duplication)
SRY	Yp11	Transcription factor	**M:** XY sex reversal, male gonadal dysgenesis; XX sex reversal in case of ectopic expression
SOX9	17q24	Transcription factor	**M:** Campomelic dysplasia, XY sex reversal, male gonadal dysgenesis **D:** XX sex reversal
FTZF1 (for SF-1)	9q33	Nuclear receptor, transcription factor	**M:** XY sex reversal, gonadal dysgenesis, adrenal insufficiency
DAX1	Xp21.3	Nuclear receptor, transcription factor	**M:** AHC, HHG **D:** XY sex reversal, gonadal dysgenesis
WNT-4	1p35	Wnt-type signaling molecule	**M:** Müllerian agenesis/regression and androgen excess in female
AMH	19q13	TGF-β-type signaling molecule	**M:** Persistent Müllerian duct syndrome in male
AMHRII	12q12-13	Serine-threonine kinase receptor for AMH	**M:** Persistent Müllerian duct syndrome in male

AHC = adrenal hypoplasia congenita, HHG = hypogonadotropic hypogonadism

characterized by adrenal insufficiency and hypogonadism [91,92]. Intriguingly, most of these mutations abrogate the function of DAX-1 as a transcriptional repressor of SF-1 [91]. Finally, aberrations in the other "ovarian-determining" or "anti-testis" gene, *WNT4,* have been associated with impaired development of the female reproductive tract. A recent report presented a loss of function mutation in *WNT4* in a patient with primary amenorrhea, absent Müllerian duct derivates, and clinical signs of androgen excess [93]. This human phenotype has significant similarities with *Wnt4*-deficient mice [40] that exhibit failure in Müllerian duct development and masculinized androgen-producing ovaries. Taken together, this data provides great insight into the genes and pathways essential for female development in mammals.

3 Ovarian function

3.1 Endocrine regulation of folliculogenesis

The follicle, the major functional unit in the ovary, is regulated in a complex manner by both endocrine and paracrine components. The endocrine hormones are produced in the hypothalamus, i.e. gonadotropin-releasing hormone, and in the anterior pituitary, i.e. follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In general, FSH and LH are secreted by the pituitary gland according to the phase of the menstrual cycle. Hormone production is regulated by direct feedback mechanisms from the gonads, which can be ectopically mimicked to suppress reproductive function. Suppression of gonadotropin secretion, and therefore folliculogenesis and ovulation, has been the main endocrine tool for female contraception for decades, while corresponding approaches for male contraception remain to be proven useful or reliable [94,95]. This section presents studies of both murine and human ovarian function.

During fetal life, a pool of primordial follicles is formed as the somatic cells surround the oocytes (Figure 4). To some extent before birth, but more significantly at puberty, these quiescent follicles coordinately enter the growth phase as the oocyte increases in size and the squamous granulosa cells become cuboidal in order to form primary follicles. The oocyte continues to grow and granulosa cells proliferate; when two granulosa cell layers surround the oocyte, a theca cell layer is formed around the follicle. In the 1960's, paracrine regulatory factors were shown to be required for folliculogenesis at the preantral follicle stage [96]; endocrine regulation becomes vital by the antral follicle stage. During antrum formation, the granulosa cells separate into two populations, i.e. cumulus cells immediately surrounding the oocyte and mural cells (Figure 4). In the last developmental stage, the dominant follicle is prepared to ovulate in each cycle.

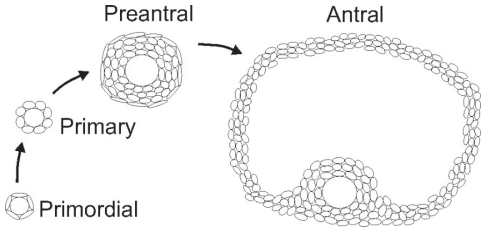

Figure 4. *Stages of folliculogenesis*. Granulosa cells surrounding the oocyte are squamous at the primordial stage, but upon recruitment at the primary stage they become cuboidal. From the preantral stage onwards, theca cells surround the follicle (presented as flat cells in the preantral follicle; omitted in the illustration of the antral follicle). Antral follicles possess two granulosa cell populations, i.e. mural cells and cumulus cells, that surround the oocyte.

FSH and LH

FSH is essential for ovarian function, upregulating a number of genes in granulosa cells [97,98], such as cyclin D2 involved in proliferation, inhibins that have both local and distant functions, aromatase enzyme required for estrogen production, and LH receptor (LHR), the expression of which precedes terminal differentiation of granulosa cells and ovulation. Correspondingly, FSH-receptor (FSHR) expression is restricted to granulosa cells, whereas LHR is expressed in both theca and granulosa cells. The gonadotropins play key roles in the regulation of steroidogenesis; LH stimulates androgen synthesis in theca cells, and FSH induces estrogen conversion from the androgen substrate in granulosa cells. This model of gonadotropin function has been named "the two gonadotropin-two cell"

concept of estradiol production in the ovary [99]. In mice null for *FSHR* or *LHR*, the serum estradiol level is dramatically decreased, the uterus is atrophic, and the ovary is significantly decreased in size [100-102]. The defects in folliculogenesis differ, however, between *FSHR*- and *LHR*-deficient mice. Both mice have no mature/ preovulatory follicles or luteal glands, but in *FSHR*-null mice the growth arrest is at the preantral stage [102], whereas in *LHR*-null mice it is at the antral stage [101]. These findings demonstrate the need for proper FSH action at the preantral stage and beyond, and LH action at the follicular maturation and ovulation stages.

From the clinical point of view, mutations in either of these receptors have a direct implication on human reproductive function [103]. An inactivating mutation in *LHR* in a woman with a normal karyotype results in a relatively mild phenotype: normal primary and secondary sexual characteristics, but amenorrhea and low levels of estradiol [104]. Histologically, follicles of all stages, other than preovulatory follicles or a corpus luteum, were detected in the ovarian sample [104]. An inactivating mutation in *FSHR* has been linked with hypergonadotropic ovarian dysgenesis characterized by poorly developed secondary sexual characteristics, streak gonads, and primary amenorrhea [105]. Ovarian maturation at puberty is arrested by inadequate FSH action in the ovary, reflected as a high number of primordial and primary follicles, but only an occasional more-developed follicle [106].

Intracellular gonadotropin pathway

In a given cell, the classical gonadotropin response employs G-proteins coupled with hormone receptor, adenylyl cyclase (AC), cyclic adenosine monophosphate (cAMP), and protein kinase A (PKA) (Figure 5). At the gene promoter level, the common signal mediator is the cAMP-responsive-element (CRE) that is bound by the phosphorylated CRE-binding protein (CREB). A number of gonadotropin- or cAMP-responsive genes, however, lack the consensus CRE site, and the response requires other endocrine cell-specific transcription factors such as SF-1, GATA-4, or GATA-6 (Figure 5) [7,67]; all

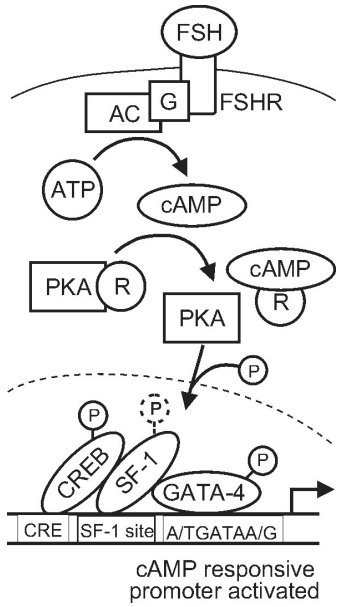

Figure 5. *The classical intracellular pathway activated by gonadotropin FSH or LH.* The solid upper line represents the cell membrane and the broken line the nuclear membrane. FSH binds its receptor FSHR, which couples with a G-protein (G). The activated G then activates adenylyl cyclase (AC), which produces the cyclic adenosine monophosphate (cAMP). cAMP binds the regulatory subunits (R) of protein kinase A (PKA), which then localize into the nucleus and phosphorylate (P) the cAMP-responsive element (CRE) -binding protein (CREB). In addition, PKA is able to phosphorylate GATA-4 and SF-1, which together with CREB activate transcription of target genes in the nucleus. However, SF-1 is constitutively phosphorylated by other kinases, and its role in this cascade is not clear.

of these are expressed in murine gonads [34,36,67,107]. Similarly to AMH activation in the fetal testis, GATA-4 and SF-1 synergistically activate genes for key enzymes in steroidogenesis [7,73]. Aromatase enzyme is needed to produce estrogens from the androgen substrate in granulosa cells. Furthermore, GATA-4 mRNA is upregulated by FSH in the mouse granulosa and Sertoli cell tumor cell *in vitro* [36], but cAMP fails to upregulate GATA-4 in primary rat Sertoli cells [108,109]. Instead, detailed *in vitro* analysis revealed that the GATA-4 function is activated in endocrine cells by phosphorylation of serine 261 by PKA in response to cAMP, depicting GATA-4 as a novel downstream effector in this pathway [7,108,109]. Interestingly, the MAPK pathway, more specifically, the extracellular signal-regulated kinase (ERK), is activated independently of PKA in response to FSH, resulting in modulation of granulosa cell steroidogenesis [110,111]. It is, thus, evident that G-protein-coupled hormone receptors, like FSHR and LHR, also use other pathways besides the one connected to PKA [112].

3.2 Paracrine interplay between oocytes and granulosa and theca cells

With no doubt about the critical roles played by endocrine gonadotropins in folliculogenesis, it has become evident that both the oocytes and the somatic cells produce a variety of paracrine factors affecting follicle development and modulating gonadotropin action [113,114]. *In vitro* studies utilizing follicle cultures show that follicular cells undergo luteinization if the oocyte is violently removed [96,115]. In addition, the oocyte supplies specific morphogens that preserve phenotype and gene expression in cumulus cells [113]. On the other hand, support by the follicular somatic cell is fundamental in providing the oocyte with the necessary nutrients and growth factors throughout folliculogenesis [116].

Members of the TGF-β superfamily have essential roles in the paracrine interplay of ovarian cells. Growth and differentiating factor 9 (GDF9), bone-morphogenic protein 15 (BMP-15; also known as GDF9B), and BMP6 are primarily oocyte-secreted [117-120], while activins, inhibins, and AMH are expressed and secreted by somatic cells [61,121-123]. The corresponding receptors for these growth factors are differentially expressed in follicular cells, further fine-tuning the three-cell interaction [123]. In general, the TGF-β signal in a given cell is mediated through specific serine/threonine kinase receptors expressed on the cell surface. The ligand-activated receptors phosphorylate the classical intracellular TGF-β signal mediators, the Smad proteins, which form complexes and localize to the nucleus to drive transcription of target genes together with cell-specific transcription factors [59]. To achieve functional diversity in cellular responses to TGF-β ligands, the corresponding receptors are able to activate kinases in a number of other pathways besides phosphorylation of Smads. As an example, the activated TGF-β receptors alternatively activate p38, a pivotal kinase in the MAPK pathway [124]. In addition, the Smad3/Smad4 complex is able to directly activate PKA [125].

Oocyte-derived GDF9 and BMP15

Considerable progress has been made in the past decade in identifying critical oocyte factors. Of these, GDF9 is a cornerstone and is predominantly expressed in mammalian oocytes [113,117-119]. Mice null for *Gdf9* have abrogated reproductive function, reflecting hypergonadotropic hypogonadism with no antral follicles and no ovulation [126]. While organogenesis is normal, folliculogenesis arrests at a single layer of cuboidal granulosa cells with no theca. The

oocytes subsequently die and the somatic cells luteinize, resembling the findings in oocytectomized follicles [127]. Thus, GDF9 may directly suppress FSH-dependent granulosa cell differentiation [128,129]. The mitogenic activity of GDF9 has been further studied by immunoneutralizing GDF9 *in vitro* to counteract its effect on granulosa cells [130]. This data indicated that GDF9 is powerful but not sufficient on its own to drive granulosa cell proliferation by the oocyte [130]. An additional mitogen could indeed be BMP15 (GDF9B), the lack of which causes only minimal ovarian histopathological defects, but a decreased ovulation rate [131]. Altogether, these findings suggest that GDF9 and BMP15 may work as heterodimers to synergistically regulate granulosa cell gene expression and proliferation, and subsequently, the ovulation rate.

Inhibin

Inhibin expression and production localizes to granulosa cells in the postnatal ovary [122]. The secreted inhibin A and inhibin B are heterodimers of a common α-subunit and βA- or βB-subunits, respectively. As endocrine hormones, the inhibins suppress production of FSH in the pituitary. In addition, accumulating evidence suggests that inhibins also play a paracrine role in the ovary [122,132]. Given that the supply of the α-subunit is critical for inhibin secretion, regulation of *inhibin-α* is fundamental and involves the endocrine FSH, the paracrine activin, and oocyte-derived factors [122,133]. Of the latter, GDF9 is one of the most likely mediators, stimulating inhibin production in human granulosa-luteal (GL) cells *in vitro* [134]. Cloning and further analysis of the *inhibin-α* promoter have demonstrated specific targets for transcriptional regulators, e.g. sites for CREB, estrogen receptors, SF-1, and GATA-4 [34,135,136]. Moreover, identification of the synergistic and cAMP-activated effects of CREB and SF-1, as well as of GATA-4 and SF-1, has shed light on *inhibin-α* regulation at the promoter level [73,135].

AMH

In addition to its essential role in sexual differentiation of the reproductive tract, AMH is a granulosa cell product capable of suppressing the follicular growth induced by FSH and epidermal growth factor [121,137-141]. The *Amh*-null mice have an increased number of follicles committed to grow, resulting in loss of the primordial follicle pool at a premature age [139,141]. Taken together, the results suggest that AMH is involved in suppressing primary growth recruitment of primordial follicles, most likely through autocrine action on granulosa cells [142]. As is the case in testicular Leydig cells [55,143], AMH may also suppress steroidogenesis in the ovary [137], e.g. by abrogating PKA-induced enzyme expression [143]. Regulation of *AMH* in postnatal testicular Sertoli cells differs somewhat from that in the fetal testis and requires FSH action through cAMP on a distal enhancer at -3068 bp of the *AMH* promoter [144,145]. In the fetal testis, the more proximal -180 bp promoter is sufficient to promote AMH expression [70]. Most recent studies show that the oocyte also contributes to *AMH* regulation in granulosa cells [146], thus having remarkable similarities to *inhibin-α* regulation. In addition, both of these genes are direct targets for SF-1 and GATA proteins [35,73,135].

AMH is evolving as a potent serum marker for ovarian reserve. In normo-ovulatory women, AMH serum levels decrease with advancing age, concomitant with a decrease in the number of antral follicles [147]. In anovulatory women with fertility problems this decline is, however, less obvious, suggesting an increased ovarian reserve and delayed ovarian aging [148,149]. With respect to assisted reproduction by *in vitro*

fertilization (IVF), AMH measurements may have prognostic value in evaluating the outcome of a given pregnancy [150,151]. Additional clinical implications for AMH include use as a marker for follow-up and as a chemotherapeutic agent in cancer.

3.3 Granulosa cell proliferation

Proliferation of granulosa cells is a key component in folliculogenesis and is regulated by endocrine, paracrine, and autocrine factors during each ovulatory cycle (Figure 6) [97,98]. Accordingly, oocyte-derived factors, FSH, and estrogens are the main players in this orchestra. Follicular growth is arrested in mice deficient in *FSH* or *FSHR* [100,152]. Of the FSH-activated genes, *cyclin D2* is of special interest given that its null mutation in mice leads to impaired granulosa cell proliferation in response to FSH [153]. In brief, the cell cycle is regulated by cyclin-dependent protein kinases that couple with the cyclin proteins to activate downstream processes and DNA replication. In the G1 phase of the cell cycle, preceding the DNA replication phase, the D-type cyclins are critical in controlling the cell-cycle clock. Of the D-type cyclins, cyclin D2 appears to be essential and specific for granulosa cells [98,153]. The upregulation of cyclin D2 by FSH also involves ERK [154]. The molecular mechanisms at the *cyclin D2* promoter are starting to unravel, involving at least the forkhead transcription factor FOXO1 and Smad proteins activated by activins [155]. The *cyclin D2* promoter also contains consensus GATA-binding site(s) [156,157], and the ERK pathway and GATA-4 activate cyclin D2 expression and proliferation at least in pulmonary cells [158]. In women, the inactivating mutation of the *FSHR* interestingly leads to an arrest of folliculogenesis [105,106], which among other possibilities may be connected to abrogation of cyclin D2-dependent proliferation.

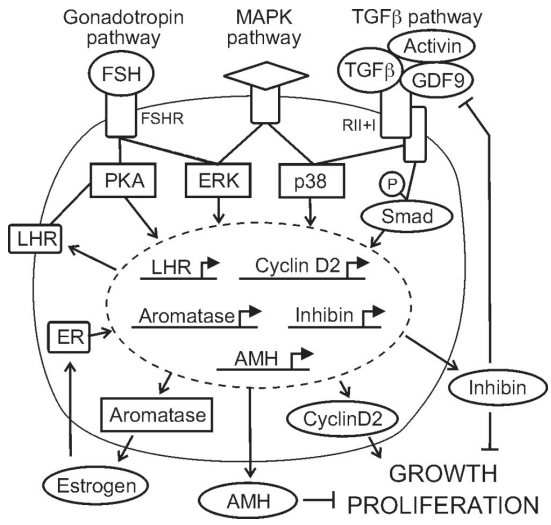

Figure 6. *Schematic summary of signaling cascades regulating expression of granulosa cell genes involved in proliferation.* Granulosa cells and oocytes secrete paracrine/autocrine ligands of the TGF-β family (e.g. activin, inhibin, AMH, TGF-β, and GDF9); ligands for the mitogen-activated protein kinase (MAPK) pathway are not presented here. However, FSHR and TGF-β receptors (types II and I) activate ERK and p38, kinases of the MAPK pathway. The classic TGF-β response involves phosphorylation of the Smad proteins, which localize to the nucleus to act as transcription factors. All of these pathways are involved in regulation of LH receptor (LHR), cyclin D2, aromatase, and secreted inhibin and AMH.

The autocrine factors activin, inhibin, and AMH have also been implicated in granulosa cell proliferation. Activin has a key role in promoting proliferation and potentiates the action of FSH by, for example, upregulating FSHR, while inhibin may antagonize the mitogenic effects of activin and possibly GDF9 [122,123,159]. Although the underlying molecular mechanisms are not fully known, AMH inhibits proliferation by suppressing the function of FSH and growth factors and the initial growth of the follicles [58,142]. The role of the oocytes is remarkable in that it activates granulosa cell proliferation as well as expression of inhibin and AMH, i.e. suppressors of proliferation. Oocyte signals are essential for FSH-independent granulosa cell proliferation in primary- and preantral-stage follicles [113,114]. Oocyte-derived GDF9 is critical for proliferation in view of the follicular growth arrest in *Gdf9*-deficient mice and the ability of GDF9 to directly stimulate DNA synthesis [126,129]. All in all, mechanisms integrating the fundamental but complex autocrine and oocyte signals in granulosa cells are just starting to become to unraveled.

3.4 Apoptosis in the ovary

Granulosa cells constantly proliferate during folliculogenesis and in each menstrual cycle. The preovulatory dominant follicle is, however, the tip of the iceberg as to the number of follicles recruited to growth. If the follicles were not adequately disposed of, the ovary would eventually fill the retroperitoneum with granulosa cell masses. Thus, self-controlled death by apoptosis is the inevitable endpoint of most of the follicles. In postnatal life, apoptosis is prominent in the somatic cells of atretic follicles [160], whereas in fetal life a great number of the newly developed oocytes undergo apoptosis [161]. In both the fetal and adult ovary, apoptosis is regulated by members of the Bcl-2 family and executed by caspase enzymes. The cyclic recruitment by FSH is key in rescuing preantral and antral follicles from undergoing apoptosis [162]. On the other hand, reduction in activity of the ERK pathway due to withdrawal of tropic hormone FSH is involved in committing the follicles to apoptosis [163]. The sex hormones seem to have adverse effects on granulosa cells in this respect; androgens promote apoptosis, while estrogens inhibit the action [164]. The initial study of GATA-4 expression in the mouse ovary interestingly suggested that GATA-4 would protect granulosa cells from undergoing apoptosis [36]. This role for GATA-4 has been supported by findings in the mouse heart, in which GATA-4 is capable of protecting the cardiac cells against cytostatic-induced apoptosis [165]. The underlying molecular mechanisms are connected to direct upregulation of anti-apoptotic gene *Bcl-X* by GATA-4 [165].

4 Ovarian granulosa cell tumors

4.1 Incidence and clinical characteristics

Ovarian cancer is the fourth-ranking malignancy in women in Finland. Granulosa cell tumors (GCTs) are the most common representative of the group of sex cord stromal tumors of the ovary, accounting for 5% of all ovarian cancers [166]. As the group's name indicates, these tumors are derived from somatic stromal cells of the gonad. The incidence of GCTs ranges from 4 to 17 cases per million worldwide, with peaks occurring at late reproductive age and menopause [167-172]. In Finland, GCT incidence has been reported to be from 4.7 to 8.7 cases per million, varying somewhat from one decade to another due to alterations in use of hormone treatments [172]. GCTs can occur at any age and in both sexes. In contrast to the predominant adult subtype of GCT, a juvenile subtype is usually diagnosed in children and adolescents [173,174]. This diagnosis is, however, mainly based on tumor morphology rather than age of the patient.

Excessive hormonal activity of tumor cells and overproduction of estrogens and inhibins are the biological features most frequently causing the clinical signs and symptoms of a GCT, both prior to menarche and in adulthood (Table 3) [175-178]. Juvenile GCT is one of the main causes of precocious puberty [173,174]. At post-menarche, various disturbances in the menstrual cycle, including prolonged fertility problems [178], usually lead to reference to a clinic. GCTs can also be discovered microscopically, e.g. as a secondary finding to an endometrial hyperplasia or carcinoma in the uterus [176,177]. Oftentimes, GCTs are hemorrhagic and large, >10-15 cm in

Table 3. Typical clinicopathological characteristics of human granulosa cell tumors (summarized from [177-181]).

Selected granulosa cell tumor characteristics		Notes
Signs and symptoms	Precocious puberty, secondary amenorrhea, meno-metrorrhagia, postmenopausal bleeding, infertility, endometrial hyperplasia or cancer, abdominal pain	
Pathologic features		
Gross appearance	Large (>10 cm), solid, cystic, hemorrhagic, "tan-yellow" (indicating steroid production)	May present in microscopic size
Microscopic	Small, round, or oval granulosa cells with "coffee bean"-like uniform nuclei; variety of histological patterns (see text); nuclear atypia rate and mitotic index usually low	May also have similarities with undifferentiated carcinoma
Immunohistochemical markers	Inhibin-α	Positive (in most cells)
	Epithelial membrane antigen	Negative (usually)
	Calretinin (a mesothelial marker)	Positive (usually)
	Anti-Müllerian hormone	Positive (diffusely)
	CD99	Positive (in some cells)
	Melan-A (a melanocytic marker)	Positive (in steroidogenic cells)

diameter, manifesting as painful abdominal mass [167,170,175].

4.2 Diagnosis, treatment, and prognosis

Although the signs and symptoms often give clues, GCT diagnosis is typically made at surgery in conjunction with an intra-operative assessment by a pathologist. Subsequent morphological analysis coupled with immunohistochemical markers aims at verifying the diagnosis (Table 3). The operation strategy depends on the clinical stage of the tumor and the patient's age and wishes to preserve fertility [176,177]. In postmenopausal women, a radical hysterectomy, including bilateral ovariectomy, is recommended even for stage I tumors. If fertility is to be preserved, only the diseased ovary is removed and lymph node biopsies performed. Adjuvant treatment is less commonly considered, as most of the tumors present at an early clinical stage (stage Ia). The chemotherapeutic approach has given varying results; whether an individual patient benefits from adjuvant therapy remains unclear, except for in the rare case of spread disease [176,177].

In contrast to epithelial ovarian cancer, GCTs are considered of to be of low malignant potential. The 10-year survival rate is favorable, varying from 60% to 95% [167,168,173,182,183]. However, GCTs have a high risk of long-term recurrence, even multiple recurrences, and may reappear as late as 37 years after the primary diagnosis and treatment [171,175,184]. Furthermore, even a microscopic primary tumor has been documented to present with a 20-cm-sized recurrence after six years [185]. These peculiar features and the limited prognostic tools challenge the clinicians when evaluating prognosis at diagnosis. None of the available tissue markers are proven useful for evaluating prognosis, leaving the clinical stage as one of the only tools in clinical practice. This stage has, however, significant limitations since the guidelines set for clinical staging of ovarian cancer by the International Federation of Gynecology and Obstetrics (FIGO) have been primarily designed for the most common epithelial ovarian cancers.

4.3 Pathologic and cytogenetic characteristics

The sex cord stromal tumors have similarities and differences in the two gonads. In addition to GCTs of adult and juvenile type, they include Sertoli cell tumors, Sertoli-Leydig cell tumors, theca cell tumors (i.e. thecomas), and a group of unclassified tumors [166,181]. The typical characteristics of ovarian GCTs are summarized in Table 3. The histological patterns of GCTs include micro- or macrofollicular, tubular, trabecular, and insular variants, which together can be considered morphologically as a differentiated type. Some of the tumors are of a diffuse or sarcomatoid type that occasionally can be misdiagnosed as an undifferentiated carcinoma, especially at an intraoperative assessment of a frozen section [177,181]. In general, the pathologic features of GCTs resemble other types of more benign cancers. Mitotic index (MI) coupled with Ki67 staining, an immunohistochemically detected marker of proliferating cells, often depict a low rate of mitosis in GCTs [166]. This is consistent with the indolent and slow growth potential of these tumors. Oddly, despite the low MI, GCTs at diagnosis tend to be large.

Tumor cell nuclei typically have a low atypia rate, suggesting the absence of complex genomic alterations. Nevertheless, a few common but nonobligatory cytogenetic changes have been detected in GCTs, including trisomy 12, trisomy 14, and monosomy 22 [186-190], and partial or complete gains in e.g. chromosomes 8, 9, and

15 [190]. While all of these areas contain genes for putative oncogenes or tumor suppressors, whether the gains or losses impact the level of gene expression has not been addressed. Epigenetic changes, such as alterations in methylation rate of gene promoters, may underlie the cytogenetic changes in cancer. Reduced expression of DNA methyl transferase, for instance, may cause chromosomal instability by genome-wide hypomethylation [191]. On the other hand, hypermethylation of specific gene promoters has beenshown to inactivate genes, such as tumor suppressor genes, a finding also reported in GCTs [188,189,192].

4.4 Molecular pathogenesis: dysregulation of normal granulosa cell function?

The characteristics of GCT cells closely resemble those of the proliferating granulosa cells in growing follicles and express many of the (normal) granulosa cell genes that have key roles in folliculogenesis [153,193-195]. The preserved granulosa cell function allows ongoing hormone production by the tumor cells. Although the specific events in GCT pathogenesis remain obscure, misregulation of granulosa cell proliferation and apoptosis is considered to inevitably contribute to the malignant transformation of granulosa cells and the development of a long-latency GCT [196,197]. Some defects behind the improper proliferation have indeed been highlighted during recent years. The regulatory factors, the normal functions of which are presented in Figure 6, are discussed below.

FSH and cyclin D2

Overactivity in the FSH signaling cascade is one of the leading hypotheses for GCT pathogenesis. FSHR and cyclin D2 expression has been shown to be strong in human GCTs [153,194]. Previous attempts have, however, failed to identify (activating) mutations in the *FSHR* or in the downstream effectors of the cascade, e.g. in G-protein, PKA, or CREB [196,198-200]; the role of GATA-4 has remained unaddressed. In mouse models, FSH deficiency is able to reduce the growth rate or penetrance of GCTs [201,202]. Of interest in this respect, the GCTs have a peak in incidence at late reproductive age and around menopause [177]. The serum gonadotropin levels at this time are high, which could aid the escape of a subpopulation of follicular cells from the normal cell cycle control, e.g. by inadequate activation of the FSH target genes. Recent studies have further supported the hypothesis, that cyclin D2 is key in promoting gonadal tumorigenesis [192,203].

MAPK, TGF-β, and Wnt signaling

In addition to FSH, mitogenic signaling through the MAPK, TGF-β, and Wnt pathways may promote the malignant transformation of granulosa cells. In fact, ERK (part of the MAPK system) has been found in an active state in *in vitro* cultured human GCT cells derived from a recurrent GCT, as well as in most aggressive primary GCTs studied [204]. This finding is consistent with the connections of FSH cascade and ERK to granulosa cell proliferation [154]. Mutations in the components of the TGF-β cascade have been found in a number of human cancers, e.g. loss of function of *Smad4* in pancreatic cancer (the gene formerly named DPC4: Deleted in Pancreatic Cancer 4) [205,206]. In one study of serous ovarian carcinomas, one-third of the tumors had very weak or no expression of Smad2 or Smad4 proteins [207]; the role of the TGF-β cascade in human GCT is largely unaddressed, except for inhibin (see below). Interestingly, the Wnt pathway has also recently been associated with ovarian tumorigenesis. Overexpression of its intracellular effector molecule β-catenin is tumorigenic in the mouse ovary; although not literally a GCT, the resulting tumors have characteristics

similar to GCTs (D. Boerboom, pers. comm. 2004). Whether the Wnt pathway and β-catenin have an impact on human GCTs remains unknown [197].

Inhibin and AMH

Loss of normal growth suppressors could further enhance granulosa cell proliferation. In the mouse, loss-of-function studies have revealed *inhibin-α* to be a tumor suppressor gene since the null mice develop sex cord stromal tumors, including ovarian GCTs [203,209]. In women, however, GCTs express inhibin-α [195,210,211], which is a key diagnostic marker in surgical pathology [181,212]. Furthermore, the GCT-secreted inhibin is utilized as a serum marker in patient follow-up [213,214]. The role of inhibin-α as a tumor suppressor in human GCT pathogenesis is, thus, constantly under debate. Lastly, AMH also has been implicated as a tissue and serum marker for GCTs as well as for Sertoli cell tumors. AMH is reported to be diffusely expressed in GCTs [179,210], and with the use of serum AMH measurements a recurrence has been detected 11 months prior to clinical presentation [215,216]. Mice deficient for both *inhibin-α* and *Amh* develop ovarian sex cord stromal tumors similar to those in *inhibin-α*-null animals [217], whereas mice deficient for only *Amh* have no reported ovarian tumors. Given the putative role of AMH as a suppressor of granulosa cell proliferation [139-141], reduced or lost AMH production could allow GCT development by abolishing local growth suppression.

AIMS OF THE STUDY

Normal ovarian development and function require specific transcriptional regulation. Previous studies have elucidated the expression and function of transcription factors GATA-4 and GATA-6, along with adrenal, testicular, and cardiac development. These factors are also expressed in the mouse ovary. Furthermore, GATA-4 has been depicted as an effector in the gonadotropin-activated cascade in endocrine cells. Based on the hypothesis that GATA transcription factors play a role in the mammalian ovary, this study was designed to determine the impact of GATA-4, GATA-6, and cofactor FOG-2 on human ovarian development, function, and tumorigenesis. Specific aims of this series of studies were:

1) To profile the expression of GATA-4, GATA-6, and their cofactor FOG-2 in the mouse and human ovary during fetal development and postnatal folliculogenesis.

2) To analyze how GATA-4, GATA-6, and FOG-2 affect ovarian development and function by studying the regulation of their expression as well as their role in activating downstream target genes *inhibin-α* and *anti-Müllerian hormone* in ovarian cells.

3) To determine whether GATA-4, GATA-6, FOG-2, and anti-Müllerian hormone have an impact on malignant proliferation of ovarian granulosa cells and on clinical behavior of granulosa cell tumors.

MATERIALS AND METHODS

1 Patients and clinical data (IV)

Study IV consists of 80 female patients diagnosed as having GCT at the Department of Obstetrics and Gynecology, Helsinki University Central Hospital, between 1971 and 2003. The patients were identified from the archives of the Department of Pathology, and available paraffin-embedded tissue samples were re-evaluated for GCT diagnosis by a gynecologic pathologist. The clinicopathologic data of tumor patients were retrospectively scrutinized from patient files, and data on survival were confirmed with the Central Statistical Office of Finland. Clinical stage was determined according to the classification scheme of FIGO. After primary surgery, the patients had been routinely monitored by at least yearly visits. Detection of late recurrences was possible since treatment of GCTs in the district is centralized to the tertiary university hospital; a few patients were tracked from other central hospitals. The study protocol was approved by the Ethics Committee of the University of Helsinki and by the National Authority for Medicolegal Affairs in Finland (TEO).

2 Tissue samples (I-IV)

2.1 Mouse (I)

All animal studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Gonads of strain CBA or NMRI mice were dissected on embryonic days (E) 12.5, 13.5, 15.5, and 17.5, and on postnatal days 1, 7, 14, 25, and 60; E10 embryos were used entirely. The samples were snap-frozen or fixed in 4% paraformaldehyde and embedded in paraffin. Sex of day 10 and 12 embryos was determined by PCR analysis for the *Sry* gene on genomic DNA [218].

2.2 Normal human ovary (II-IV)

Ovaries from eight fetuses (age 13–22 and 33 weeks) with normal karyotype were obtained after spontaneous or therapeutic abortions because of maternal disease, and from six neonates (fetal age 23–39 weeks at birth) who died within 48 h of birth because of perinatal asphyxia or infection. Adult ovarian tissue samples were obtained from women (age 22–45 years) undergoing ovariectomy because of either uterine myomas (n = 4) or focal endometrial cancer (n = 4) in Study II, and because of cervical cancer without preoperative irradiation (n = 4) in Study III. In addition, normal ovarian samples (n = 3), removed because of cervical cancer (patients' age <35 years), were used as normal reference tissue in Study IV. The samples were fixed in 4% buffered formaldehyde (II) or formalin (III and IV) and embedded in paraffin for histological analyses, *in situ* hybridization, and immunohistochemistry, or snap-frozen and stored at -70ºC for RNA extraction and Northern blot analysis (II and III). These studies were approved by the local Ethics Committees of the Universities of Oulu and Helsinki.

2.3 Human tumor samples and tissue microarray (III, IV)

For mRNA analysis (III), tumor samples were obtained from 24 women who underwent surgery for either GCT (n=15) or thecoma (n=9) at the Department of Obstetrics and Gynecology, Helsinki University Central Hospital. In addition, the tumor samples of the 80 GCT patients (IV) were subtyped and evaluated for the degree of nuclear atypia by a gynecologic

pathologist according to established criteria [219]. Tumors having micro- or macrofollicular, tubular, trabecular, and insular characteristics were considered a differentiated subtype (n=56), separate from more diffuse (sarcomatoid) tumors (n=24). From each tumor, four areas with the highest mitotic activity were sought, and the number of mitotic figures in ten high-power fields was counted in each to establish the mitotic index (MI; cut-off low or high). A tissue microarray was constructed from the samples as described previously [220]. In brief, four core tissue biopsies were obtained from each of the 80 primary GCTs, and the specimens were arranged in one recipient paraffin block.

3 Cell cultures (I, III)

3.1 Cell lines and transient transfections (I)

293T, COS-7, NIH 3T3, and KK-1 cells were cultured using DMEM, supplemented with 10% fetal bovine serum and antibiotics (Gibco, Grand Island, NY, USA). The KK-1 mouse granulosa cell line was established from the ovarian tumor of *inhibin-α* promoter-driven SV40-T-antigen transgenic mice [221]. Twenty-four hours before transfection, the cells were plated on 12- or 6-well dishes. KK-1 cells were transfected with Lipofectamine or Lipofectamine 2000 (Gibco BRL, Grand Island, NY, USA), and the other cells with FUGENE6 (Roche Molecular Biochemicals, Mannheim, Germany), with minor modifications to the manufacturers' protocols. The transfected plasmids included the luciferase reporter-coupled promoters of *MIS* [70], wildtype *inhibin-α*, and *inhibin-α* with mutated GATA-binding sites [34], as well as the expression vectors for GATA-4 [222], and FOG-2 [12]. In the *inhibin-α*–luciferase plasmids, the original pTKGH reporter plasmid [34] was replaced with pGL2 by subcloning to the *Hin*d III sites and thereafter by sequencing to verify orientation. In all experiments, pCMVβ (Clontech, Palo Alto, CA, USA), a β-galactosidase expression vector, was cotransfected to monitor transfection efficiency. The *inhibin-α* promoter-transfected cells were stimulated with recombinant TGF-β (R&D Systems, Minneapolis, MN, USA) for 24 h prior to harvest, which was carried out 40-48 h after transfections in all assays. The determined luciferase-enzyme activity was normalized to the β-galactosidase activity, and each experiment was performed in triplicate at least three times to ensure reproducibility. The results were presented as mean ± standard error of three independent experiments.

3.2 Human granulosa-luteal cell culture and treatments (III)

Human granulosa-luteal (GL) cells were obtained by follicular aspiration from regularly menstruating women undergoing oocyte retrieval for IVF. In ovarian-hyperstimulated patients, the oocyte retrieval was carried out 36-37 h after human chorionic-gonadotropin (hCG) administration. For the *in vitro* experiment, the cells from two to four patients were pooled, enzymatically dispersed, and separated as previously described [223]. The cells were then recovered for RNA extraction or plated at a density of 2-5 x 10^5 cells/well on six-well dishes, and cultured in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, and antibiotics (Gibco). Hormone treatments were performed between days 3 and 7 of culture for different time periods. Before treatments, the cells were transferred to DMEM supplemented with 2.5% fetal calf serum. The cells were treated with 30 ng/ml of recombinant human FSH (rhFSH) (Organon International BV, Oss, The Netherlands), 30 or 100 ng/ml of purified hCG (obtained from the National Hormone and Pituitary Distribution Program,

NIDDK, NIH), or 0.5-1.0 mM 8-bromo-cAMP (8-Br-cAMP) (Sigma Chemical Co., St. Louis, MO, USA). Each experiment was performed at least three times with duplicate or triplicate cultures to ensure reproducibility.

4 mRNA expression (I-IV)

4.1 Cloning of human GATA-4, GATA-6, and FOG-2 cDNAs for templates (III)

Human 575-bp GATA-4, 712-bp GATA-6, and 342-bp FOG-2 cDNAs were synthesized by RT-PCR from human GL cell RNA using specifically designed oligonucleotides: for GATA-4, 5'-CTC-CTT-CAG-GCA-GTG-AGA-GC and 5'-GAG-ATG-CAG-TGT-GCT-CGT-GC (GenBank accession no. NM002052); for GATA-6, 5'-ATG-ACT-CCA-ACT-TCC-ACC-TCT and 5'-CAG-CCT-CCA-GAG-ATG-TGT-AC (GenBank accession no. NM005257); for FOG-2, 5´-CAG-AGT-CGA-CAG-CAA-CTT-CC and 5´-GCC-TGC-TGG-ACT-CAA-TTC-AG (GenBank accession no. AA975109). The GATA-4 and GATA-6 cDNAs were subcloned into the pGEM-7Zf(+/-) vector (Promega, Madison, WI, USA), and the FOG-2 cDNA into the pGEM-T Easy vector (Promega), and sequenced to verify the sequence and orientation.

4.2 RNA extracts and Northern analysis (I-III)

Total cellular RNA was extracted by the guanidine isothiocyanate-cesium chloride method [224] or by a commercial kit (RNeasy cat# 74104, Qiagen, Hilden, Germany), quantified by absorbance at 260 nm, size-fractionated in 1.5% agarose-formaldehyde, and transferred to a Biodyne Transfer membrane (Pall Europe Ltd., Portsmouth, England). The filter was UV-cross-linked prior to hybridization. cDNA probes were synthesized from templates (Table 4) with a Prime-a gene kit (Promega) and labeled with [^{32}P]- -deoxy-CTP (3000 Ci/mmol; Amersham, Arlington Heights, IL, USA). The probes were purified with Nuck Trap columns (Stratagene, La Jolla, CA) or with Nick columns (Pharmacia, Uppsala, Sweden) and used at 1–3 x 10^6 dpm/ml in hybridization solution containing 50% formamide, 6 x SSC buffer, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumine, 100 g of salmon sperm DNA/ml, 100 g of yeast RNA/ml, and 0.5% SDS. Northern blot filters were hybridized for 16 h at 42ºC, washed with 1 x SSC-0.1% SDS, and exposed to X-ray films with Trimax intensifying screens (3M, Ferrania, Italy) at -70°C.

4.3 *In situ* analysis (I-IV)

Paraffin-embedded sections were deparaffinized in xylene, rehydrated in decreasing alcohol series, and treated with proteinase-K (Sigma) before hybridization. The sections were subjected to *in situ* hybridization as described elsewhere [225] with some modifications. The tissue sections were washed in PBS, acetylated, dehydrated, and then incubated with 1 x 10^6 cpm of [^{32}P]-labeled (1000 Ci/mmol; Amersham) antisense and sense riboprobes (Table 4) in a total volume of 80 µl at 60°C for 18 h. After washes, RNA digestion, and dehydration, the sections were dipped in NTB2 nuclear emulsion (Kodak, Rochester, NY, USA) and exposed in the dark at 4°C for 1 or 2 weeks. After developing, the sections were counterstained with hematoxylin and eosin.

5 Protein expression (I-IV)

5.1 Nuclear protein extracts and Western analysis

KK-1 cells were stimulated with recombinant TGF-β (R&D Systems), harvested, and the nuclear proteins extracted as described previously [226], employing two steps with two lysis buffers on ice; first for 15 min with 10 mM Hepes, 1.5 mM MgCl2, 10 mM KCL, 1 mM dithiotreitol, 0.5 mM PMSF, and a protease inhibitor cocktail, followed by rapid centrifugation to pellet the nuclei; second for 20 min with 10 mM Hepes, 15% (vol/vol) glycerol, 1.5 mM MgCl2, 0.2 mM EDTA, 420 mM NaCl, 1 mM dithiotreitol, 0.5 mM PMSF, and a protease inhibitor cocktail, followed by rapid centrifugation to pellet the nuclear debris. Protein aliquots of 40 μg were subsequently separated by 7.5% SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA). The nonspecific antibody binding on the membrane was blocked with 5% skim milk in 0.1% Tween-TBS buffer, and GATA-4 or control Actin proteins were detected with primary antibodies (Table 4) for 1 h at room temperature followed by a secondary antibody (Jackson Laboratories, West Grove, PA, USA), and then visualized by an enhanced chemiluminescence plus kit (Amersham Biosciences, Buckinhamshire, UK).

5.2 Immunohistochemistry (I-IV)

Paraffin-embedded sections were deparaffinized, hydrated, and treated with 10 mmol/l citric acid in a microwave oven for

Table 4. Probes and antibodies used in the study.

Gene/Protein	*	Source of cDNA or riboprobe template	Type of antibody	Dilution in IHC	Source
GATA-4	m	Ref. [222]	Rabbit-antimouse (human-reactive)	1:200	Santa Cruz
	h	For *in situ* III; for NB ref. [227]		1:400	
GATA-6	m	-	Rabbit-antihuman (mouse-reactive)	1:200	Santa Cruz
	h	For *in situ* III; for NB ref. [36]		1:50	
FOG-2	m	Ref. [12] (a 800 bp fragment)	Rabbit-antimouse (human-reactive)	1:500	Santa Cruz
	h	III		1:100	
AMH	m	-	Goat-antihuman (mouse-reactive)	1:200	Santa Cruz
	h	-		1:100	
SF-1	h	-	Rabbit-antimouse (human-reactive)	1:800	Upstate
Bax	h	-	Antirabbit	1:500	Pharmingen
Bcl-2	h	-	Antimouse	1:25	DAKO
Ki-67	h	-	Mouse-antihuman	1:150	DAKO
Cyclophilin	h	Ref. [228]	-	-	-
GAPD	h	Ref. [229]	-	-	-
18S	m	Ambion	-	-	-
Actin	m	-	Goat-antihuman	(WB)	Santa Cruz

* Indicates mouse (m) or human (h) studies; IHC = immunohistochemistry, NB = Northern blotting, WB = Western blotting; all antibodies are polyclonal, except Bcl-2 and Ki-67, which are monoclonal.

10-20 min for antigen retrieval. Endogenous peroxidase activity was then blocked with 3% hydrogen peroxide for 5 min, and nonspecific binding was blocked by using 1.5% normal serum in 0.1% Tween-PBS for 30-60 min. The primary antibodies (Table 4) were exposed for 1 h at 37°C, or overnight at 4°C in 0.1% Tween-PBS. An avidin-biotin immunoperoxidase system (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) was utilized to visualize the bound antibody with diaminobenzidine (DAB, Sigma Chemicals, St. Louis, MO, USA) as a substrate for the peroxidase enzyme in the presence of 0.03% hydrogen peroxide. The sections were counterstained with hematoxylin. Nonimmune serum or PBS replaced the primary antibody to obtain a negative control for each antibody. Double immunohistochemistry for FOG-2 and MIS (I) was carried out so that the FOG-2 protocol was finished with nickel-DAB (Vector Laboratories), followed by exposure of AMH antibody on the same slides, which was then visualized by DAB. Ki-67 analysis was carried out with a Dako TechMate 500 automated staining machine.

6 *In situ* DNA 3'-end labeling (II)

Apoptosis in the ovarian samples was analyzed by employing an *in situ* DNA 3'-end labeling kit (Oncor, Gaithesburg, MD, USA). Paraffin-embedded sections were deparaffinized and hydrated, and the cell membranes were permeabilized by incubating the sections with proteinase K (Boehringer Mannheim GmbH, Mannheim, Germany) in PBS for 15 min. Endogenous peroxidase activity was blocked with 5% hydrogen peroxide for 5 min. DNA fragmentation was identified by applying terminal transferase enzyme with digoxigenin-labeled nucleotides to the sections for 1 h under coverslips. Anti-digoxigen antibody was used to recognize the digoxigen-labeled nucleotide chains attached to the 3'-ends of DNA in the samples. A color reaction was produced with DAB in the presence of 0.03% hydrogen peroxide, and the sections were counterstained with hematoxylin.

7 Light microscopy and scoring of results (I-IV)

In all cases, the tissue samples that were subjected to RNA *in situ* hybridization, immunohistochemistry, or *in situ* DNA 3'-end labeling were evaluated with light microscopy; the RNA *in situ* sections under both bright and dark-field optics. In Study II, the results were scored into negative, moderately positive, positive, strongly positive, and very strongly positive classes. In Study IV, the immunohistochemically stained sections of a normal adult ovary (n=3) and of the tissue microarray were scored blinded to the clinical data and separately by two researchers, with a consensus reached in cases of disparate scoring. When heterogeneous staining was detected, the strongest and weakest stainings of the four tissue cores of individual tumors in the microarray were omitted from the analysis. Granulosa cells from normal ovaries were scored to establish the immunoreactivity of normal proliferating granulosa cells of primary to medium-sized antral follicles. Scoring classes were as follows: 1) For the nuclear antigens (GATA-4, GATA-6, FOG-2, and SF-1) negative/weak for 0-20% positive nuclei, or up to 100% positive nuclei with very weak intensity; intermediate for 20-80% positive nuclei with moderate or strong intensity; high for >80% positive nuclei with moderate or strong intensity. 2) For the cytoplasmic antigen (AMH) negative for 0% positive cells; weak for 1-20% positive cells or <80% positive cells with weak intensity; intermediate for >20% positive cells with moderate intensity; high for >20% positive cells with strong intensity or >80% of

positive cells with moderate to strong intensity.

8 Statistical analysis (I, III, IV)

The results of transient transfection assays were analyzed by ANOVA, followed by Duncan's multiple range test to reveal significant differences (I). In GL-cell experiments, the data were analyzed by Student's *t*-test to reveal significant differences between untreated and hormone-stimulated cultures (III). In Study IV, the three or four categorical scoring results were combined into two to represent normal expression reflecting immunoreactivity in normal granulosa cells, and reduced expression compared with normal immunoreactivity. The resulting nominal variables were analyzed with contingency tabling (2x2) and chi-square or Fisher's exact tests. In addition, a logistic regression model was employed for multivariate analysis, in which each of the clinical measures was the dependent variable. The analyses were carried out using Exstatics or Statview 5.0.1 softwares for Macintosh®. $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

1 GATA-4 and FOG-2 in ovarian development (I, II)

Mammalian gonadogenesis is a complex orchestration of events requiring cooperation between a number of genes and corresponding proteins. The Y chromosome is the primary determinant of the path taken, with the rest depending on hormones, paracrine factors, intracellular signal mediators, and transcription factors that activate genes in the nucleus of a given cell. Previous studies have shown that transcription factors GATA-4 and GATA-6 are expressed in the fetal mouse ovary and testis [34-36]. These factors functionally synergize with other cell-specific transcription factors, SF-1 being an important example [7]. In preliminary studies, FOG-2, the closest cofactor of GATA-4, was also detected in fetal mouse gonads [12,13]. Little was, however, known about the expression and role of GATA-4 and FOG-2 in the earliest phases of ovarian development.

1.1 Expression and role in bipotential mouse gonads

We assessed the expression of GATA-4 and FOG-2 in the bipotential urogenital ridge (I). Mouse embryos were sacrificed, the sex verified, and the urogenital ridge identified from serial sections of paraffin-embedded tissue. *In situ* hybridization analysis revealed GATA-4 and FOG-2 mRNA to be readily expressed in the bipotential urogenital ridge of both sexes at E10.5 (Figure 2A-D in I). Immunohistochemistry verified the corresponding proteins in the somatic cells of the urogenital ridge, whereas the germ cells (identified by morphology) presumably remained negative (Fig 3A-B in I). At E12.5, when Sry expression was about to cease, the gonads hardly showed any signs of differentiation; GATA-4 and FOG-2 expression was still evident in the somatic cells of both sexes. In addition to our findings in mice, GATA-4 expression has been detected the undifferentiated rat and porcine gonads [230,231], suggesting a role in the formation of the mammalian gonad from the urogenital ridge. *Gata4*-deficient mice die prior to gonadogenesis [19,20], and a different approach confirmed that GATA-4:FOG-2 interaction is essential for gonadogenesis [74]. Accordingly, abrogation of their cooperation resulted in reduced Sry expression, absent Sox9 and AMH expression, and subsequent failure in early testicular differentiation; the phenotype of female transgenic mice has not been reported in detail [74]. In fact, Sry promoter seems to be a direct GATA-4 target, and forced expression of a dominant negative GATA-4 protein in the urogenital ridge of male mice may impair testis development (R. S. Viger, pers. comm. 2004). Thus, GATA-4 presumably assists the genes promoting testis differentiation of the bipotential gonad.

1.2 GATA-4 and FOG-2 in somatic cells during fetal life

Previous studies have depicted GATA-4 expression as being downregulated in the fetal mouse ovary after the bipotential stage [35]. Our survey, however, revealed a somewhat different pattern; GATA-4 expression as well as FOG-2 expression, remained at a high level in the somatic cells of the mouse ovary over the entire fetal period (Figures 1-3 in I). In the fetal testis, FOG-2 was only weakly detectable in E13.5 and absent in E15.5 Sertoli cells (I, and [232]). Further, AMH expression was evident in the FOG-2-negative Sertoli cells (Figure 4 in I). mRNA *in situ* and Northern analysis verified strong GATA-4 and FOG-2

expression in the fetal ovary, whereas in the fetal testis the FOG-2 levels were significantly lower than those postnatally (Figures 1 and 2 in I). With respect to the findings of Viger *et al.* [35], reasons such as lack of an antigen retrieval method in immunohistochemistry or analysis of a different mouse strain may underlie the contrasting results that they obtained on GATA-4 expression in fetal gonads.

In addition to mouse tissues, GATA-4 and FOG-2 expressions were analyzed in the fetal human ovary (II). Northern and *in situ* analysis of GATA-4 revealed mRNA expression in week 18, 22, 24, 27, 35, and 39 fetal ovaries (Figure 3 in II). Further, GATA-4 protein was very strongly expressed in pregranulosa/stromal cells at weeks 13 and 14 (Table 1 and Figure 2G-I in II), when primordial follicles were not yet evident. During weeks 16-33, GATA-4 protein expression was somewhat reduced in the follicular granulosa cells. Besides GATA-4 expression *in situ* analysis indicated FOG-2 mRNA expression in week 21 and 24 ovaries, and by immunohistochemistry the FOG-2 protein was localized to somatic and occasional germ cells at weeks 14-39 (Anttonen *et al.,* unpublished data). The putative role of GATA-4 in protecting follicles from apoptosis, i.e. atresia [36,165] was also addressed in the fetal ovary (II). While the oocytes represented the majority of apoptotic cells, occasional somatic cells, pregranulosa or follicular, underwent apoptosis throughout gestation and correspondingly expressed the apoptosis-promoting Bax (Table 1 and Figures 1 and 2 in II). The high GATA-4 expression in somatic cells may be interpreted as indicating a survival role for GATA-4.

In the fetal human ovary, the SF-1 expression profile differs significantly from that in the mouse, and, similarly to GATA-4, it is not dimorphic in the gonads (I, and [233,234]). Following sexual differentiation, SF-1 is widely expressed in ovarian somatic cells [233]. These findings imply that GATA-4, SF-1, and perhaps FOG-2 are pivotal in granulosa cell survival, from their differentiation in fetal life onwards. Although high GATA-4 expression in the fetal ovary has been consistently verified in four mammalian species (I, II, and [230,231]), the ultimate role of GATA-4 in fetal development of the mammalian ovary requires further analysis.

1.3 FOG-2 as a putative *AMH* repressor in the fetal ovary

In the fetal testis, *AMH* is activated as part of the testicular cascade, enabling adequate development of the male reproductive organs from the sex ducts. As discussed previously (Section 2.2 in Review of the Literature), *AMH* activation in the early mouse testis involves Sox9, SF-1, and GATA-4 [41,58,62], and in the early mouse ovary these activators are either not expressed, e.g. Sox9, or downregulated, e.g. SF-1, beyond the indifferent stage (Figures 3 and 7). However, the situation is different with GATA-4 (I), indicating that the ovary must possess tools to hamper *AMH* activation by GATA-4 and SF-1 synergism in the bipotential gonad. Given the dimorphic expression of FOG-2 in fetal somatic cells, we addressed one possible mechanism by utilizing transient transfection assays with the *AMH* promoter construct and expression plasmids for GATA-4 and FOG-2 (I). We found that FOG-2 was able to downregulate the activation of the *AMH* promoter by GATA-4 in cultured granulosa cells (KK-1 cell line) (Figure 5 in I), suggesting that FOG-2 could indeed have this kind of function in the fetal ovary (I). Corresponding results have also been obtained in primary rat Sertoli cell cultures [72], in which the activity of the *AMH* promoter was repressed by overexpression of FOG-2. Furthermore, a

truncated GATA-4, which works as dominant negative competitor for the endogenous GATA-4, was able to downregulate the AMH promoter activity. The results of Tremblay *et al.* [72] also indicate that without a need to bind the GATA site in the promoter, GATA-4 may promote AMH activation directly through the SF-1 protein.

A mammalian female lacks the Y chromosomal *Sry*, which is necessary for testis development [37,41] and may act to facilitate testicular gene activation by epigenetic mechanisms [46]. The *Sry* promoter is directly activated by SF-1 and GATA-4, and both factors are expressed in the bipotential urogenital ridge of both sexes (Figure 7) (I, and [41]). Regulation of *AMH* involves the same factors as well as the Sry-driven Sox9, which is key in activating the *AMH* promoter in the testis [62,66]. Sox9 levels are negligible in the fetal ovary, and sustained expression of FOG-2 and DAX-1 could provide additional tools for guaranteeing that AMH remains unexpressed (Figure 7). However, more robust analysis utilizing transgenic mice is required to consistently relate our findings on FOG-2 to gonadogenesis and ovarian development *in vivo*.

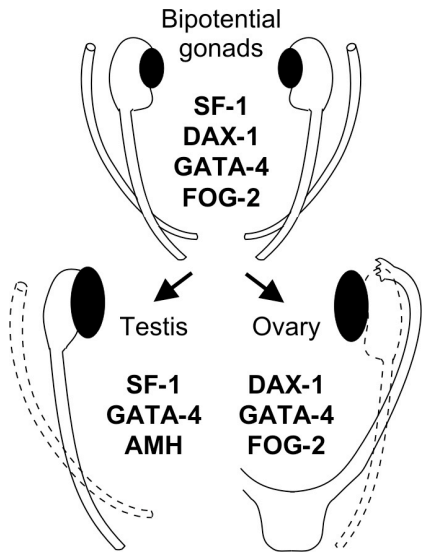

Figure 7. *Expression and proposed function of GATA-4, FOG-2, SF-1, and DAX-1 in AMH regulation and sex differentiation in the mouse (see also Figures 2 and 3)*. Beyond the bipotential stage, expression of *AMH* activators GATA-4 and SF-1 is retained in the testis, whereas only GATA-4 is retained in the ovary. Expression of *AMH* repressors FOG-2 and DAX-1 is downregulated in the testis, but retained in the ovary to suppress *AMH*.

2 GATA-4, GATA-6, and FOG-2 in ovarian function (I, III, IV)

Ovarian folliculogenesis is regulated by endocrine and paracrine mechanisms. The intracellular response of a given cell to hormonal stimulation employs secondary ligand-specific messengers that activate transcription factors to drive gene expression. Previous studies have shown expression of GATA transcription factors in mouse endocrine organs, i.e. in the adrenals, testis, and ovary [31,34-36]. Over the course of our studies, GATA-4 appears to have evolved as an effector in the gonadotropin cascade (Figure 5) [7]. Furthermore, GATA transcription factors and FSH share several endocrine target genes in the ovarian granulosa cells, including LHR, AMH, inhibin, and aromatase [7,34,64,235].Taken together, these findings prompted studies on the expression and function of GATA and FOG factors in the human ovary.

2.1 Expression patterns during folliculogenesis

Granulosa cells of primordial follicles, formed during fetal life in human, preserve the oocytes until their extinction at menopause; folliculogenesis is activated around puberty. We found no GATA-4 expression in primordial follicles, but at the onset of growth (i.e. initial recruitment) and transformation into primary follicles via an intermediary stage, GATA-4 expression was initiated in granulosa cells (Figure 8 in next page and Figure 2 in III). With further development of the follicles, granulosa cells of preantral and antral follicles uniformly expressed GATA-4 protein. Immunoreactivity for GATA-4 was localized to cumulus and mural granulosa cells, theca cells, and stromal cells, whereas oocytes remained negative. *In situ* mRNA analysis confirmed the GATA-4 expression pattern (Figure 1A-B in III).

Expression patterns for GATA-6 and FOG-2 proteins somewhat resembled each other during human folliculogenesis (Figure 8). Granulosa cells of primary follicles had negligible or weak immunoreactivity, but in later follicular stages GATA-6 and FOG-2 proteins were expressed in most follicular granulosa cells. FOG-2 was also expressed in fallopian tubes and occasional oocytes (unpublished observations). *In situ* analysis for GATA-6 mRNA (Figure 1C-D in III) revealed expression also in the luteal glands. In addition to the results on full ovarian tissue samples, mRNA expression for GATA-4, GATA-6, and FOG-2 was observed in freshly isolated human granulosa cells of preovulatory follicles from women undergoing IVF treatments (Figure 4 in III). Thus, both mRNA and protein of GATA-4, GATA-6, and FOG-2 were expressed in granulosa and theca cells of growing human follicles (III and IV).

FOG-2 expression was also surveyed in the postnatal mouse ovary and was generally detected in parallel to GATA-4 expression, i.e. in granulosa cells from primary follicles onwards (I). However, a few granulosa cells in the primordial follicles readily expressed FOG-2 while GATA-4 was negative, but in antral follicles FOG-2 expression was more often downregulated while GATA-4 expression was retained (Figure 6 in I). In addition, FOG-2 was coexpressed with GATA-6 in luteal glands and in the mucosa of fallopian tubes, which remained GATA-4 negative (Figure 7 in I). The FOG-2 expression pattern in mouse and human specimens suggests a broader role for FOG-2 in the mammalian reproductive tract, than merely being a GATA cofactor. In support of this, recent evidence shows that FOG-2 may belong to a family of other transcriptional repressors [236].

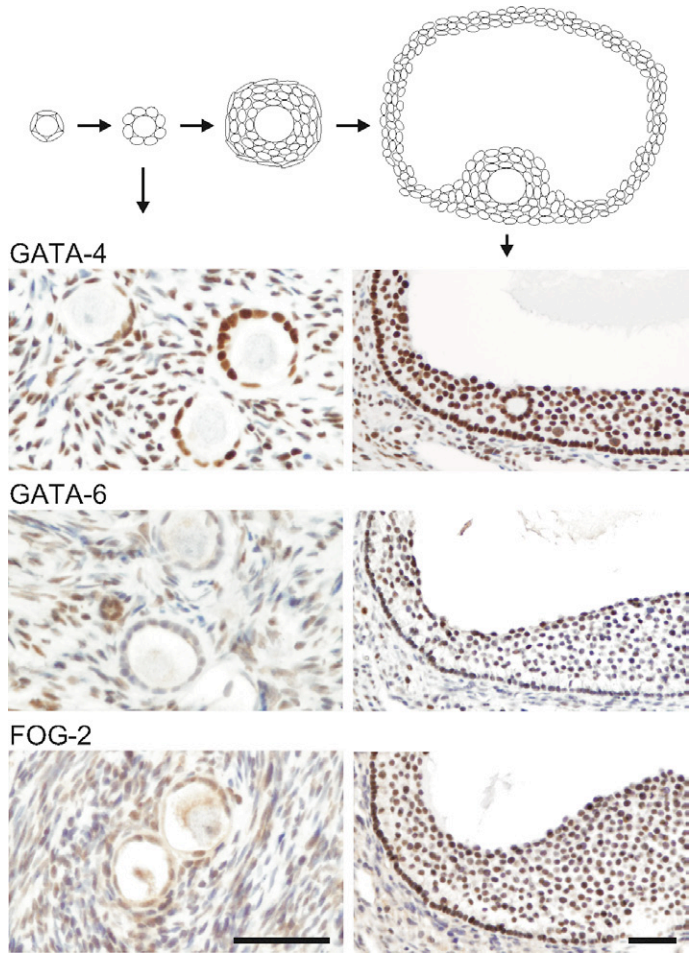

Figure 8. *Expression of GATA-4, GATA-6, and FOG-2 in human folliculogenesis*. Primary follicles are presented in the left panels, and a small antral follicle in the right panels. Brown indicates positive immunohistochemical staining in nuclei for these antigens. Sections are counterstained with hematoxylin, resulting in blue nuclei. GATA-4 expression is highly positive in follicles at both stages, whereas GATA-6 and FOG-2 expression is somewhat weaker or negative at the primary stage but positive at the antral stage. The scale bar for both sets of panels represents 50 µm.

2.2 Gonadotropin regulation of GATA factors

Gonadotropins have a fundamental impact on folliculogenesis; especially FSH is required for development from preantral to antral follicles [97,162]. FSH and forskolin (mimics FSH effects) were previously shown to be able to upregulate GATA-4 mRNA levels up to 1.7-fold in murine granulosa tumor cells (NT-1 cell line), which resemble normal granulosa cells [36]. In addition, treatment of premature three-week-old mice with gonadotropin from pregnant mare serum (PMSG) enhanced follicular expression of GATA-4 and GATA-6 transcripts [36]. To address this phenomenon in humans, we utilized cultures of isolated preovulatory human granulosa cells (III). These cells are derived from women undergoing hyperstimulation treatments to provide oocytes for IVF, they luteinize during *in vitro* cultures, and are thus called granulosa-luteal (GL) cells. Treatments with recombinant human FSH, hCG, or 8-Br-cAMP (a cell membrane permeable analog of cAMP) had no effect on steady-state levels of GATA-4 and FOG-2 in GL cells (III). Instead, GATA-6 mRNA levels were modestly but significantly upregulated by hCG (Figure 5

in III). In addition, 8-Br-cAMP upregulated GATA-6 mRNA levels within 2 h of stimulation of GL cells. These effects on GATA-6 rather than on GATA-4 may well reflect the luteinized phenotype of GL cells, given that only GATA-6 is expressed in luteal glands, both in the human and in the mouse ovary (I and III).

Several lines of evidence indicate an essential role for GATA factors in the gonadotropin pathway. In addition to granulosa cells, postnatal testicular Sertoli and Leydig cells express GATA-4, and Leydig cells and adrenal cortical cells express GATA-6 [31,34,234,237]. In testicular Sertoli and Leydig tumor cells, mRNA levels for GATA-4 are modestly but significantly upregulated *in vitro* by hCG and/or FSH [34,36], and in human adrenocortical cells stimulation with $(Bu)_2$-cAMP (analog of 8-Br-cAMP) resulted in a 3-fold increase in GATA-6 mRNA levels [238]. Furthermore, Tremblay *et al*. found that in primary Sertoli cells the GATA-4 protein levels remained stable when stimulated by cAMP, and instead the serine 261 of the GATA-4 protein was phosphorylated by PKA, resulting in enhanced transcription-driving activity of GATA-4 [7,108,109]. In the case of GL cells, GATA-4 may well be phosphorylated upon FSH, hCG, or cAMP stimulation, despite the lack of increase in GATA-4 mRNA (III).

In women with an inactivating mutation in the *FSHR*, GATA-4 expression is negative in the large pool of primordial follicles [239]. However, granulosa cells of primary follicles in these patients readily express GATA-4 (unpublished observations), suggesting that either FSH or FSHR is not required for the initial activation of GATA-4 expression, which instead may involve oocyte-derived factors. Of these, GDF9 is the most plausible candidate given its mitogenic activity and growth arrest of follicles in GDF9 deficiency [126-130]. From a clinical point of view, the phenotype of *Gdf9*-null mice closely reflects the changes in hypergonadotropic hypogonadism [113]. In *FSHR*-null mice with a corresponding phenotype, follicle growth is arrested at the preantral stage, resulting in decreased estradiol levels due to lack of antral follicles [100,102]. In addition, GATA-4 expression in these follicles is impaired in 10-day-old animals compared with age-matched wildtype animals [102]. Taken together, the temporal and spatial presence of GATA-4 and GATA-6 in the mammalian ovary (I-IV, and [36,230,231]) provides granulosa and theca cells with an essential nuclear effector for the gonadotropins to drive cell-specific gene expression, including expression of receptors for themselves [235].

2.3 Activation of granulosa cell gene *inhibin-α* requires GATA

Inhibin-α, essential in the ovarian function, is predominantly expressed in the granulosa cells of growing follicles [122]. Providing a major negative feedback mechanism from the gonads to the pituitary gonadotropin production, FSH activates inhibin expression in granulosa cells [122,133]. Along with characterizing GATA-4 expression in the mouse ovary and testis, *inhibin-α* was shown to be a direct target gene for GATA factors in the gonads [34,36]. Accordingly, GATA-4 specifically binds its target sequence in the *inhibin-α* promoter (Ketola and Parviainen, unpublished data) and transactivates the promoter in synergy with SF-1, as analyzed by *in vitro* transient transactivation assays of granulosa and Leydig cell lines [34,73]. Further, when the GATA-binding sites in the *inhibin-α* promoter were mutated, GATA-4 was unable to activate the promoter [34]. In addition, in GL cells, the treatments that upregulated GATA-6 mRNA levels were also able to upregulate *inhibin-α* mRNA

levels, indicating indirectly the need for GATA factors in this process (III).

Our most recent analysis has revealed that, in addition to a key role in the FSH cascade, GATA-4 is involved in the oocyte-derived TGF-β signal to activate *inhibin-α* in granulosa cells (Anttonen *et al.,* unpublished data). GATA-4 enables the activation of *inhibin-α* promoter by TGF-β, as indicated by the lack of a TGF-β effect on the promoter when GATA-4 was not overexpressed in non-GATA/non-SF-1-expressing COS cells (Figure 9A). In transient transfection assays of KK-1 granulosa cells, which endogenously express GATA-4 and SF-1, TGF-β readily stimulated the *inhibin-α* promoter, whereas mutations in the GATA-binding sites in the promoter resulted in abrogation of the TGF-β effect (Figure 9B). Furthermore, similarly to FSH, TGF-β was able to upregulate GATA-4 protein expression in KK-1 cells (Figure 9C).

Taken together, the results suggest that GATA-4 serves as a link between endocrine and oocyte-derived paracrine activation of inhibin production and is essential for the granulosa cell function. With respect to the yet unknown mechanisms activating GATA-4 expression in granulosa cells of primary follicles, of interest is the activation of GDF9 expression in oocytes at the same stage [117-119], and GDF9 utilizing the same signaling cascade as TGF-β in granulosa cells [134,240]. These findings provide evidence that initiation of GATA-4 expression in granulosa cells involves the oocyte-derived factors.

2.4 GATA factors integrate essential signaling pathways in granulosa cells

Several endocrine target genes for GATA transcription factors have been identified while this study was underway [34,64,73,235,241,242]. In granulosa cells,

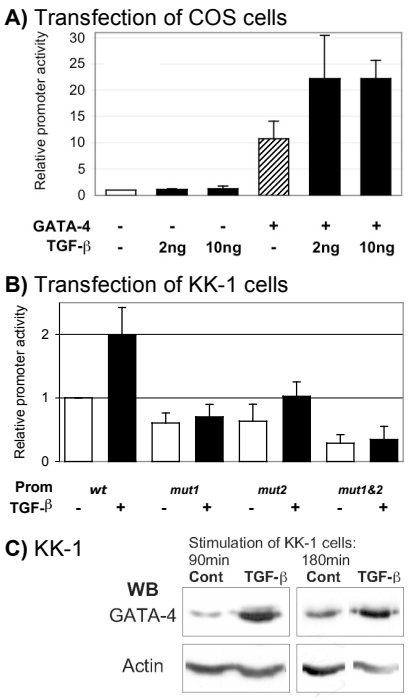

Figure 9. *Role of GATA-4 and GATA motifs in activation of inhibin-α by TGF-β.* **A)** COS cells were transfected with *inhibin-α* promoter and GATA-4 expression plasmid, and stimulated with 0, 2, or 10 ng/ml TGF-β. TGF-β stimulates the promoter only in the presence of GATA-4. **B)** KK-1 granulosa cells were transfected with a wild-type (wt) *inhibin-α* promoter, as in A, or with a promoter possessing mutations in either or both of the GATA motifs (mut1, mut2, mut1&2), and stimulated with 0 or 10 ng/ml TGF-β. Mutations in the GATA-binding sites abrogate the activation of *inhibin-α* promoter by TGF-β. **C)** TGF-β upregulates GATA-4 protein levels in KK-1 granulosa cells, as analyzed by Western blotting of nuclear protein extracts. Actin is a control for equal loading of samples.

regulation of GATA targets LHR, AMH, inhibin, and aromatase involves cooperative action between FSH, TGF-β and MAPK pathways (simplified presentation in Figure 10). Evidence for the role of GATA-4 in the FSH pathway is consistent, and our recent data also suggest a role for GATA-4 in mediating the TGF-β signal in granulosa cells (Figure 9). TGF-β is already known to employ GATA-4 in T helper cells and cardiac cells to transactivate *interleukin 5* and *Nkx2.5* genes, respectively [243,244]. The direct involvement of GATA-4 in the MAPK cascades has been established in cardiac cells, in which the induction of transcriptional activity of GATA-4 is mediated by ERK and p38 MAPK, which phosphorylate serine 105 in the GATA-4 protein [27-29]. The capability of these kinases to phosphorylate and activate GATA-4 in gonadal cells remains to be studied. In granulosa cells, however, the insulin-like growth factor activated cascade that involves MAPK has been demonstrated to employ GATA-4 to activate transcription of *StAR* (steroidogenic acute regulatory protein), yet another endocrine GATA target gene [245]. In addition, the present analysis of transcription factor expression in normal human ovaries indicate roles for GATA-4 and GATA-6 in granulosa cells (II-IV). GATA-4 and GATA-6 seem to orchestrate the endocrine, paracrine, and autocrine signals essential for ovarian function (Figure 10).

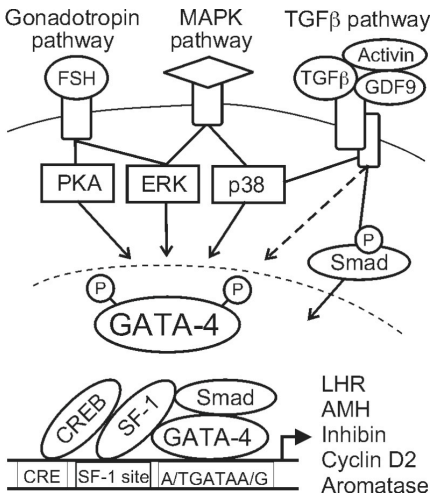

Figure 10. *Proposed function of GATA-4 in integrating endocrine, paracrine, and autocrine regulation of granulosa cells (see also Figures 5 and 6)*. FSH and TGF-β upregulate GATA-4 expression. GATA-4 is phosphorylated (P) by PKA and putatively by ERK. GATA-4 is also employed by the TGF-β pathway to facilitate autocrine/paracrine regulation by activin, TGF-β, or the oocyte-derived GDF9. p38 is activated by TGF-β and may also phosphorylate GATA-4. Note that the listed target genes are either activated or suppressed by these pathways.

3 Granulosa cell proliferation and tumorigenesis (III, IV)

Folliculogenesis involves a balance of granulosa cell proliferation and apoptosis. This balance is essential to avoid inadequate proliferation of ovarian somatic/stromal cells and development of sex cord stromal tumors, such as granulosa or theca cell tumors, and epithelial cancer. In view of the expression profiles and the proposed functions of GATA-4 and GATA-6 in the human ovary (Figures 8 and 10), we addressed their contribution to granulosa cell proliferation as reflected by granulosa cell tumors (GCTs).

3.1 Expression profiles in granulosa cell tumors

mRNA analysis revealed that GATA-4, GATA-6, and FOG-2 are expressed in GCTs as well as in thecomas (theca cell tumors) (Figure 3A-B in III). In our first GCT study (III), all tumors examined expressed GATA-4 (n=15) and FOG-2 (n=12), and most also expressed GATA-6 (9/10). In addition, all thecomas expressed GATA-6 (n=4) and FOG-2 (n=7), and most also GATA-4 (8/9). In this analysis, differences in mRNA levels were obvious but not quantified. Confirming the mRNA analysis, GATA-4 protein was also expressed in these tumors (Figure 3C-D in III). The findings suggest that these factors are required for granulosa and theca cell differentiation of the given tumors, and prompted a further analysis to profile their expression in a larger number of tumors (IV).

Primary GCT tissue samples of 80 patients were collected from the archives of the pathology unit and embedded in a tissue microarray that enables high throughput studies on protein expression in tissue samples (IV) [220]. In addition, we scrutinized the detailed clinical histories of all of the patients at Women's Hospital. The protein expression profiles for GATA-4, GATA-6, SF-1, FOG-2, and AMH were analyzed by immunohistochemistry, and the immunoreactivity levels in tumors were scored based on comparisons with levels in normally proliferating granulosa cells of primary/preantral and small/medium-sized antral follicles (Figure 11 in next page and Figure 2 in IV). The detailed analysis revealed that the majority of GCTs express GATA-4, GATA-6, SF-1, and FOG-2 at levels comparable with those in the normal granulosa cells (Table 5). The high expression of GATA-4 resembles the expression level in normal granulosa cells, whereas for the rest of the factors the normal level is intermediate or high. The findings support the hypothesis that GATA-4, GATA-6, SF-1, and FOG-2 play a role in granulosa cell tumors. Moreover, the variations in immunoreactivity levels, i.e. normal sustained vs. reduced (abnormal) expression levels, enabled further analysis regarding the clinical behavior of GCTs (clinical features of patients presented in Table 1 in IV).

Table 5. Protein expression profiles in primary granulosa cell tumors.

	Scoring result, total n=80		
Antigen	Negative or weak	intermediate	high
GATA-4	5 (6%)	40 (50%)	35 (44%)
GATA-6	23 (29%)	46 (57%)	11 (14%)
SF-1	13 (16%)	50 (63%)	17 (21%)
FOG-2	21 (26%)	41 (51%)	18 (23%)
AMH	55 (69%)	17 (21%)	8 (10%)

3.2 AMH as a suppressor of granulosa cell tumor growth

In contrast to the other factors studied, AMH expression was more often reduced in GCTs, being weak in 40% and negative in 29% of the GCTs (Table 5 and Figure 11). Thus, 69% of GCTs exhibited significantly reduced

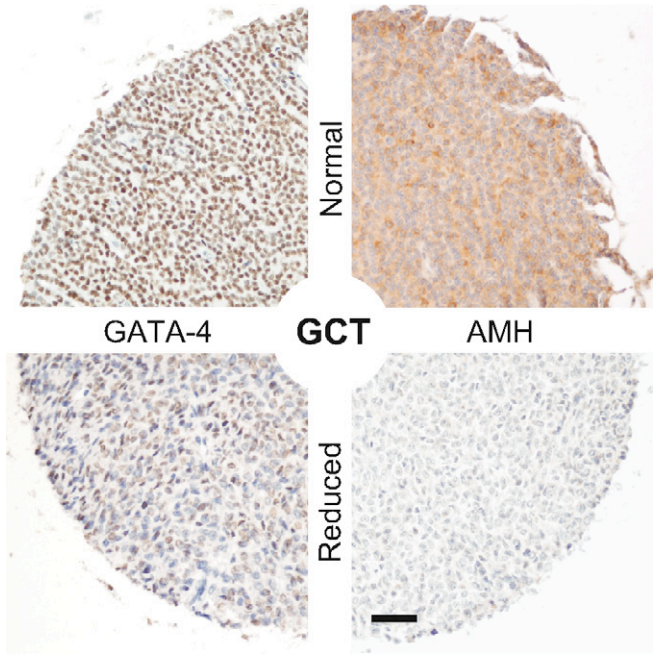

Figure 11. *Examples of GATA-4 and AMH expression in two granulosa cell tumors.* Immunohistochemical staining of GATA-4 (left panels; nuclear stain) and AMH (right panels; cytoplasmic stain) in tumor samples embedded in a tissue microarray. Upper panels (from one tumor) show an example of expression resembling normal granulosa cells, and lower panels (from one tumor) show expression level that is reduced level. Sections are counterstained with hematoxylin; scale bar represents 50 µm.

AMH expression, compared with expression levels in granulosa cells of growing follicles (IV, and [121]). Subsequent statistical analysis proved that these tumors, especially the AMH negative ones, are significantly larger in size (Figure 3A in IV). Considering the function of AMH in suppressing both the initial growth recruitment of follicular granulosa cells and the growth factor/FSH-stimulated granulosa cell proliferation [138-141], the reduced AMH expression in GCTs could well be one of the defects causing misregulated proliferation of granulosa cells leading to GCTs. Decreased AMH production by tumor cells could promote inadequate proliferation of the cells in the immediate neighborhood, given that GCTs are reported to express receptors for AMH [246]. The potential of AMH to act as a growth inhibitor of ovarian, breast, and prostate cancer cells is of great interest [58,247-249], and further analyses of the prevalence of AMH receptors in GCTs and the usefulness of AMH as a therapeutic agent for GCT are warranted.

The reduced (i.e. negative/weak) GATA-6 expression found in 29% of the GCTs was also associated with an increase in tumor size (Figure 3A in IV). GATA-6 expression was associated with AMH expression as well, suggesting that, instead of or in addition to GATA-4, GATA-6 contributes to the regulation of *AMH* in normal and malignant granulosa cells. Postnatal regulation of the *AMH* promoter has been addressed in Sertoli cells, where its activation was found to be dependent on FSH, SF-1, and GATA-4 [62,72,144,145]. The proposed role of FOG-2 as a downregulator of *AMH* in the fetal ovary (Figure 7) may not be so obvious in the postnatal ovary considering that FOG-2 and AMH expressions significantly overlap in preantral follicles in mouse (Figure 6 in I). Nevertheless, since *AMH* activation in granulosa cells involves the oocyte-derived factors [146], GATA-4 may contribute in a similar manner as in the case of *inhibin-α* (Figure 9).

3.3 GATA-4 expression is associated with aggressive tumor behavior

Our results suggest that GATA-4 facilitates granulosa cell proliferation and aggressive behavior in a subgroup of GCTs. An aggressive GCT is characterized by advanced clinical stage, large size, recurrence, and death of the GCT. Firstly, GATA-4 expression was initiated at the same time as granulosa cells started to proliferate (presented in Section 2.1). Secondly, 94% of the GCTs expressed GATA-4 at a intermediate or high level, and 44% at a high level resembling the level found in normal granulosa cells (Table 5, and Figures 8 and 11). Thirdly, in the clinical survey, the high GATA-4 expression was significantly associated with clinical stage Ic or higher and with risk of recurrence (Figure 3B-C in IV). High GATA-4 expression was detected in 18 of the 30 tumors of stage Ic, II, or III. One should note here, that in stage Ic ovarian cancer the disease is no longer limited to within the ovary, stage II cancer often has pelvic extension, and stage III cancer has microscopically confirmed peritoneal or lymph node metastasis. In addition to clinical stage, tumor size is suggested to be useful in evaluating prognosis of a GCT patient [177], but in our patient series both of these failed to correlate with risk of recurrence, as an indicator of prognosis. By contrast, the high sustained GATA-4 expression in the primary GCT was significantly associated with recurrence risk in all 80 patients as well as in the 50 patients followed up for at least 10 years (Figure 3C in IV; characteristics of recurred tumors in Table 2 in IV). We were, however, unable to associate any of the parameters studied with overall survival because of the low number of deaths due to GCT in these patients, a feature characteristic of this tumor type.

Adrenocortical carcinomas have similarities with GCTs in that their genesis seems to be gonadotropin-dependent.
Hypergonadotropism is involved in inducing tumorigenesis in certain mice strains after gonadectomy as well as in the *inhibin-α* null mice [33,250]. Interestingly, GATA-4 is upregulated along with tumorigenesis in these mice, whereas normal adrenocortical cells express GATA-4 only during fetal development [31-33]. In contrast to fetal life, the proliferation rate is low in postnatal adrenals, suggesting that the appearance of GATA-4 has an effect on adrenocortical proliferation. In addition, human adrenal carcinomas express GATA-4 [31,251]. Given also the common embryonic origin of adrenocortical cells and ovarian granulosa cells, i.e. the urogenital ridge, GATA-4 may serve as an essential element in proliferation of adrenal and ovarian cells. With respect to clinical practice, additional analyses are, however, required to assess whether GATA-4 has prognostic value in the human GCTs.

3.4 Granulosa cell tumor pathogenesis may involve GATA-4

A proposal for the factors having an impact on granulosa cell tumorigenesis and tumor growth is presented in Figure 12 (evolved from Figures 6 and 10). Cyclin D2 is of special interest considering its role in granulosa cell proliferation and tumorigenesis [98,153,154,203]. The *cyclin D2* promoter interestingly contains consensus GATA-binding site(s) [156,157], and although direct promoter analysis remains to be carried out, GATA-4 is able to upregulate cyclin D2 expression in pulmonary cell lines in stimulations that employ the ERK pathway [158]. In addition to supporting proliferation putatively through *cyclin D2* activation, GATA-4 may protect the granulosa cells from undergoing apoptosis (II) by activating anti-apoptotic genes such as *Bcl-X* [36,165]. Taken together, the prominent expression of GATA-4 in GCTs may serve as a link for FSH and ERK signaling, both active in

GCTs, to promote the granulosa cell proliferation leading to malignancy. One possibility is that GATA-4 is activated by an as yet unknown mutation in the *GATA4* gene.

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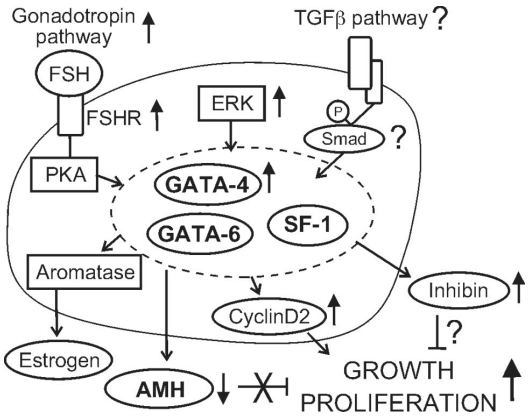

Figure 12. *Simplified illustration of granulosa cell tumor molecular pathogenesis, in light of the results presented (see Figures 6 and 10 for comparison).* Symbol ↑ represents upregulation or active state in GCTs and symbol ↓ represents downregulation in GCTs. GATA-4 may enhance the tumorigenic actions of FSH and ERK, putatively by activating *cyclin D2*. GATA-6 and SF-1 may have an impact on *aromatase* and *AMH* expression. Lastly, the reduced AMH expression may impair the necessary autocrine suppression of granulosa cell proliferation. The impact of inhibin-α on human tumorigenesis remains unclear.

CONCLUSIONS AND FUTURE PROSPECTS

Unraveling expression profiles of transcriptional regulators during development and tumorigenesis in the mammalian ovary provides the physiological context to address functional aspects. This information may ultimately help to improve diagnostic and treatment strategies for human disease. In addressing the aims of this series of studies, new questions and goals emerged to be addressed in future research. The main conclusions are as follows:

1. Transcription factor GATA-4 is readily expressed in the coelomic epithelium of the mouse urogenital ridge, derived from the mesendodermal layer. Expression of GATA-4 as well as cofactor FOG-2 occurs in the ovarian somatic cells throughout fetal and postnatal life in both mice and humans, indicating a role for these two factors in sustaining differentiated cell lineages in the ovary. During gonadal differentiation, FOG-2 may support ovarian determination by inhibiting the AMH expression that determines the testis formation.

2. Upon activation of folliculogenesis, GATA-4 expression is activated in the granulosa cells of primary follicles in the human ovary, and continues in these cells until follicular atresia or luteinization of the ovulated follicles, suggesting a role for GATA-4 in supporting granulosa cell proliferation. GATA-4 is necessary for *inhibin-*α expression in granulosa cells, whereas GATA-6 is involved in steroidogenic function of the granulosa and luteal cells. In general, the GATA transcription factors may prove to be essential in orchestrating the regulation of granulosa cell functions by endocrine and paracrine effectors.

3. Misregulated granulosa cell proliferation may lead to malignant tumors. Most of the resulting granulosa cell tumors express GATA-4, supporting the role of GATA-4 in granulosa cell differentiation and proliferation. Furthermore, GATA-4 expression remains at a high level and comparable with that in normal granulosa cells, especially in the more aggressive granulosa cell tumors that present in advanced clinical stage and recur during follow-up. By contrast, AMH, which is expressed in healthy granulosa cells, is usually downregulated in granulosa cell tumors. This may improve the growth potential of the tumors, given that AMH is a known growth inhibitor in cancer cells.

Open questions and putative means to address these in future research include:

1. Would abrogation of GATA-4 function in the early gonad abrogate ovarian development and differentiation? The most probable answer is yes; to prove this requires the development of an ovarian somatic cell-specific *Gata4*-null mutation in mice. In fact, a testicular Sertoli cell-specific model has already been developed, but the results are as yet unreported (R. S. Viger, pers. comm. 2004). With respect to human disorders in reproductive tract development, mutation of *GATA4* could be involved in a subset of the syndromes.

2. Is FOG-2 an anti-testis or ovarian-determining factor? This can only be speculated about prior to development of a mouse model in which FOG-2 is ectopically expressed in the fetal testis. Mutation analysis in well-characterized human reproductive tract development disorders could also help to answer this question.

3. Is GATA-4 essential for healthy ovarian function? Based on our findings and the results of other studies, several lines of evidence suggest that GATA-4 is mandatory for ovarian function. A mouse model in which *Gata4* is abrogated in postnatal granulosa cells would confirm this. An alternative approach would be to downregulate GATA-4 expression in cultured ovaries by, for example, adenoviral means.

4. Is the high GATA-4 expression in granulosa cell tumors caused by activating mutations in the gene? If the answer by sequencing *GATA4* in tumor DNA turned out to be yes, GATA-4 would be shown to be one of the as yet unknown links in the overactive FSH cascade during tumorigenesis.

5. Are receptors for AMH prominently expressed in granulosa cell tumors? If yes, could AMH act as a therapeutic agent for this cancer? The first question could be answered by employing the recently developed AMH receptor II recognizing antibody with the granulosa cell tumor tissue microarray. To answer the second question, a pilot experiment with an *in vitro* culture of primary granulosa cell tumors and treatment with recombinant AMH would be useful.

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