GATA TRANSCRIPTION FACTORS DURING TESTICULAR DEVELOPMENT AND DISEASE

Ilkka Ketola

Program for Developmental and Reproductive Biology
Biomedicum Helsinki
University of Helsinki
Finland

and

Pediatric Graduate School
Hospital for Children and Adolescents
University of Helsinki
Finland

ACADEMIC DISSERTATION

Helsinki University Biomedical Dissertations No. 24

To be publicly discussed with permission of
The Medical Faculty of the University of Helsinki,
in the Niilo Hallman Auditorium of the Children's Hospital,
on 21 February, 2003, at 12 noon

Helsinki 2003

Supervisors

Professor Markku Heikinheimo
University of Helsinki

and

Professor Juha S. Tapanainen
University of Oulu

Reviewers

Professor Raimo Voutilainen
University of Kuopio

and

Docent Jorma Palvimo
University of Helsinki

Official opponent

Professor Heikki Ruskoaho
University of Oulu

ISBN 952-91-5575-1 (paperback)
ISBN 952-10-0956-X (PDF)
ISSN 1457-8433
Helsinki University Printing House
Helsinki 2003

To my family

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1 ORIGINAL PUBLICATIONS

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

I) Ketola I, Rahman N, Toppari J, Bielinska M, Porter-Tinge SB, Tapanainen JS, Huhtaniemi IT, Wilson DB and Heikinheimo M. Expression and regulation of transcription factors GATA-4 and GATA-6 in developing mouse testis. Endocrinology 1999 140:1470-1480.

II) Ketola I, Pentikäinen V, Vaskivuo T, Ilvesmäki V, Herva R, Dunkel L, Tapanainen JS, Toppari J and Heikinheimo M. Expression of transcription factor GATA-4 during human testicular development and disease. Journal of Clinical Endocrinology and Metabolism 2000 85:3925-3931.

III) Ketola I, Anttonen M, Vaskivuo T, Tapanainen JS, Toppari J and Heikinheimo M. Developmental expression and spermatogenic stage specificity of transcription factors GATA-1 and GATA-4 and their cofactors FOG-1 and FOG-2 in the mouse testis. European Journal of Endocrinology 2002 147:397-406.

IV) Ketola I, Toppari J, Vaskivuo T, Herva R, Tapanainen JS and Heikinheimo M. Transcription factor GATA-6, cell proliferation, apoptosis and apoptosis related proteins bcl-2 and bax in human fetal testis. Journal of Clinical Endocrinology and Metabolism, in press.

In addition, some unpublished data are presented.

2 ABBREVIATIONS

AR	Androgen receptor
DHT	5α-dihydrotestosterone
E	Embryonal day
EDS	Ethylene dimethanesulfonate
EMSA	Electrophoretic mobility shift analysis
FGF-9	Fibroblast growth factor-9
FOG	Friend of GATA
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing homone
hCG	Human chorionic gonadotropin
hpg	Hypogonadal
IHC	Immunohistochemistry
Insl3	Insulin-like hormone-3
ISH	In situ hybridization
LH	Luteinizing hormone
MIS	Müllerian inhibiting substance
mRNA	Messenger ribonucleic acid
P	Postnatal day
PGC	Primordial germ cell
SCF	Stem cell factor
SCOS	Sertoli-cell-only syndrome
SF-1	Steroidogenic factor-1
SRY	Sex-determining region of chromosome Y
StAR	Steroidogenic acute regulatory protein
UGR	Urogenital ridge
WT-1	Wilms' tumor gene-1

3 ABSTRACT

Members of the GATA family of zinc-finger transcription factors are expressed in a variety of tissues. They are well conserved through evolution and are considered to play critical roles in regulating the development and function of several organisms from flies to mammals. The six members of the GATA family are called GATA-1 to GATA-6. They share high structural homology among their zinc-finger regions that are functionally indispensable. Friends of GATA, FOG-1 and FOG-2, are multitype zinc-finger proteins that modulate the transcriptional activity of GATA factors.

GATA factors are essential for hematopoiesis, ventral morphogenesis, heart development, and endoderm formation. GATA-1, GATA-4, GATA-6 and their cofactors FOG-1 and FOG-2 are expressed in the gonads. In humans and in experimental animal studies they have been linked to hematological diseases and tumor formation. Given the developmentally non-redundant role of GATA-1, GATA-4, and GATA-6 and their cofactors FOG-1 and FOG-2, and their abundant expression in gonads, this study aimed to further evaluate their role in testicular development and function.

GATA and FOG mRNAs and proteins were detected by Northern and Western blotting, and temporospatial expression was studied by use of mRNA in situ hybridization and immunohistochemistry. Cell cultures were employed in order to study in vitro hormonal regulation. Testicular samples from genetically hypogonadal mice, after GnRH receptor antagonist-treatment and after chemical abolition of Leydig cells, served for the study of hormonal regulation in vivo. The gene activation studies utilized in vitro co-transfections.

Transcription factor GATA-4 and FOG-2 were expressed in the undifferentiated mouse urogenital ridge, implying a role for them even during the earliest stages of testicular development. Expression of GATA-4 persisted in Sertoli and Leydig cells throughout the fetal and postnatal development. Gonadotropins and androgens regulated GATA-4, but their action was not required for basal expression of GATA-4 at any time of development. GATA-4 regulated the inhibin α gene that is a crucial hormone subunit for proper spermatogenesis. Human Sertoli and Leydig cell tumors exhibited robust GATA-4 expression, suggesting a role for GATA-4 in tumorigenesis or in associated processes such as enhanced cell proliferation. FOG-2 expression ceased in fetal testis, but became upregulated in the somatic cells of the newborn testis. Later, along with advancing spermatogenesis, FOG-2 was downregulated in somatic cells, but its expression reappeared stage-specifically in germ cells.

In human fetal testis, the expression pattern of GATA-6 partially overlapped with that of GATA-4. Differences in the expression of these related factors may reflect distinct functions in the human testis. The early expression of GATA-6 suggests that it may play a role in testicular differentiation.

GATA-1 was not expressed in mouse fetal testis. In contrast, FOG-1 was expressed in fetal Sertoli cells, indicating that it may act as a cofactor for GATA-4 during late fetal testicular development. Postnatally, GATA-1 and FOG-1 were co-expressed in Sertoli cells. In adult testis, their expression was stage-specific, whereas the expression of GATA-4 was constant regardless of the stage of the spermatogenetic wave. Thus, in mouse postnatal testis, FOG-1 most likely modulates the transcriptional activity of GATA-1.

In conclusion, testicular GATA factors are expressed in a distinct, but partially overlapping manner during testicular development from the fetal period to adulthood. GATA-4 is hormonally regulated and regulates the inhibin α gene. The expression pattern of testicular GATA factors suggests that they are important regulators of testicular development and function. FOG-1 and FOG-2 are likely to modulate their actions.

4 INTRODUCTION

Precise gene expression is the basic requirement for development and growth of living organisms. Our genetic information is stored in DNA, which is replicated in order to perpetuate genetic material from one generation to the next. Gene expression is the transformation of DNA information into functional molecules. Transcription, synthesis of RNA from a DNA template, is the first stage of gene expression and the principal stage at which it is controlled. Transcription factors are proteins that regulate transcription through complex mechanisms.

The members of the GATA family of transcription factors are regulatory proteins that control gene expression and developmental processes in various tissues. They are well conserved through evolution from yeast to mammals. Vertebrates possess six known GATA factors, GATA-1 to GATA-6. These are essential for normal hematopoiesis, heart development, and lung and gut morphogenesis. They are expressed in a distinct but partially overlapping manner in a number of tissues, including the gonads. Function of the GATA factors is modulated by their interaction with other transcription factors, transcriptional co-activators, and co-repressors. The FOG proteins FOG-1 and FOG-2, as the best characterized group of cofactors, either enhance or repress the activity of GATA factors depending on cellular context. Besides evolutionarily conserved expression patterns, GATA and FOG proteins regulate gene expression in a functionally conserved manner.

In all vertebrates gonadal development is remarkably similar. Development of the testis or ovary from a bipotential gonadal primordium is a process common to mammals, birds, and reptiles. In mammals, several genes are known to be important for sex determination. Among these, the Y-linked testis-determining gene SRY determines the sexual fate. The presence of SRY in the genital ridge triggers a differentiation cascade that eventually results in testis development. Mutations that adversely affect the function of SRY protein are responsible for disorders associated with male-to-female sex reversal. Genetic analyses of human gonadal dysgenesis and animal studies have revealed that sex determination results from a complex interplay between a number of different genes.

Following sex determination, fetal testis produces hormones that ensure development of the male-type ductal system and external genitalia. At puberty, major morphological changes occur within the testis. Supportive cells, namely Sertoli cells, proliferate, testes grow in size, and steroid-producing Leydig cells mature, reactivating the production of testosterone. These changes are aimed at facilitating germ cell proliferation and subsequent sperm production. Under the influence of gonadotropin-releasing hormone (GnRH), the pituitary gonadotropins FHS and LH regulate the postnatal function of testes. Inhibin, secreted from Sertoli cells, and testosterone, as well, inhibit by feedback mechanism gonadotropin secretion.

Gene expression data provide an important resource for defining gene function and for identifying hierarchies and networks of genes that regulate specific developmental programs. Testicular development is a tightly regulated process, requiring temporally and spatially controlled expression of a number of genes. Those genes act in concert in a manner that subsequently results in the adult-type, functionally mature testis. GATA factors are involved in various developmental processes. The study assessed the expression of gonadal GATA proteins and their cofactors during testicular development and studied their expression during normal development as well in developmental testicular diseases and in tumors.

5 REVIEW OF THE LITERATURE

5.1 Testicular Development and Function

The gonad arises as an identical primordium in all embryos and has the capacity to develop into either testis or ovary. The sex chromosomes control differentiation of the gonads. In mammals, the genetic sex of the embryo is established at fertilization with the inheritance of an X or Y chromosome from the father. Gonads of an XY individual develop as testes and those of XX as ovaries. The development of the internal genitalia duct system and external genitalia is determined by the hormones and hormone-like substances produced by the developing gonads. Testosterone and Müllerian inhibiting substance (MIS, also known as anti-Müllerian hormone, AMH), both secreted by the fetal testis, are needed for development of the male phenotype. Absence of these hormones leads to female-type sexual differentiation (Figure 1).

The testis is comprised of four cell lineages: the germ cells, connective tissue cells, steroid-producing cells, and supporting cells such as Sertoli and myoid cells. Sertoli and germ cells surrounded by myoid cells form seminiferous tubules in which the spermatogenesis takes place. Before puberty, they are called testicular cords. In the interstitium, between the tubules, Leydig cells are the steroid-producing cells (Figure 2).

5.1.1 Fetal period

The gonads appear as a paired structure within the intermediate mesoderm. This region is called the urogenital ridge and gives rise to the adrenals, kidneys, and gonads. The sub-region of the urogenital ridge where the gonad arises is called the genital ridge (Capel 2000). The epithelium of the coelomic cavity lines the urogenital ridges and serves as the source of multiple gonadal cell lineages (Karl and Capel 1998, Schmahl et al. 2000). The ductal system arises from a structure adjacent to the genital ridge called mesonephros that regresses during embryonal development, in the mouse by the embryonal day (E) 12.5.In the mouse, primordial germ cells (PGCs) are first seen in the yolk sec at E7 (Ginsburg et al. 1990). The exact cellular origin of PGCs is unknown. Their ancestors are thought to arise from a pool of epiblast cells at E5-6.5. PGCs migrate to the gonadal part of the urogenital ridge and enter that area between E9.5 and E11, and they may contribute to Sertoli cell differentiation (Adams and McLaren 2002). Primordial germ cells proliferate mitotically, yielding a total of about 20 000 cells from the initial pool of 50 cells (McLaren 2000). PGCs differentiate to gonocytes (prespermatogonia) that undergo mitotic arrest until birth when they resume proliferation (Sutton 2000, de Rooij 2001).

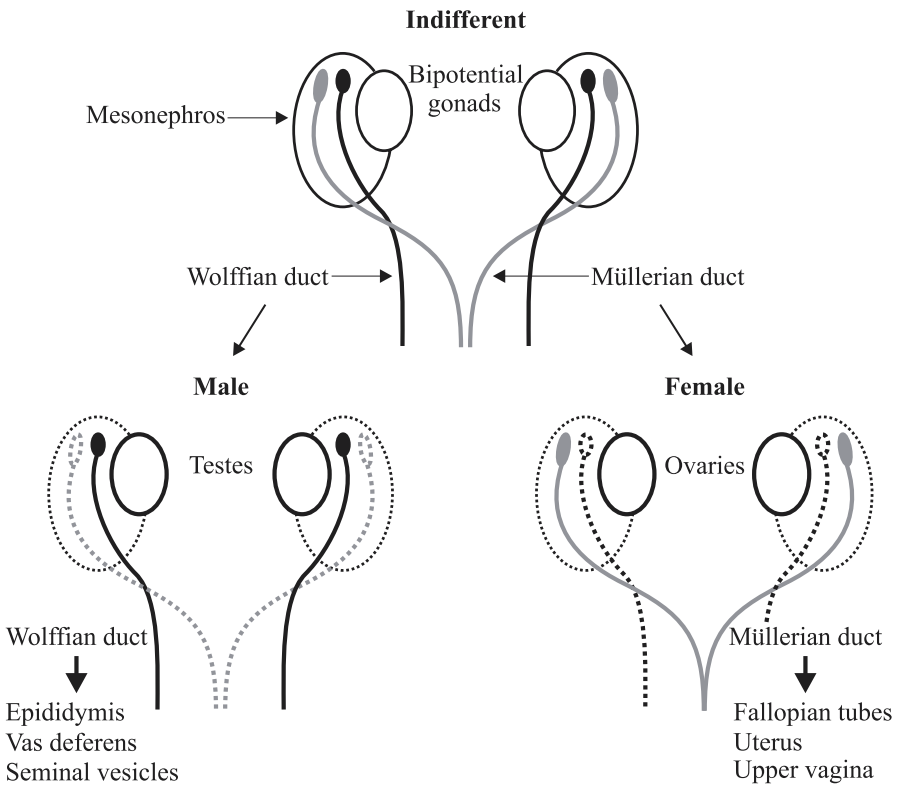

Figure 1. Development of the internal genitalia of the male and female from indifferent gonadal primordia. Fetal testes produce MIS and testosterone. MIS causes the regression of Müllerian ducts, and testosterone and its derivative dihydrotestosterone are required for the development of male-type external genitalia (Rey and Picard 1998).

Male and female mouse genital ridges are morphologically identical at E11. During that time, cells of the coelomic epithelium proliferate intensively and migrate into the undifferentiated gonad. Those cells differentiate to Sertoli cells that surround germ cells to form testicular cords (Karl and Capel 1998). After the initial cord formation at E12.5, it is possible to distinguish the sex of an embryo by morphology (Kaufman and Bard 1999). Sertoli cells are believed to act as the organizing center of the male gonad, and are essential for normal fetal testicular development (Magre and Jost 1984, Magre and Jost 1991, Buehr et al. 1993, Martineau et al. 1997, Merchant-Larios and Moreno-Mendoza 1998, Schmahl et al. 2000, Yao and Capel 2002). Leydig cell precursors originate from the adjacent mesonephros and migrate into the genital ridge by E11.5 (Buehr et al. 1993, Merchant-Larios and Moreno-Mendoza 1998), or they are derived from the cells of the coelomic epithelium (Karl and Capel 1998, Yao and Capel 2002).

Table 1. Timeline for mouse and human testis differentiation. (Data from Carlson 1999, Kaufman and Bard 1999).

Mouse, Day	Human, Week	Stage of testicular development
E9.5-11	4-6	Primordial germ cells migrate from yolk sac to posterior body wall, where they induce formation of genital ridges.
E11	6	Cells from coelomic epithelium and mesonephros proliferate and form the sex cords surrounding germ cells. Genital ridges sexually indifferent.
E12.5	7-8	Sertoli and germ cells form testis cords (seminiferous tubules in adult). Leydig cells differentiate. Testicular morphology recognizable.
E13.5	7	Descent of testes and differentiation of external genitalia begin, completed during the 7^{th} to 9^{th} months. Testicular descent of mice completed postnatally.

The mesonephros regresses during embryonal development. In males, however, it gives rise to rete testis, epididymis, and mesonephric (Wolffian) ducts. The rete testis forms a collecting drainage system continuous with the testicular cords (seminiferous tubules in the adult). The mesonephric (Wolffian) ducts give rise to the epididymis and other ducts which are needed for maturation, nutrients, fluid, and delivery of sperm (Sainio et al. 1997). Testosterone produced by Leydig cells promotes the differentiation of Wolffian-duct derivatives (Rey and Picard 1998). The Sertoli-cell product MIS induces the regression of paramesonephric (Müllerian) ducts, which are the progenitors of female oviducts, uterus, and the upper portion of the vagina (Figure 1) (Behringer et al. 1994).

The testis is differentiated, and testicular morphology is clearly recognizable by E13.5. The testes, still situated in the upper lumbar region, begin to descend, guided by gubernaculum testis into the pelvis and eventually into the scrotum (Hutson 1985, Hutson et al. 1997, Kubota et al. 2002). Around E14.5, testis is encapsulated by the fibrous tunica albuqinea, and the external genitalia are differentiating. By E17.5, the testes and internal genital duct system are similar to those in the newborn (Kaufman and Bard 1999, Kaufman 2001) (Table 1).

5.1.2 Postnatal period

The testis of a newborn or an infant is a functionally immature organ. Postnatal development of the testis aims at a functionally/sexually mature organ capable of producing spermazoa (sperm) and testosterone.

During the postnatal period, the number of Sertoli cells increases significantly. Sertoli cell proliferation is most active at puberty, and by adulthood, fully differentiated Sertoli cells finally cease proliferation (Orth 1982, Cortes et al. 1987, Vergouwen et al. 1991, Vergouwen et al. 1993,

Sharpe et al. 2000). They are essential for spermatogenesis, and their proliferation is required to provide the structural and functional framework over which germ cells will subsequently proliferate and differentiate (Griswold 1998, Chemes 2001). Immediately after birth, gonocytes differentiate into spermatogonia. At puberty, spermatogenesis begins, and spermatogonia differentiate into mature spermatozoa (Figure 2) (de Kretser et al. 1998, de Rooij 2001). The number of Leydig cells remains fairly constant from the late fetal period throughout infancy. In early puberty, Leydig cells initiate their proliferation and differentiate into mature adult-type testosterone-producing cells (Nistal et al. 1986, Habert et al. 2001).

The main regulators of testicular function are gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Figure 3). These are secreted from the pituitary gland under the control of hypothalamic gonadotropin-releasing hormone (GnRH) (Griffin and Wilson 1998). Sertoli cells are the main targets of FSH, and LH acts on Leydig cells. The fetal development of Sertoli and Leydig cells is independent of gonadotropins. Postnatally, gonadotropins stimulate Sertoli proliferation and are essential for normal differentiation and proliferation of adult-type Leydig cells (Orth 1984, Baker and O'Shaughnessy 2001, Heckert and Griswold 2002).

LH stimulates Leydig cells to produce testosterone that is needed for normal spermatogenesis (O'Donnell et al. 1996, McLachlan et al. 2002). The effect of testosterone and some other regulatory factors on spermatogenesis occurs through Sertoli cells; disruption of Sertoli cell-germ cell interactions leads to spermatogenic defects (Griswold 1998, Syed and Hecht 2002). The role of FSH in male reproductive function is controversial. While testosterone alone is sufficient to maintain spermatogenesis, FSH may not be an absolute requirement for male fertility (Singh et al. 1995, Tapanainen et al. 1997, Plant and Marshall 2001).

The most important negative feedback regulators of gonadotropin secretion are testosterone and inhibin. Testosterone acts mainly on the hypothalamus to suppress GnRH secretion (Griffin and Wilson 1998). Sertoli cell-specific inhibin B, a heterodimer of the α and β-B chains, inhibits FSH secretion from the pituitary gland (Plant and Marshall 2001). Inhibin B also serves as a efficient positive marker for spermatogenesis and Sertoli cell function (Anderson and Sharpe 2000). Testosterone and its metabolite estradiol have a suppressive feed-back effect on FSH secretion (Griffin and Wilson 1998).

Whereas inhibin and testosterone act as endocrine regulators of gonadotropin secretion, activin serves as a paracrine or autocrine regulator within the testis and pituitary (de Kretser et al. 2001). Activin and inhibin share common β-subunits (A or B), and activin is a hetero- or homodimer of β-chains. In the testis, activin modulates androgen production locally and has an influence on the proliferation of Sertoli and germ cells. In the pituitary, activin enhances FSH secretion.

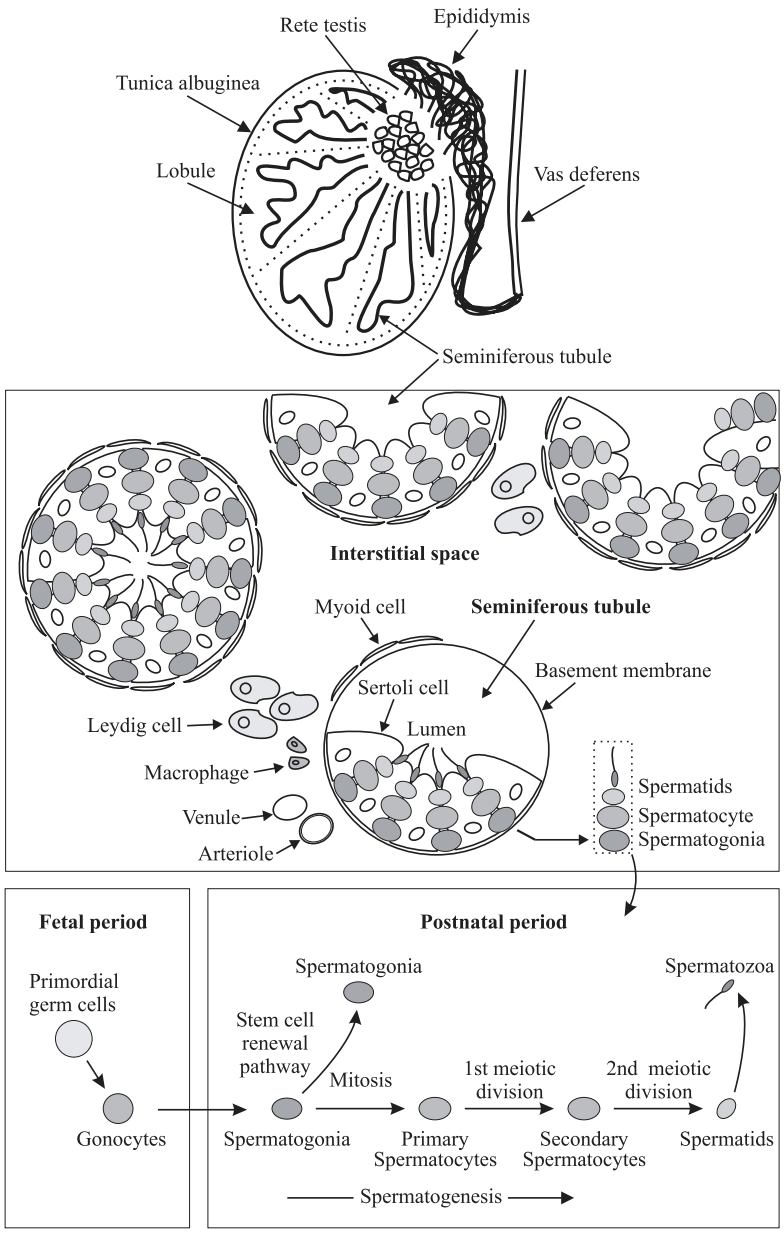

Figure 2. Schematic view of testis structure and the differentiation program of male germ cells. Above: General structure of the human testis. Lobules are filled with seminiferous tubules (only one shown). **Center:** Cross-section of seminiferous tubules in which spermatogenesis takes place. Germ cells are at different phases of differentiation in a given tubule. Sertoli cells are supportive cells facilitating maturation of germ cells. Basement membrane and myoid cells separate seminiferous tubules from the interstitial space. **Below:** Differentiation program of male germ cells. During the fetal period, primordial germ cells differentiate into gonocytes, which differentiate into spermatogonia right after birth. At the beginning of puberty, spermatogonia proliferate, to generate undifferentiated stem cells and to proceed along the germ cell differentiation pathway into spermatozoa. Final panel modified from (Sassone-Corsi 1997).

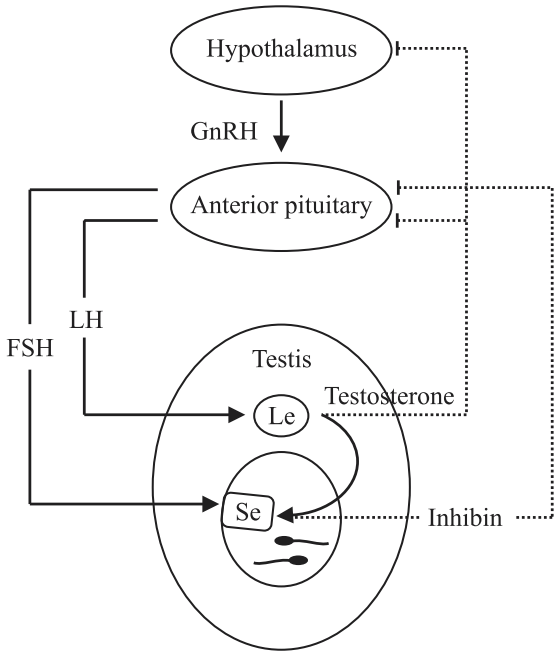

Figure 3. Schematic view of the function of the hypothalamic-pituitary-gonadal axis. Solid lines mark stimulatory, and dotted lines inhibitory effect. Le = Leydig cell; Se = Sertoli cell.

5.2 Genes Essential for Gonadal Development

Sex determination results from a complex interplay between different genes that are conserved in many species. In mammals, several genes are known to be important for sex determination (Figure 4). They orchestrate the expression of genes that further mediate gonadal development. The development of testis or ovary from a bipotential gonad throughout vertebrates is remarkably similar.

5.2.1 Establishment of the urogenital ridge

The Wilms' tumor suppressor gene WT-1 is required to establish the bipotential gonads and kidneys (Kreidberg et al. 1993, Moore et al. 1999). This gene is expressed in gonads of both sexes throughout fetal development (Pelletier et al. 1991b, Armstrong et al. 1993). Mutations in the WT-1 gene result in complete XY sex-reversal, possibly through reduced SRY activity (Pelletier et al. 1991a, Barbaux et al. 1997, Hammes et al. 2001, Hossain and Saunders 2001).

Another gene expressed early is steroidogenic factor-1 (SF-1, also known as Ad4BP) (Lala et al. 1992, Morohashi et al. 1992), required for the formation of gonads and adrenals (Luo et al. 1994, Achermann et al. 1999, Bakke et al. 2001). SF-1 is already expressed in the gonadal primordium of both sexes, and in the Sertoli and Leydig cells of the testis (Ikeda et al. 1994, Shen et al. 1994, Schmahl et al. 2000). It is not necessary for the initial formation of genital ridges; rather, it seems to be required for the differentiation or the maintenance and growth, or both, of the somatic cells already present there. Later, in the fetal testis, SF-1 regulates MIS in concert with WT-1 and SOX-9 (Shen et al. 1994, De Santa Barbara et al. 1998, Nachtigal et al. 1998, Arango et al. 1999, Watanabe et al. 2000, Shen and Ingraham 2002) and with a number of genes involved in steroidogenesis (Parker and Schimmer 1997).

5.2.2 Testis differentiation

While WT-1 and SF-1 are required for the establishment of the urogenital ridges of both sexes, SRY (Sex determining Region on Y chromosome) is a Y-linked gene that acts dominantly to trigger testis development from indifferent urogenital ridge. Deletions or mutations of the SRY gene lead to XY female development (Gubbay et al. 1990, Lovell-Badge and Robertson 1990, Sinclair et al. 1990, Gubbay et al. 1992, Hawkins et al. 1992). Even reduced SRY expression causes XY sex-reversal or ovotestis (Laval et al. 1995, Nagamine et al. 1999, Hammes et al. 2001, Washburn et al. 2001). A female mouse carrying an SRY transgene develops a male (Koopman et al. 1991). No target genes for SRY are known. SRY is first expressed in the male mouse urogenital ridge around E10. Its expression peaks in Sertoli cells at E11.5 and declines sharply thereafter, indicating that SRY initiates the differentiation of gonadal supporting cell precursors to develop as testicular Sertoli cells rather than as ovarian granulosa cells (Hacker et al. 1995, Albrecht and Eicher 2001). Sertoli cell signaling, in turn, is thought to be essential for further testis development (section 5.1.1).

A close relative of SRY is SOX-9. It is highly conserved at the amino acid level and is expressed in testes of all vertebrates (Kent et al. 1996, Morais da Silva et al. 1996, Bowles et al. 2000, Nagai 2001). Mutations in SOX-9 result in XY sex-reversal, whereas ovarian development is normal, demonstrating that SOX-9 is necessary for testis determination (Foster et al. 1994, Wagner et al. 1994, Koopman 1999). Furthermore, SOX-9 expression in ovaries results in female-to-male sex-reversal (Huang et al. 1999, Bishop et al. 2000, Vidal et al. 2001). SOX-9 may represent an ancestral sex-determining gene, and mammals have evolved SRY as a Y-linked switching mechanism (Nagai 2001). Based on expression pattern, SOX-9 serves as a putative target gene for SRY, although definitive proof for this is still lacking. After Sertoli cell differentiation, SOX-9 regulates the expression of MIS and SF-1 (De Santa Barbara et al. 1998, Shen and Ingraham 2002).

The Sertoli cell product MIS is not essential for testis formation but is required for the formation of the male-type ductal system that eventually effects fertility (Behringer et al. 1994). MIS is

expressed in Sertoli cells through fetal development (Munsterberg and Lovell-Badge 1991, Hacker et al. 1995), and very low levels of AMH permit its function, which favors male-type development (Arango et al. 1999). SOX-9 regulates MIS expression with co-ordinated interactions between SF-1, WT-1, and GATA-4 (Foster et al. 1994, De Santa Barbara et al. 1998, Nachtigal et al. 1998, Arango et al. 1999, Tremblay and Viger 1999). DAX-1 down-regulates MIS transcription, repressing the synergistic action of SF-1 and WT-1 (Nachtigal et al. 1998).

5.2.3 Anti-testis genes

The nuclear receptor DAX-1, considered an anti-testis gene, is proposed to act as an SRY antagonist (Swain et al. 1998, Yu et al. 1998). DAX-1 gene duplication results in male-to-female sex-reversal (Bardoni et al. 1994, Zanaria et al. 1994), and mutations in DAX-1 lead to hypogonadotropic hypogonadism (Muscatelli et al. 1994, Tabarin et al. 2000). DAX-1 is expressed in the gonads of both sexes (Ikeda et al. 1996, Swain et al. 1996, Tamai et al. 1996). It is coexpressed with SF-1 along the developing hypothalamic-pituitary-gonadal axis (Guo et al. 1995, Ikeda et al. 1996, Swain et al. 1996, Zazopoulos et al. 1997), and DAX-1 may repress SF-1-mediated activity in those organs (Ito et al. 1997, Zazopoulos et al. 1997, Wang et al. 2001).

A member of the WNT family of secreted proteins, WNT-4 has been suggested to suppress male development, because it down-regulates steroid production (Vainio et al. 1999). In human males, duplication of the region of chromosome 1 that includes WNT-4 leads to sex-reversal (Jordan et al. 2001).

5.2.4 Other genes important for gonadal development

Fibroblast growth factor-9 (FGF-9) is widely expressed in mouse embryos, and male mice lacking FGF-9 exhibit sex-reversal and phenotypes range from testicular hypoplasia to complete sex-reversal (Colvin et al. 1999, Colvin et al. 2001). FGF-9 regulates SRY-dependent processes such as cell proliferation and migration into the gonad, and Sertoli cell differentiation. Mouse knockout studies have revealed several transcription factors that may play important roles in gonadal development; these include Lim1, Lhx9, Emx2, and M33 (Shawlot and Behringer 1995, Miyamoto et al. 1997, Katoh-Fukui et al. 1998, Birk et al. 2000). Their gonadal phenotype varies from complete gonadal agenesis to sex reversal. More profound studies are needed to evaluate their function in gonadal development.

For normal spermatogenesis, the correct gonadal position is essential. Disruption of the INSL-3 gene (also known as relaxin-like factor) causes bilateral cryptorchidism, i.e., failure of the testis to descend from its embryonal retroperitoneal position into the scrotum (Zimmermann et al. 1999, Adham et al. 2000).

The genes described herein represent some of the most important genes known to be involved in sex-determination and gonadal development. Many genes have sexually dimorphic expression patterns in the gonad, and some of these undoubtedly contribute to testis formation and function (Wertz and Herrmann 2000). Human gonadal dysgeneses serve as invaluable in vivo models for study of gonadal development, since murine models may not accurately reflect the physiological situation (Swain and Lovell-Badge 1999, Morrish and Sinclair 2002, Parker and Schimmer 2002). Despite sex differences, gonadal development also provides an excellent model for study of organogenesis and its genetic control.

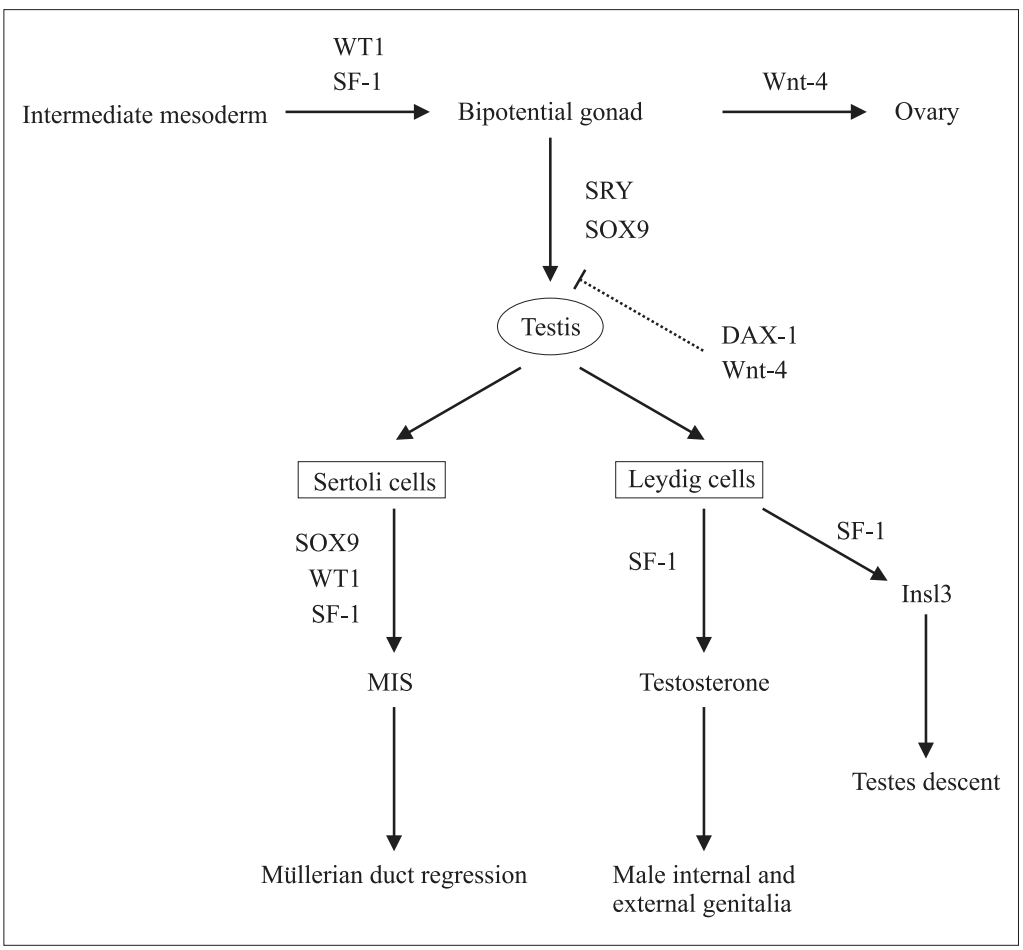

Figure 4. Molecular events in mammalian sex determination and testicular differentiation involving genes. Solid lines indicate activating effects, and dotted line, inhibition.

5.3 Diseases of the Human Testis

Abnormalities of testicular function comprise a heterologous group of disorders that cause different consequences depending on the phase of sexual development in which they are manifested. Defects range from rare syndromes with underandrogenization to normal virilization with reduced fertility. Tumors of the testis are the second most common malignancy, after leukemia, in men between age 20 and 35. The epidemiological evidence suggests that testicular cancer, undescendent testis, and impaired spermatogenesis are biologically closely associated (Skakkebaek et al. 1998).

5.3.1 Hypogonadism

Male hypogonadism refers to a failure in testicular function that eventually may lead to infertility (Table 2). The most common developmental defect of the testis is Klinefelter's syndrome, 47XXY (Berkovitz and Seeherunvong 1998). Klinefelter males have typically divergent testicular histology and small testes, and as a result, reduced testosterone production. In hypogonadotropic hypogonadism such as Kallman's syndrome (Hardelin 2001), testosterone production is reduced due to a lack of stimulatory hormones. In patients with androgen resistance, the impact of androgens is reduced. Complete androgen resistance results in XY male-to-female sex-reversal, whereas the mildest forms of androgen resistance may result in infertility with otherwise normal genitalia (Sultan et al. 2002). In cryptorchidism, one or both testes have failed to descend. Spermatogenesis requires the lower temperature that is present in the scrotum, but the temperature-dependent mechanism is unknown. Varicocele, cryptorchidism, and Klinefelter's syndrome account for 45% of known conditions in men with infertility (Greenberg et al. 1978, Griffin and Wilson 1998)

Table 2. Abnormalities of testicular function. (Modified from Griffin and Wilson 1998, Huhtaniemi and Dunkel 2000).

Hypothalamic-pituitary	Tumor
	Trauma
	Anabolic steroids
	Kallman's syndrome
	Idiopathic hypogonadal hypogonadism (IHH)
Testicular	Developmental and structural defects
	Germinal cell aplasia:
	cryptorchidism, varicocele
	Klinefelter's syndrome
	Acquired:
	viral orchitis, radiation, environmental toxins
	Systemic diseases:
	liver and renal diseases, AIDS
Others	Androgen resistance

5.3.2 Tumors

Germ cell tumors, the most common type of testicular tumors (Table 3), are presumed to be derived from primordial germ cells. Besides testis, germ cell tumors can originate in extragonadal sites such as the mediastinum and brain. Stromal tumors, e.g., Sertoli and Leydig cell tumors, account for the minority of testicular tumors (Kaplan et al. 1986). Germ cell and stromal tumors may be hormonally active and cause endocrinological symptoms including gynecomastia and azoospermia.

Table 3. Classification of testicular tumors. (Table modified from Griffin and Wilson 1998; data from Mostofi 1980).

I) Germ cell tumors (95%)
A) Single-cell-type tumors
Seminomas
Yolk sac tumors
Teratomas
B) Combination tumors
II) Tumors of gonadal stroma (1-2%)
Leydig cell
Sertoli cell
Primitive gonadal structures
III) Gonadoblastomas
Germ and stroma cells

5.4 The Gata Family of Transcription Factors

Transcription factors are *trans*-acting molecules that bind to specific *cis*-acting DNA sequences on promoters and/or enhancers of genes (Lewin 1997, Berg et al. 2002). The members of the GATA family of transcription factors form a group of these regulatory proteins which control gene expression in multiple tissues. GATA transcription factors are related by their homologous DNA-binding domains. GATA factors are well conserved through evolution; they are found in various organisms ranging from cellular slime mold to humans. Even some plants are proposed to have GATA homologs (Lowry and Atchley 2000). Vertebrate GATA factors descend from a common ancestral sequence, whereas the evolutionary pathway among nonvertebrate GATA factors is much different from that within vertebrates (Lowry and Atchley 2000).

All GATA factors contain one or two DNA-binding zinc finger domains of the distinctive form Cys-X_2-Cys-$X_{17\text{-}18}$-Cys-X_2-Cys (X represents any amino acid, and the subscript denotes their number). These recognize a consensus DNA sequence, (A/T)GATA(A/G), known as GATA motif (Ko and Engel 1993, Merika and Orkin 1993), which is an essential *cis*-element present in the promoters and enhancers of a variety of genes (Orkin 1992).

The function of GATA factors is modulated by their interaction with other transcription factors, transcriptional co-activators, and co-repressors, the best-characterized group of cofactors being the FOG proteins FOG-1 and FOG-2 that exist in species from flies to human. GATA and FOG proteins not only share evolutionary conserved expression and structural homology but also fulfill functionally conserved functions (Cantor and Orkin 2001, Fossett and Schulz 2001, Fossett et al. 2001).

5.4.1 Mode of action

All GATA proteins are approximately 50 kDa in size. Vertebrate GATA proteins contain two highly conserved zinc fingers and the N-terminal transcriptional activation domain. The zinc fingers in particular are shown to play essential roles in GATA-mediated gene activation. The C-terminal zinc finger is needed for DNA binding, and the N-terminal finger stabilizes this interaction and mediates interactions with other factors such as FOG proteins (Martin and Orkin 1990, Trainor et al. 1996, Tsang et al. 1997, Svensson et al. 1999, Tevosian et al. 1999). Within the zinc finger regions, the amino acid sequence is almost identical between the divergent GATA factors. The DNA binding region of mouse GATA-4 is 70% identical with that of mouse GATA-1, and mouse and human GATA-4 zinc fingers are 100% identical (Huang et al. 1995).

All the GATA factors bind to GATA or GATA-like sequences. However, subtle differences exist in their individual binding affinities for various promoters. Those differences and interactions with other factors such as FOGs may allow precise programming of GATA function despite their overlapping expression pattern in multiple tissues (Ko and Engel 1993, Merika and Orkin 1993, Yamagata et al. 1995, Mackay et al. 1998, Sakai et al. 1998, Charron et al. 1999, Morrisey et al. 2000, Kowalski et al. 2002).

GATA-1 is the founding member of GATA family and has received the most extensive study (Tsai et al. 1989). Given that all GATA factors share structural homology, predictions as to the properties and function of any GATA factor can be made to some extent based on the studies performed on GATA-1. Indeed, results from studies in diverse developmental contexts with GATA factors other than GATA-1 suggest that all GATA factors function in a quite similar manner.

In vitro studies suggest that many properties of the GATA family of proteins are shared and interchangeable. In mouse GATA-1-deficient embryonic stem (ES) cells, GATA-3, GATA-4, and even chicken GATA-1 are able to compensate for the hematological GATA-1 defect (Blobel et al. 1995). In these cells, even chimeric molecules, in which both zinc fingers of mouse GATA-1 were replaced with the zinc fingers of human GATA-3 or with the single finger of the fungal GATA factor, display rescue activity. However, in vivo experiments have failed to demonstrate that GATA factors are functionally equivalent. In mice deficient in GATA-1, transgenic expression of GATA-2 or GATA-3 has rescued the embryonic lethal phenotype of the GATA-1 mutation, but

adult transgenic mice developed anemia (Takahashi et al. 2000). Furthermore, the embryonal lethality of most GATA knockout mice indicates that GATA factors do not share complete functional redundancy in vivo (Table 4).

Table 4. Homozygous null mutations of GATA factors.

Homozygous null mutation	Consequence
GATA-1	Lethal at E10.5-11.5; hematopoietic failure
GATA-2	Lethal at E9.5-11.5; hematopoietic failure
GATA-3	Lethal at E11-12.0; neurogenic defect
GATA-4	Lethal at E7-10.5; defective ventral morphogenesis and heart tube formation
GATA-5	Viable, genitourinary malformations in females
GATA-6	Lethal at E5.5-7.5; block in endoderm differentiation

5.4.2 Vertebrate GATA factors

Vertebrates have six GATA transcription factors, designated GATA-1 to GATA-6 according to the order in which they were identified (Evans and Felsenfeld 1989, Tsai et al. 1989, Yamamoto et al. 1990, Arceci et al. 1993, Laverriere et al. 1994, Morrisey et al. 1996, Morrisey et al. 1997).

The GATA proteins are divided into two subgroups based on their expression pattern. GATA-1, GATA-2, and GATA-3 are expressed mainly in blood-forming cells and are essential for normal hemopoiesis (Pevny et al. 1991, Tsai et al. 1994, Pandolfi et al. 1995, Fujiwara et al. 1996, Ting et al. 1996, Shivdasani et al. 1997, Vyas et al. 1999).

GATA-4, GATA-5, and GATA-6 are expressed in visceral and parietal endoderm, heart, lung, liver, pancreas, adrenals, gonads, gut epithelium, smooth muscle cells, and some other tissues. Gene disruption studies on mice have revealed that these proteins are important for ventral morphogenesis, heart, genitourinary tract and endoderm formation, and lung maturation (Kuo et al. 1997, Molkentin et al. 1997, Morrisey et al. 1998, Koutsourakis et al. 1999, Molkentin et al. 2000, Liu et al. 2002b).

GATA-1

GATA-1 was originally identified in hematopoietic cell lineages (Martin et al. 1990, Romeo et al. 1990, Orkin 1992, Zon et al. 1993) and was found to be essential for erythroid and megakaryocytic cell differentiation (Pevny et al. 1991, Fujiwara et al. 1996, Shivdasani et al. 1997, Vyas et al. 1999). In addition to hematopoietic cells, GATA-1 is expressed in Sertoli cells of the testis (Ito et al. 1993, Yomogida et al. 1994) (Table 5). GATA-1 gene transcription in Sertoli cells is directed by the testis-specific promoter 8 kb upstream to that in erythroid cells. The five exons that encode GATA-1 protein are commonly used by testis and erythroid transcripts (Ito et al. 1993, Onodera et al. 1997a, Onodera et al. 1997b).

GATA-1 is expressed in postnatal Sertoli cells in a stage-dependent manner, and maturing germ cells may negatively control its expression (Yomogida et al. 1994). Furthermore, FSH via cAMP reduces GATA-1 expression in testicular cells (rat Sertoli cells and mouse Leydig tumor cell line MA-10) (Zhang et al. 2002). In cell culture studies, GATA-1 transactivates a number of testicular genes, including inhibin α- and β-B-subunits, MIS, StAR, and aromatase (Feng et al. 1998, Feng et al. 2000, Robert et al. 2002). The physiological relevance of these findings remains unclear, since GATA-1 knockout animals die in utero before proper testis development (Fujiwara et al. 1996).

GATA-2

GATA-2 is preferentially expressed in the hematopoietic cell lineages (Tsai et al. 1989, Orkin 1992). It is indispensable for normal hematopoiesis, neurogenesis, and genitourinary development (Tsai et al. 1994, Zhou et al. 1998, Nardelli et al. 1999). In mice, impaired GATA-2 expression disturbs Wolffian and Müllerian duct development, but testes develop normally. In the fetal ovary, GATA-2 is expressed in the germ cells between E11.5 and 15.5, as judged by digoxygenin in situ hybridization (Siggers et al. 2002). No GATA-2 expression has been detected in the testis.

GATA-3

Disruption of the GATA-3 gene leads to embryonic lethality due to noradrenaline deficiency. It also results in abnormalities of the nervous system and of kidney development, in aberrations in fetal liver hematopoiesis and in block of T-cell differentiation (Pandolfi et al. 1995, Ting et al. 1996, Lim et al. 2000). In humans, GATA-3 haplo-insufficiency causes the HDR syndrome that results in hypoparathyroidism, deafness, and renal anomaly (Van Esch et al. 2000). GATA-3 is expressed in the Wolffian duct and mesonephros, but no reports exist on gonadal expression (Labastie et al. 1995, Debacker et al. 1999). Interestingly, GATA-3 serves a role also in adipocyte differentiation (Tong et al. 2000).

GATA-4

GATA-4 is found in a number of different tissues. During embryonal development, it is expressed in the primitive (yolk sac) endoderm, heart, gut, liver, and gonads, and in adult heart, intestine, and gonads (Arceci et al. 1993, Kelley et al. 1993, Heikinheimo et al. 1994, Laverriere et al. 1994, Huang et al. 1995, White et al. 1995, Morrisey et al. 1996).

The role of GATA-4 in the heart is the most extensively studied. GATA-4-deficient mice die embryonally between E7.0 and E10.5 because of severe folding abnormalities (Kuo et al. 1997, Molkentin et al. 1997). GATA-4 is essential for heart development through combinatorial interactions with other transcription factors (Nemer and Nemer 2001). It is also linked to hypertrophy-associated gene expression (Molkentin 2000, Hautala et al. 2001, Marttila et al. 2001,

Kerkela et al. 2002, Yanazume et al. 2002). GATA-4 is also required for proper differentiation of gastric epithelium (Jacobsen et al. 2002).

Besides the heart, GATA-4 is essential for testicular development, possibly by regulating SRY expression (Tevosian et al. 2002). GATA-4 activates several gonads-expressed genes that encode steroidogenic enzymes (StAR, aromatase), hormones (MIS, inhibin alpha, inhibin/activin beta-B), and transcription factor SF-1 (Viger et al. 1998, Ketola et al. 1999, Silverman et al. 1999, Tremblay and Viger 1999, Feng et al. 2000, Watanabe et al. 2000, Tremblay et al. 2001, Tremblay and Viger 2001a, Tremblay et al. 2002). DAX-1 represses MIS transcription in Sertoli cells by disrupting transcriptional synergism between GATA-4 and SF-1 (Tremblay and Viger 2001b). This synergism may represent a mechanism for the regulation of SF-1-dependent genes in other target tissues such as the pituitary and adrenals. SF-1, FOG-1, and FOG-2 modulate transcriptional activity of GATA-4 through distinct mechanisms depending on the cellular context (Tremblay et al. 2001, Robert et al. 2002, Anttonen et al. 2003).

GATA-5

GATA-5 is expressed in the developing heart, lung, gut, and gonadal ridge (Laverriere et al. 1994, Morrisey et al. 1997). It shares high amino acid-level sequence identity with murine GATA-4 and GATA-6, but not with other GATA factors. GATA-5 regulates specific cardiac and gastric genes (Gao et al. 1998, Charron and Nemer 1999, Nemer and Nemer 2002). Female mice with homozygous deletions for GATA-5 exhibit pronounced genitourinary abnormalities that include vaginal and uterine defects and hypospadias. Male mice are unaffected (Molkentin et al. 2000).

GATA-6

During the embryonal period, GATA-6 has been detected in the primitive streak, visceral endoderm, heart, stomach, liver, gut, atrial smooth muscle cells, developing bronchi, and urogenital ridge. It is expressed in adult heart, stomach, gut, lung, pancreas, and ovary (Laverriere et al. 1994, Jiang and Evans 1996, Morrisey et al. 1996, Narita et al. 1996, Suzuki et al. 1996, Huggon et al. 1997). Human GATA-6 is a 499-amino-acid protein almost identical in the two zinc finger-binding domains with other human GATA proteins (Suzuki et al. 1996, Huggon et al. 1997).

GATA-6 is essential for visceral endoderm formation (Morrisey et al. 1998, Koutsourakis et al. 1999) and for proper lung development (Keijzer et al. 2001, Liu et al. 2002b, Yang et al. 2002). Based on in vitro studies, GATA-6 regulates genes expressed in the heart, lung, and gut (Gao et al. 1998, Charron et al. 1999, Liu et al. 2002a, Robert et al. 2002). In the gonads, GATA-6 has been proposed to upregulate MIS, StAR, inhibin α, and aromatase genes, and its activity is down-regulated by FOG-1 and FOG-2 (Robert et al. 2002). Due to early embryonic lethality before gonadal development, the in vivo relevance of these findings remains unclear (Morrisey et al. 1998, Koutsourakis et al. 1999).

Table 5. Expression of GATA factors in mouse gonads. ND = not defined.

	Fetal			Adult	
	Urogenital ridge	Testis	Ovary	Testis	Ovary
GATA-1	-	-	-	+	-
GATA-2	+	-	+	-	-
GATA-3	-	-	-	-	-
GATA-4	+	+	+	+	+
GATA-5	+	ND	ND	-	-
GATA-6	+	+	+	+	+

5.4.3 Friends of GATA, FOG-1, and FOG-2

The transcriptional activities of GATA factors are modulated by their interactions with other transcription factors and with transcriptional coactivators and repressors (Krause and Perkins 1997). FOG-1 is the multitype zinc finger protein first demonstrated to interact with GATA-1, and it serves as a cofactor for GATA-1-mediated transcription (Tsang et al. 1997). It is coexpressed with GATA-1 in the hematopoietic tissues and in adult liver and testis. FOG-1 itself and its interaction with GATA-1 are essential for normal hematopoiesis (Tsang et al. 1998, Crispino et al. 1999, Deconinck et al. 2000, Chang et al. 2002).

FOG-2 was originally described as a cofactor for GATA-4 (Holmes et al. 1999, Lu et al. 1999, Svensson et al. 1999, Tevosian et al. 1999). It is a1151-amino acid nuclear protein that contains eight zinc fingers structurally related to those of FOG-1. FOG-2 is predominantly expressed in embryonal mouse heart, urogenital ridge, and neuroepithelium. In the adult, it is expressed in the heart, brain, and testis. FOG-2 interacts with all known GATA factors, either activating or repressing their transcriptional activity, depending on the promoter and cell type in which they are tested. FOG-2 and its interaction with GATA-4 are essential for heart morphogenesis and testicular development (Svensson et al. 2000, Tevosian et al. 2000, Crispino et al. 2001, Tevosian et al. 2002). FOG-1 and FOG-2 function in hematopoiesis and during cardiogenesis are conserved in Drosophila, Xenopus, and mammals (Cantor and Orkin 2001, Fossett and Schulz 2001, Fossett et al. 2001).

5.4.4 Clinical implications of GATA and FOG families

Human studies aid in evaluation of the relevance of in vivo and in vitro animal studies. When the findings of these studies are combined, new therapeutic approaches may become possible. GATA factors have been linked to the pathogenesis of various human diseases: A mutation in the GATA-1 DNA-binding site in the platelet glycoprotein Ibβ gene promoter results in a rare bleeding disorder, Bernard-Soulier syndrome (Ludlow et al. 1996). Mutations in GATA-1 zinc fingers that disturb DNA binding or interaction with FOG-1 may lead to anemia, trombocytopenia, and to thalassemia (Nichols et al. 2000, Freson et al. 2001, Mehaffey et al. 2001, Yu et al. 2002). Mutations

in GATA-1 may also constitute one step in the pathogenesis of the megakaryoblastic leukemia in Down syndrome (Wechsler et al. 2002). GATA-3 has been proposed to play a role in asthmatic airway inflammation (Nakamura et al. 1999, Ray and Cohn 1999, Christodoulopoulos et al. 2001). GATA-4 may be involved in the pathogenesis of some yolk sac, adrenal, gastrointestinal, and ovarian tumors (Kiiveri et al. 1999, Pehlivan et al. 1999, Siltanen et al. 1999, Laitinen et al. 2000, Lin et al. 2000, Lassus et al. 2001). Furthermore, GATA-4 haploinsufficiency may contribute to congenital heart disease in patients with monosomy of 8p23.1 (Pehlivan et al. 1999).

5.5 Role of Apoptosis in Testis

Apoptosis, also known as programmed cell death, is an evolutionarily conserved process that plays an essential role in the regulation of tissue development and homeostasis. It is involved in normal development and in the pathogenesis of diverse diseases (Jacobson et al. 1997, Raff 1998). Apoptotic cells commit controlled suicide in order to remove structures or cells that are no longer needed. In the testis, apoptosis serves a role in regulating the growth and survival of germ cells which eventually results in normal spermatogenesis (Dunkel et al. 1997, Matsui 1998, Sinha Hikim and Swerdloff 1999, Kierszenbaum and Tres 2001). Furthermore, regression of Müllerian ducts occurs through apoptosis (Roberts et al. 1999). The Bcl-2 family of proteins is a major regulator of germ cell apoptosis, either by supporting cell survival or promoting cell death (Figure 5). The balance between germ cell survival and death is a prerequisite for spermatogenesis and testis development. Bcl-2 and bax have well-established roles in those processes (Rodriguez et al. 1997, Russell et al. 2002). Additionally, the balance and dimerization of pro- and anti-apoptotic factors controls cell death and survival (Yang et al. 1995, Rucker et al. 2000, Yan et al. 2000b, Yan et al. 2000c).

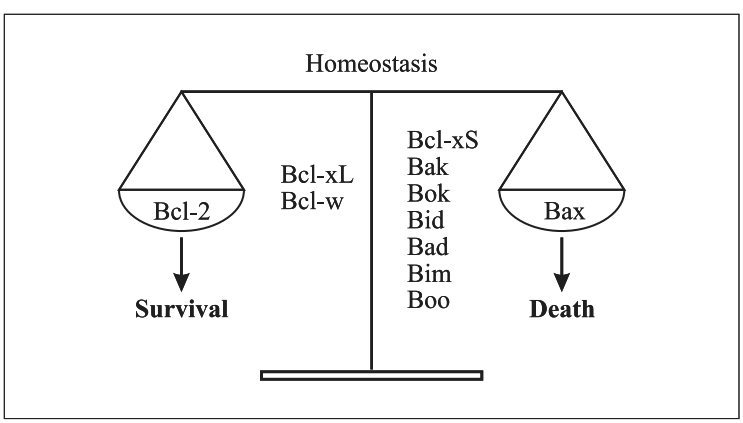

Figure 5. Regulation of apoptosis by Bcl-2 family of proteins. Modified from (Sinha Hikim and Swerdloff 1999). Bcl-2 family members on the left promote cell-survival and on the right are pro-apoptotic.

GATA factors are proposed to play roles in apoptotic processes. Best characterized is the effect of GATA-1 on the production of red blood cells (De Maria et al. 1999, Orkin and Weiss 1999). Caspases, which are death-promoting enzymes, inactivate GATA-1, which results in apoptotic death of maturing red blood cells. In the ovary, from fetal until adult life GATA-4 may protect granulosa cells from apoptosis (Heikinheimo et al. 1997, Vaskivuo et al. 2001). In embryonic stem cells, cardioblast differentiation is blocked and cells are lost through apoptosis in the absence of GATA-4 (Grepin et al. 1997). GATA-6 downregulation leads to apoptosis within the embryonic endoderm and in colorectal cancer cells (Morrisey et al. 1998, Shureiqi et al. 2002). No reports have appeared concerning GATA factors and their possible role in apoptosis of the testis.

6 AIMS OF THE STUDY

The aims of the study were to study:

1) Temporal and spatial cell-specific expression patterns of transcription factors GATA-1, GATA-4, GATA-6 and their co-factors FOG-1 and FOG-2 during testicular development from the fetal period to adulthood.

2) Regulation of GATA-4 and GATA-6 in testis by both in vitro and in vivo approaches.

3) Gonadal genes that are regulated by testicular GATA factors.

4) Role of gonadal GATA factors in human testicular diseases.

7 SAMPLES AND METHODS

These studies were accepted by the Ethics Committees of the Children's Hospital of the University of Helsinki and the University of Oulu, and conducted according to recommendations of the Declaration of Helsinki. Animal studies were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Table 6. Samples and methods of the studies.

Study	Samples	Methods
I	Mouse and rat testis hpg mouse testis GnRH receptor antagonist-treated rat EDS-treated rat Gonadal tumor cell lines Sertoli (MSCFSHR-1) Leydig (BLT-1, mLTC-1) Granulosa (KK-1)	Rnase protection: GATA-4, GATA-6 Northern blot: GATA-4 ISH: GATA-4, GATA-6 Pem, H+/K+ -ATPase IHC: GATA-4, GATA-1 In vitro co-transfection: Inhibin α promoter GATA-4 expression vector
II	Normal human testis Fetal, prepubertal, pubertal, adult Testis samples from developmental testicular diseases and after hormonal treatment Testicular tumors	Northern blot: Western blot: GATA-4 IHC:
III	Mouse testis E10.5 UGR to adult	ISH: FOG-2 IHC: GATA-1, GATA-4 FOG-1, FOG-2
IV	Human fetal testis	ISH: GATA-4, GATA-6 IHC: GATA-4, GATA-6 Bcl-2, Bax, Ki-67 In situ DNA 3'-end labeling

ISH = mRNA in situ hybridization
IHC = immunohistochemistry
UGR = urogenital ridge

7.1 Tissue samples

7.1.1 Human testicular samples

Fetal and premature newborn testicular tissue samples (n = 16) at weeks 12 to 40 came from abortions induced for socio-medical reasons and from autopsy specimens. Normal juvenile (n = 9) or pubertal (n = 1) testis biopsy samples were obtained from boys diagnosed with acute lymphoblastic leukemia (ALL) without testicular involvement. Testis samples from cryptorchid boys (n = 33) were obtained from diagnostic biopsies during orchidopexy before (n = 19) or after (n = 14) hCG treatment. Undescended testes were classified as either inguinal or high scrotal. Testicular tissue from patients with androgen resistance (n = 11) came from therapeutic gonadectomies. Testis samples also came from prostate cancer patients having received GnRH-agonist (n = 2) or GnRH-agonist-antiandrogen (n = 1) treatment before orchidectomy. One biopsy sample was obtained from a 26-year-old man with Kallman's syndrome who had received human menopausal gonadotropin (HMG) treatment for 2.5 years. Testicular samples from adult patients with prostate cancer (n = 7) served as controls. Sertoli (n = 1), Leydig (n = 5), and germ cell tumors (n = 6) came from patients undergoing gonadectomy.

7.1.2 Animal samples, models, and cell culture

Samples from mouse embryos, and fetal and postnatal testes were obtained by mating B6SJLF1/J, CBA, or NMRI mice. For estimating embryonal age, the noon of the day on which the copulating plug was found was considered embryonal day 0.5. Precise staging of dissected embryos was performed according to *The Atlas of Mouse Development*. PCR analysis of the Zfy or SRY gene was conducted in order to determine the sex of E10.5 and E12.5 embryos. To study hormonal effects, testicular tissue was harvested from a hypogonadal (hpg) mouse strain, 3-week-old Sprague-Dawley rats treated with gonadotropin-releasing hormone (GnRH) receptor antagonist azaline B, and 3-month old Sprague-Dawley rats after treatment with ethane-1,2-dimethane sulphonate (EDS).

Immortalized Leydig cell tumor lines BLT-1 and mLTC-1 were cultured in plastic dishes on Dulbecco's Modified Essential Media (DMEM) with GlutaMAX®/F12 1:1 buffered with HEPES (20 mmol/L) and supplemented with 10% heat-inactivated fetal bovine serum, glucose (4.5 g/L), and gentamicin (100 mg/L). Cells were used for immunohistochemistry after 2 to 3 days in culture. For hormone stimulation, cells were cultured for 24 h in the presence of 1, 10, and 100 µg/L recombinant hCG, 10 µg/L progesterone, 20 µg/L aminoglutethimide (AMG), or a combination of 100 µg/L recombinant hCG and 20 µg/L aminoglutethimide (AMG).

7.2 Experimental methods

7.2.1 RNase protection assay

RNase protection assays were performed with a commercially available kit according to manufacturer's recommendations (Ambion), with 10 µg of total testicular RNA. The antisense riboprobes: GATA-4, GATA-6, and ß-actin, were 32P-labeled.

7.2.2 Northern hybridization

Total RNA was isolated with the Qiagen RNeasy Mini Kit or the guanidinium thiocyanate method. Denatured total RNA (10or 20 µg) was subjected to electrophoresis on a 1%, 1.2%, or 1.5% denaturing agarose gel and then transferred onto nylon membranes. These membranes were hybridized with ^{32}P-labeled RNA probes for mouse GATA-4 and GATA-6 or with synthetic oligonucleotide probes for human GATA-4. For increasing sensitivity of the hybridization for human GATA-4, the two different oligomers were labeled simultaneously and pooled for hybridization. Hybridization was performed at 60°C overnight, and the membranes were washed three times for 20 min each at 60°C with 1x SSC/0.1 % SDS**.** Hybridization signals were detected by autoradiography or by phosphoimager. Intensities of the specific bands were quantified with Tina® software and normalized to 28S and to 18S ribosomal RNAs in the gel stained with ethidium bromide. A specific probe for ribosomal 28S mRNA also served as a loading control.

7.2.3 mRNA in situ hybridization

Tissue samples were washed in PBS and then fixed in 4% paraformaldehyde or formalin and embedded in paraffin, or they were frozen in liquid nitrogen in cryopreservation solution. In situ hybridization was carried out with minor modifications as described elsewhere (Wilkinson 1992). In brief, tissue sections (8-10 µm) were deparaffinised, permeabilized, dehydrated, and then incubated with 1 x 10^6 cpm of ^{33}P-labeled antisense or sense riboprobes in a total volume of 80 µl and incubated overnight at temperatures of 60 to 62°C. Hybridization signals were detected after a 5- to 21-day exposure to emulsion at 4°C.

7.2.4 Western blotting

Tissue sections were homogenized on ice in homogenization buffer. After centrifugation at 17 000 *g* at 4°C for 30 min, the supernatants were collected and their protein concentrations were determined by Bio-Rad DC protein assay. Proteins (40 µg) were loaded onto 10% SDS-

polyacrylamide gel, and electrophoresis was performed at 160 V. The proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by electrophoresis for 2 h at 4°C in transfer buffer at 100 V. The transfer was checked by staining with 0.2% Ponceau S in 3% trichloroacetic acid. GATA-4 protein on the membrane was detected by an affinity-purified rabbit polyclonal antibody to GATA-4 at dilution 1:1000, followed by horseradish peroxidase-conjugated secondary antibody. The bound secondary antibody was located with the ECL detection kit.

7.2.5 Immunohistochemistry

Testicular samples or cultured Sertoli and Leydig cells were fixed in 4% paraformaldehyde, formalin, or Stieve's fixative and embedded in paraffin, or they were frozen in liquid nitrogen in cryopreservation solution. Tissue sections (6-8 µm) were deparaffinized in xylene and hydrated gradually through graded alcohols. If needed, endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. Samples were subjected to immunohistochemistry by the antibodies described in Table 7. Antibodies were at 1:25-1:500 dilution. Samples with primary antibody were incubated at 37°C for 1h or at 4°C overnight. An avidin-biotin immunoperoxidase system served to visualize bound antibody; 3-amino-9-ethylcarbazole or 3,3'-diaminobenzedine (DAP) served as the chromogen, and the development reaction occurred in the presence of 0.03% H_20_2. Samples were analyzed by light/darkfield and phase-contrast microscopy. Whenever possible, in immunohistochemistry and in situ hybridization, the expression patterns for respective antibodies and transcripts were studied for adjacent tissue sections.

Table 7. Antibodies

Antigen		Clonality	Source
GATA-1	Rat-antimouse	MC	Santa Cruz Biotechnology
GATA-4	Rabbit-antihuman	PC	See Arceci et al. 1993
	Goat-antimouse	PC	Santa Cruz Biotechnology
GATA-6	Rabbit-antihuman	PC	Santa Cruz Biotechnology
FOG-1	Goat-antimouse	PC	Santa Cruz Biotechnology
FOG-2	Rabbit-antimouse	PC	Santa Cruz Biotechnology
Bcl-2	Mouse-antihuman	MC	DAKO
Bax	Rabbit-antihuman	PC	Pharmingen
Ki-67	Mouse-antihuman	MC	DAKO

Antibodies were used at 1:100 – 1:500 dilution, bax at 1:25
MC = monoclonal
PC = polyclonal

7.2.6 In situ DNA 3'-end labeling

Apoptosis was qualitatively identified in the testes by use of an in situ DNA 3'-end labeling (TUNEL) kit (Oncor). Paraffin sections of testes were rehydrated through an alcohol series, and permeabilized in proteinase K, and endogenous peroxidase activity was inhibited by 5% hydrogen peroxide. DNA fragmentation was identified by applying terminal transferase enzyme with digoxigenin-labeled nucleotides to the samples and incubating for 1 h under coverslips. Antidigoxigenin antibody served to recognize the digoxigenin-labeled nucleotide chains attached to the 3'-ends of sample DNA. A color reaction was produced with 3,3'-diaminobenzedine in the presence of 0.03% hydrogen peroxide.

7.2.7 Transfections

Leydig tumor cell line mLTC-1 and granulosa cell line KK-1 were split at a density of 1×10^5 the day prior to transfection and then transfected with DOTAP Liposomal Transfection Reagent (Boehringer) with a slight variation from manufacturer's instructions. The 211-bp fragment of inhibin α was cloned into the reporter plasmid pTKGH (Nichols Institute Diagnostics). The first, second, or first and second GATA sites in the inhibin α promoter were mutated by replacing the G with a C, by site-directed mutagenesis with the Gene Editor Site Directed Mutagenesis System (Promega). For each well, the appropriate pTKGH reporter plasmid was mixed with the expression vector pMT2-GATA-4 or with the control vector pMT2. The DNA was then diluted with 20 mM Hepes buffer and added to the DOTAP, and the transfections were carried out according to manufacturer's instructions; cells were incubated in total 72 h. Aliquots were removed for use in the hGH radioimmunoassay (Nichols Institute Diagnostics). All transfections were carried out in triplicate and repeated at least 3 times.

7.2.8 Statistics

Statistical analyses were performed on the basis of 3 independent experiments. Data were subjected to one-way analysis of variance and by the SuperANOVA program, followed by Duncan's New Multiple Range and Fisher's Protected LSD post-hoc tests. All p-values less than 0.05 were considered significant.

8 RESULTS AND DISCUSSION

8.1 Expression of GATA and FOG factors during testicular development

It is of utmost importance to know the temporal and spatial expression pattern of a given gene in order to understand its function. In vitro gene activation studies are invaluable tools to explore gene function. These studies lack, however, the normal cell context and composition, and this may result in misjudgments. Detailed in vivo studies of gene expression are needed to reconcile discrepancies with cell culture models, thus defining the interactions that actually may occur and regulate gene expression in any given tissue.

Previous studies indicate that GATA-1, GATA-4, and GATA-6 are expressed in the gonads (Arceci et al. 1993, Yomogida et al. 1994, Narita et al. 1996, Suzuki et al. 1996), but their testicular expression patterns have not been studied in detail. Findings show expression of GATA-1 outside the hematopoietic system to be restricted to the Sertoli cells of the testis, and GATA-4 and GATA-6 to be present in the ovary and testis. In the ovary, GATA-4 and GATA-6 have been expressed in a distinct, but partially overlapping manner (Heikinheimo et al. 1997). GATA-1 already had a well-established role in the regulation of hematopoiesis (Pevny et al. 1991), and GATA-4 and GATA-6 were proposed to regulate heart differentiation and function (Heikinheimo et al. 1994, Ip et al. 1994, Molkentin et al. 1994, Thuerauf et al. 1994, Morrisey et al. 1996). Taken that GATA-1, GATA-4, and GATA-6 are expressed in the gonads and that they are potent regulators of gene expression in various tissues, they could well be important regulators of testicular function.

8.1.1 Expression in urogenital ridge and fetal testis (I-IV)

Expression of GATA-1 during the earliest stages of testicular development was then unresolved, as was the cell-specific expression of GATA-4 and GATA-6 in the testis. When FOG-1 and FOG-2 were discovered, their cell-specific expression as to the gonads was not yet addressed (Tsang et al. 1997, Svensson et al. 1999, Tevosian et al. 1999). Therefore, in this thesis, the temporal and spatial expression of GATA-1, GATA-4, and GATA-6 in the testis, as well as that of their cofactors FOG-1 and FOG-2 from the early embryonic period to adulthood is explored in detail.

GATA-1 and FOG-1 were absent from the undifferentiated genital ridge of the male **mouse**, as studied by immunohistochemistry (III) (Figure 6 and Table 8). With further fetal testicular development, GATA-1 remained undetectable throughout embryogenesis, whereas FOG-1 protein was evident in the Sertoli cells after testicular differentiation at E15.5, and this expression persisted until term. GATA-4 and FOG-2 mRNAs and proteins were detected in the urogenital ridge of E10.5

male embryos (III). GATA-4 expression was obvious throughout fetal testicular development, and localized in the Sertoli, Leydig, and myoid cells as well as cells of the tunica albuginea (I, III). Conversely, the expression of FOG-2 mRNA and protein diminished in Sertoli cells, parallel to advancing fetal testicular development. Some interstitial cells and cells in the tunica albuginea, however, expressed FOG-2 mRNA and protein also in late fetal testis (II). As judged by mRNA in situ hybridization, GATA-6 transcripts were detected at E13.5 in the testicular cords, and their expression persisted there throughout fetal development (I).

The resolution of in situ hybridization did not allow us to determine the precise cell types expressing GATA-6 within the cords (I). Because GATA-6 antibodies were unavailable at that time, we were unable to assess whether GATA-6 protein was present in Sertoli or germ cells. Later, we conducted GATA-6 immunohistochemistry on fetal testis samples. In contrast to the findings in man, GATA-6 protein was localized in testis cords and seminiferous tubules in the germ cells, and not in Sertoli cells (unpublished results).

Table 8. Expression of GATA and FOG factors during mouse testicular development.
ND = not defined.

Age	GATA-1	GATA-4	GATA-6	FOG-1	FOG-2
Genital ridge	-	+	ND	-	+
E17.5	-	+	+	+	-
Newborn	-	+	+	+	+
Adult	+	+	+	+	+

In **human** fetal testis, GATA-4 and GATA-6 mRNAs and proteins were present between gestational weeks 12 and 40 (II, IV). Sertoli and Leydig cells expressed both GATA-4 and GATA-6, but myoid cells were positive only for GATA-4. A subset of the Sertoli and Leydig cells did not express GATA-6. Immunoreactivity for GATA-4 and GATA-6 was most intensive at the beginning of the second trimester and declined towards term. Gonocytes were negative for both these factors at all ages studied (II, IV).

Expression of GATA-4 overlapped in human fetal testis with that observed in the mouse. In contrast, the expression of GATA-6 protein in testicular cords differed between man and mouse (IV; our unpublished results). In a recent study, GATA-6 expression was detected in mouse fetal Sertoli cells by digoxygenin in situ hybridization (Robert et al. 2002). Unfortunately, the data presented was inadequate to clarify the cell types that expressed GATA-6. The reason for these contradictory results for GATA-6 Sertoli and germ cell expression remains unclear. It may reflect differences between mouse strains or methods, but further efforts should be made to clarify the expression of GATA-6 in mouse testis. Of interest to note, GATA-6 mRNA has already been detected in the undifferentiated genital ridge (Morrisey et al. 1996). Thus, GATA-6 may play a role in the regulation of gonadal development.

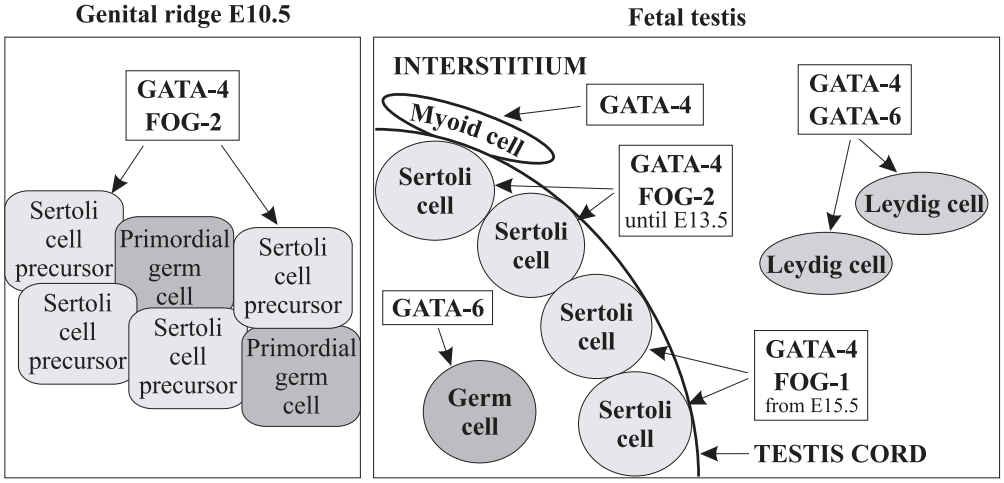

Figure 6. Schematic illustration of GATA-4, GATA-6, FOG-1, and FOG-2 expression in mouse genital ridge and fetal testis.

8.1.2 Fetal expression of GATA and FOG factors in relation to gonadal developmental genes

GATA-4 and FOG- 2 are co-expressed in the genital ridge before testicular cords have formed, and their expression overlaps with that of several key regulators of gonadal development and sexual differentiation. GATA-4 and FOG-2 are expressed simultaneously with WT-1 and SF-1 in the urogenital ridge and just previous to expression of SRY, SOX-9, MIS, and DAX-1 (Swain and Lovell-Badge 1999, Capel 2000, Morrish and Sinclair 2002). Given that all those factors have a crucial impact on testicular development, the expression patterns of GATA-4 and FOG-2 strongly indicate roles for them in early testicular development. Furthermore, GATA-4 RNA and protein are expressed in cells of the coelomic epithelium of the primitive streak embryo at E7.0 (Heikinheimo et al. 1994). Some of these cells will eventually give rise to the testis Sertoli cells, and expression of GATA-4 may be needed for initiating and/or maintaining the differentiation pathway towards a mature Sertoli cell. If this is the case, GATA-4 is one of the earliest Sertoli cell markers.

FOG-1 was originally identified as a cofactor for GATA-1 (Tsang et al. 1997). In the fetal testis, FOG-1 is expressed at E15.5, co-localizing with GATA-4 in Sertoli cells. Given that GATA-1 is not expressed in fetal testis, it is plausible that in fetal testis, FOG-1 interacts not with GATA-1, but rather with GATA-4. Indeed, recent studies have shown that FOG-1 and FOG-2 are capable of interacting with known testicular GATA factors (Robert et al. 2002). In fetal testis, FOG-2 expression gradually ceases, but it is abundantly expressed throughout the fetal period in the ovary (III) (Anttonen et al. 2003). This sexually dimorphic expression pattern after gonadal differentiation may indicate that after the very early phases of testicular differentiation, FOG-2 promotes female rather than male development.

8.1.3 Expression in postnatal testis (I-III)

Newborn testes are functionally immature organs. In mammals, the period before puberty or sexual maturity ranges from weeks to several years. In mice, testicular development starts shortly after birth, and the last phases of the neonatal period overlap with the beginning of puberty. In human males, however, the period before the onset of puberty ranges from 9 to 13 years (Chemes 2001). Nevertheless, during that time, considerable changes in testicular morphology occur. The testis increases in size as Sertoli cells proliferate, Leydig cells mature to adult-type steroid-producing cells, and finally spermatogenesis begins, ultimately to produce fertile spermatozoa. Given that these GATA and FOG transcription factors are likely to play important roles in fetal testicular development, they may be the regulators of postnatal development and function, as well. In order to reveal the temporal and spatial expression patterns of these factors in the postnatal testis, we conducted mRNA in situ hybridization and immunohistochemical analyses of testicular samples from newborn to adult testis (I-IV).

GATA-4 and GATA-6 transcripts were detectable in the testis of the newborn **mouse**, and this expression persisted throughout adulthood (I) (Figure 7 and Table 8). The expression of GATA-4 protein localized to Sertoli and Leydig cells without any stage-specificity. Diverging from the GATA-4 expression pattern, GATA-6 was expressed in spermatogonia and Leydig cells, as seen by immunohistochemistry (unpublished results). In prepubertal **human** testes, GATA-4 protein was expressed in Sertoli cells and spermatogonia, whereas only a few Leydig cells were faintly GATA-4 positive. After puberty, GATA-4 was expressed in the Sertoli cells, was upregulated in the Leydig cells, and downregulated in the germ cells (II).

FOG-1 protein was detectable in the Sertoli cells of the newborn **mouse**, and the expression persisted there throughout postnatal testicular development (III). From P7 onwards, FOG-1 expression was similar to that of GATA-1. In the adult testis, Sertoli cell expression of FOG-1 and GATA-1 was stage-specific; these were predominantly expressed in the Sertoli cells of stage VII to XII seminiferous tubules (III).

FOG-2 mRNA and protein were abundantly expressed in the testis of the newborn **mouse**. They were present in the Sertoli and Leydig cells, and in the cells of the tunica albuginea. Some of the gonocytes were also immunoreactive for FOG-2, but this was never noted after the first postnatal week. Along with advancing spermatogenesis, FOG-2 expression in the somatic cells ceased, whereas it was upregulated in germ cells. In adult testis, midpachytene spermatocytes up to step 9 spermatids were positive for FOG-2 predominantly in stage VII to XII seminiferous tubules (III).

Comparison of the expression patterns of GATA-1 and MIS reveals an inverse relationship in their Sertoli cells expression, suggesting that GATA-1 may downregulate MIS in male mice during the establishment of puberty (Beau et al. 2000). At that time, GATA-1 co-localized with FOG-1 in their Sertoli cells, and the expression was stage-specific later in adult testis. This indicates that

FOG-1 may act as a co-activator or co-repressor for GATA-1. In the adult testis, Sertoli cell expression of inhibin α and inhibin βB-subunits (Kaipia et al. 1992, Majdic et al. 1997), FSH receptor (FSHR) (Heckert and Griswold 1991, Heckert and Griswold 1993), stem cell factor (SCF) (Hakovirta et al. 1999), and androgen receptor (AR) (Bremner et al. 1994, Zhou et al. 2002) is stage-specific (Table 9). The stage-specificity of these gene products suggests that they may be target genes for those GATA/FOG family members. Of note, GATA-4 expression is not stage-specific (I-III). Nevetherless, GATA-4 activates and binds to promoters of genes of stage-specific expression. Stage-specific expression of GATA/FOG protein may thus not be an ultimate requirement for the activation of a given gene.

A profound change occurs in the expression of FOG-2 parallel to advancing spermatogenesis, namely, somatic cells cease to express FOG-2, and in the adult testis, it is expressed stage-specifically in spermatocytes and spermatids. FOG-2 and GATA-6 are the only GATA and FOG family members expressed in both male germ and somatic cell lineages. Tyrosine-kinase receptors, namely c-kit and its truncated form tr-kit, are also expressed in germ cells (Manova et al. 1990, Manova and Bachvarova 1991) and in Leydig cells. The c-kit receptor and its ligand SCF are essential for maintenance of PGCs, and postnatally they regulate spermatogenesis and Leydig cell development (Mauduit et al. 1999, Rossi et al. 2000, Yan et al. 2000a, Kierszenbaum and Tres 2001). Because c-kit receptors have essential GATA-binding sites in their promoters (Rossi et al. 1992, Yamamoto et al. 1993), an intriguing possibility is that GATA-6 and FOG-2 may act in concert with c-kit and its derivatives within the germ and Leydig cells.

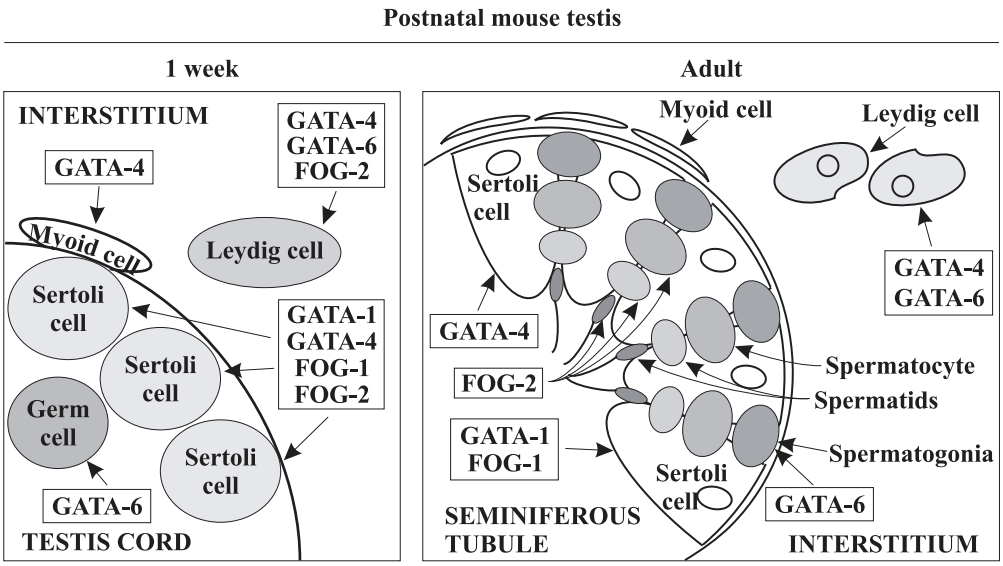

Figure 7. Schematic illustration of GATA-1, GATA-4, GATA-6, FOG-1, and FOG-1 expression in mouse testis.

Table 9. Genes with stage-specific expression in Sertoli cells.

Gene	Highest expression stage		Putative regulation by GATA factors
Inhibin α	XIII-I	mRNA; rat (Bhasin et al. 1989) Protein stage-specific, stages not addressed (Majdic et al. 1997)	GATA-4 and GATA-1 transactivate
Inhibin βB	XIII-III	mRNA; rat (Kaipia et al. 1992) Protein stage-specific, stages not addressed (Majdic et al. 1997)	GATA-4 and GATA-1 transactivate
FSH-receptor	XIII-II	mRNA; rat (Heckert and Griswold 1991)	GATA sites in promoter (Huhtaniemi et al. 1992) Inverted GATA-element required for promoter activity (Kim and Griswold 2001) GATA-4 binds to promoter (unpublished results, Figure 8)
Stem cell factor	II-VI VII-VIII	mRNA; rat (Hakovirta et al. 1999) Protein; mouse (Vincent et al. 1998)	GATA site in promoter (Taylor et al. 1996)
Androgen receptor	II-VII VI-VII III	Protein; rat (Bremner et al. 1994) Protein; mouse (Zhou et al. 2002) Protein; human (Suarez-Quian et al. 1999)	GATA site(s) in promoter (Song et al. 1993; Mizokami et al. 1994)

In prepubertal **human** testis, GATA-4 was expressed in spermatogonia (II). This was the only period of testicular development at which GATA-4 expression was detectable in germ cells. During the first months of postnatal life, fetal gonocytes resume their mitotic activity and differentiate into spermatogonia (de Rooij 2001). After this initial differentiation, there appear incomplete spermatogenic waves before the onset of puberty (Chemes 2001). The expression of GATA-4 in a subset of spermatogonia may relate to this premature germ cell differentiation/proliferation. The same expression pattern was not observed in the **mouse** testis. However, in another study, GATA-4 expression was detected in the germ cells of the adult mouse, not in Sertoli or Leydig cells (Viger et al. 1998). It is therefore possible that some minor species and strain-specific differences exist in the expression patterns of GATA and FOG proteins. The reason for and significance of these differences remain unclear.

In **human** testis, a few prepubertal Leydig cells showed weak GATA-4 expression, whereas robust GATA-4 expression was detected after puberty. The precursors of adult type Leydig cells are hormonally inactive before puberty (Chemes 2001). At the initial stages of puberty, Leydig cell precursors activate and differentiate into steroid-producing cells. This testosterone production

coincides with increasing GATA-4 expression, suggesting a possible link between GATA-4 and androgen production. This is supported by findings in fetal testes, as well. GATA-4 expression is at its highest in Leydig cells when they are hormonally most active, just before midgestation (Tapanainen et al. 1981). Furthermore, results from in vitro gene activation studies indicate that GATA-4 activates genes essential for steroidogenesis (section 8.3.4).

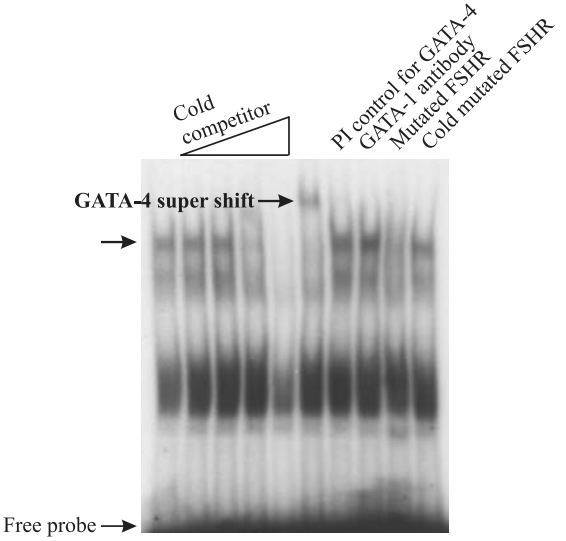

Figure 8. Mouse FSH receptor promoter binding GATA-4 protein. EMSA performed with ^{32}P-labeled double-stranded FSHR oligonucleotide corresponding to nucleotides –543/-528. This promoter sequence of FSHR includes a putative GATA binding site just upstream of the second major transcription initiation site. Arrow on the left indicates the complex formed by ^{32}P-labeled FSHR probe and GATA-4 protein from a mouse Sertoli cell tumor line (MSCFSHR-1) nuclear extract (10 µg). GATA-4, but not GATA-1, antibody bound to FSHR/GATA-4 complex (GATA-4 super shift). When the first G was mutated to C at the GATA site, no FSHR/GATA-4 complex was formed (mutated FSHR). A cold competitor was used in 0.1, 1, 10, and 50-fold excess (triangle) and 50-fold excess in cold mutated FSHR; goat normal serum served as PI control for GATA-4.

8.2 Regulation of testicular GATA factors

8.2.1 Cell culture (I)

It has been demonstrated that FSH treatment of the mouse Sertoli cell line MSC-1 results in a modest increase in the steady state level of GATA-4 mRNA but not that of GATA-6 (Heikinheimo et al. 1997). Given these findings, we used Northern hybridization in order to study the regulation of GATA-4 in Leydig cell lines in which it was abundantly expressed (I). In two Leydig cell tumor lines, mLTC-1 and BLT-1, hCG stimulation (100 µg/L) resulted in a modest but significant upregulation of GATA-4 mRNA expression, whereas progesterone (10 µg/L) significantly downregulated GATA-4 mRNA levels. After blocking steroidogenesis with aminoglutethimide (20 µg/L), no significant alteration in GATA-4 mRNA levels was observable (I). No GATA-6 mRNA expression could be detected in these cells before or after hormonal treatments (I).

In summary, stimulation with gonadotropins resulted in modest changes in the steady state levels of GATA-4 mRNA in gonadal cell lines (I) (Heikinheimo et al. 1997). These results indicate that gonadotropins may upregulate GATA-4 expression in Sertoli and Leydig cells, i.e., in the cells expressing receptors for these pituitary gonadotropins in the testis (Griffin and Wilson 1998). Our

findings thus indicate that GATA-4 may be involved in the gonadotropin-induced signaling cascade leading to cAMP-activated gene expression.

Not only GATA-4 but also GATA-1 may be regulated by gonadotropins. In the primary cultures of rat Sertoli cells, FSH suppressed GATA-1 mRNA but not that of GATA-4 (Zhang et al. 2002). The same effect on GATA-1 was also seen with cAMP in the MA-10 Leydig cell line, indicating that gonadotropin regulation of GATA factors may occur through the cAMP pathway. Although some results on GATA-4 regulation are controversial, testicular GATA factors seem to be differentially regulated by gonadotropins. Cell lines used in in vitro studies may exhibit different settings or variable amounts of other proteins that further modulate the impact of hormones on GATA expression. Furthermore, tumor cell lines and purified primary cell cultures lack the normal signaling control from the adjacent tissues, and this may also contribute to somewhat controversial results.

8.2.2 Regulation of GATA-4 and GATA-6 in vivo (I)

In order to gain insight into the hormonal regulation of GATA-4 and GATA-6 in vivo, we used several models of **genetically or pharmacologically manipulated animals**. The hormonal regulation of these factors was studied by disrupting the hypothalamic-pituitary-gonadal axis at various sites: hpg mice (Cattanach et al. 1977) exhibit congenital and functionally complete gonadotropin deficiency; azaline B treatment (Campen et al. 1995) results in postnatal gonanadotropin deficiency, and EDS treatment (Bartlett et al. 1986) destroys Leydig cells, leading to testosterone depletion with subsequent Sertoli cell dysfunction (Table 10).

Table 10. Murine models in Study (I).

Models	Site of action	Consequence of treatment, or genetic defect
Hpg mouse	No GnRH secreting neurons in hypothalamus	Congenital lack of gonadotropins
Azaline B treatment	GnRH receptor antagonist in pituitary	Block in gonadotropin secretion
EDS treatment	Destruction of Leydig cells in testis	No testosterone production

GATA-4 expression in animal models was studied by in situ mRNA hybridization and immunohistochemistry, and GATA-6 expression by in situ mRNA hybridization (I). Congenital lack of gonadotropins or the treatment applied did not totally abolish GATA-4 expression in the seminiferous tubules or interstitium. GATA-4 expression persisted in Sertoli and Leydig cells of hpg mice, and in Sertoli cells of azaline B-treated rats. EDS treatment did not affect GATA-4 expression in Sertoli cells. However, the expression intensity of GATA-4 in the testes of hpg mice was reduced, indicating downregulation of GATA-4 expression by the total absence of gonadotropins. On the contrary, no detectable changes appeared in GATA-6 expression in hpg mice or after chemical treatment of azaline B- and EDS-treated rats.

Human studies on GATA-4 expression offered the possibility to assess indirectly the in vivo regulation of GATA-4 (II). Results of those studies were in line with results in animal models. In fetal Sertoli cells, that strongest GATA-4 expression coincided with high serum FSH levels (Kaplan et al. 1976) suggests that GATA-4 may be under gonadotropic control also in human beings. GATA-4 was in low or absent from Sertoli and Leydig cells of patients with androgen resistance, suggesting that congenital lack of androgen action influences GATA-4 expression. Furthermore, after hormonal treatment for prostate cancer, GATA-4 expression remained unchanged in Sertoli cells (GnRH agonist and GnRH agonist plus antiandrogen). These results agree with findings in rodent testes after GnRH receptor antagonist treatment or chemical abolition of Leydig cells.

In the light of current findings, the normal testicular response to gonadotropins and androgen action are both needed for normal GATA-4 expression. However, neither gonadotropin nor androgen action nor both are prerequisites for basal expression of GATA-4 and GATA-6. Gonadotropin stimulus may be more important for GATA-4 expression during fetal development than postnatally, taken that changes in the expression of GATA-4 were seen only in congenital gonadotropin- and androgen defects. Furthermore, it is most likely that not only gonadotropins and androgens, but other hormones and factors are also involved in the regulation of GATA-4. For example, progesterone downregulated GATA-4 expression in Leydig cell tumor lines (I). Alternatively, GATA-4 may well be constitutively expressed in testis somatic cells, making the response to hormonal stimulus modest. The effects of hormonal stimuli on GATA-4 expression possibly are mediated indirectly through cofactors which are hormonally inducible (Silverman et al. 1999). Because in situ hybridization and immunohistochemistry are insufficiently sensitive to detect minor changes in RNA and protein expression, and methods like real-time RT-PCR should be applied to enhance sensitivity.

In the ovary, GATA factors are probably under more stringent hormonal control than in the testis. This suggestion is based on in vivo studies in which pregnant mare's serum gonadotropin (PMSG) and estrogen enhanced follicular expression of GATA-4 and GATA-6; induction of ovulation with hCG caused a decrease in GATA-4 mRNA expression (Heikinheimo et al. 1997). Ovarian function is more dependent on gonadotropins than is testis function (Aittomaki et al. 1995). It has been demonstrated that a mutation in the FSH receptor (FSHR) gene causes hereditary hypergonadotropic ovarian failure, which results in infertility. In the male, lack of FSH function is incompatible with testicular maturation and fertility (Tapanainen et al. 1997). Previously, FSH was considered essential for pubertal initiation of spermatogenesis and maintenance of normal sperm production in adults. However, males homozygous for an inactivating FSHR mutation suffer from various degrees of spermatogenic failure, calling into question the essential role of FSH in the initiation of spermatogenesis. In the ovary, the inactivating FSHR mutation (Ala189Val) results in very low or negligible GATA-4 expression (Vaskivuo et al. 2002). It has been impossible to study testicular GATA-4 expression in men with this FSHR mutation.

8.3 Role of GATA and FOG factors in testicular function

8.3.1 GATA-4 regulating inhibin α gene (I)

The 5' region of the mouse inhibin α gene has promoter activity essential for its function. This region includes a pair of consensus GATA-binding sites between nucleotides -153 and -110 (Su and Hsueh 1992). Mutation analysis of the GATA elements in rat inhibin α promoter demonstrated that mutations of either one or both of the two GATA sites reduce basal and cAMP-stimulated activity of the inhibin α promoter (Feng et al. 1998). Furthermore, in human and rodent testis, inhibin α is expressed in Sertoli and Leydig cells overlapping with GATA-4 expression (Bhasin et al. 1989, Tone et al. 1990, Eramaa et al. 1992, Majdic et al. 1997, Anderson et al. 2002) (I-IV). FSH and LH are the main regulators of inhibin α in Sertoli and Leydig cells (Ying et al. 1986, Lee et al. 1989). In those cells, FSH and LH slightly upregulate GATA-4, providing a plausible mechanism for hormonal control of inhibin α expression.

Electrophoretic mobility shift analysis (EMSA) demonstrated that GATA-4 specifically binds to both GATA sites of inhibin α promoter. When the first G on the GATA site was mutated to C, binding activity was totally abolished (Figure 9, unpublished results). Furthermore, GATA-4 transactivated mouse inhibin α promoter in Leydig (mLTC-1) and granulosa (KK-1) tumor cell lines in in vitro transactivation experiments (I) (Figure 10). Mutations of one or both of the GATA binding sites of inhibin α promoter fully abolished GATA-4-induced transactivation. Results of in vitro experiments do not necessarily reflect the physiological situation, but data from other studies support a potential link between GATA-4 and the regulation of inhibin α gene in the testis.

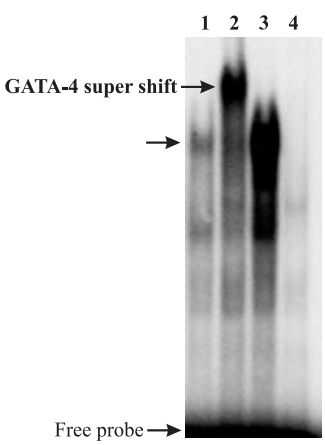

Figure 9. GATA-4 binds to inhibin α promoter. Lane 1: EMSA with ^{32}P-labeled double-stranded inhibin α promoter oligonucleotide (-155/-140) probe and 10 µg protein from nuclear extract of mouse Sertoli cell tumor line (MSCFSHR-1). Lane 2: Super shift with GATA-4 antibody. Lane 3: GATA-1 antibody. Lane 4: Mutated GATA binding site of inhibin α promoter (G → C). Results were identical with (-121/-104) inhibin α promoter oligonucleotide. Both of these promoters contain GATA-binding sites.

All in all, findings on the temporal and spatial expression of GATA-4 and its hormonal regulation, as well as results from transactivation reporter gene assays, support a potential link between GATA-4 and regulation of inhibin α expression in testes.

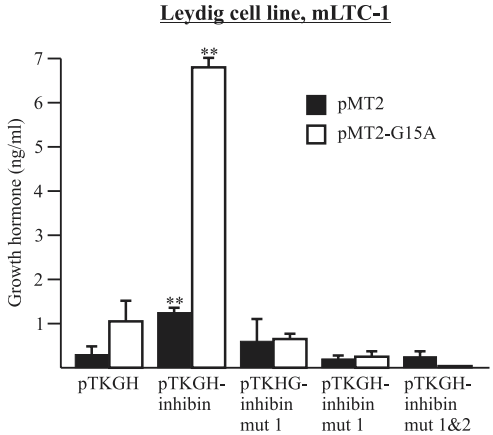

Figure 10. Cotransfection of GATA-4 expression vector (pMT2-G15A) and inhibin α promoter Growth Hormone (GH) reporter plasmid (pTKGH-inhibin) into mLTC-1 tumor cell lines. Activation of inhibin α promoter by GATA-4 is abolished by mutating the first (mut 1), second (mut 2), or first and second (mut 1&2) GATA sites of the promoter. Bars indicate mean (±SEM) GH concentration (ng/ml). ** $P = 0.0001$ (between GH concentrations).

Other researchers have used another Leydig cell tumor line, MA-10, in corresponding transactivation studies; their results reveal that GATA-1, but not GATA-4, transactivates rat inhibin α promoter (Feng et al. 1998). Nucleotide sequences of inhibin α promoter of the mouse and rat are very much alike, and sequences are identical between the two GATA sites (Su and Hsueh 1992), meaning that species-specific differences in promoters do not explain differing findings regarding inhibin α promoter activation. Two recent studies, however, may explain in part these controversial findings on inhibin α promoter activation. Besides Sertoli cells, also Leydig cells from 3-week-old juvenile rats expressed GATA-1 mRNA (Zhang et al. 2002), whereas in adult rat testis, only Sertoli cells expressed GATA-1 (Feng et al. 1998, Robert et al. 2002). These findings and the gene transactivation studies suggest a role also for GATA-1 in the regulation of inhibin α transcription, at least during certain periods of testicular development.

Besides gonadal cell lines, GATA-1 and GATA-4 transactivate inhibin α promoter in the CV-1 non-gonadal cell line, and this observation provides another piece of evidence for roles for both GATA-1 and GATA-4 in the regulation of inhibin α. African green monkey kidney cell line, CV-1, expresses none of the GATA factors endogenously. In this cell line, both GATA-1 and GATA-4 transactivate inhibin α promoter (Tremblay and Viger 2001a, Robert et al. 2002). Because non-gonadal cell lines such as CV-1 may lack other factors perhaps involved in inhibin α-signaling pathways in the testis, interactions that otherwise modulate GATA-1 and GATA-4 activity in vivo thus may be neglected. On the other hand, the activation of inhibin α gene by GATA-1 and GATA-4 in CV-1 cells indicates that these factors directly activate inhibin α gene transcription.

8.3.2 FOG-1 and FOG-2 repression of inhibin α gene activation by GATA-4

Testicular GATA factors are expressed in a partially overlapping manner during testicular development. The cell-specific differences in gene regulation by testicular GATA proteins may well be explained by interactions between GATA-binding proteins and their cofactors. The most

notable are FOG proteins, which were originally identified as GATA-specific cofactors in yeast two-hybrid analyses. FOG-1 and FOG-2 either enhance or repress the promoter activity of the cardiac or hematopoietic genes regulated by GATA factors (Fossett and Schulz 2001, Cantor and Orkin 2002). This provides a plausible mechanism for fine-tuning the expression of GATA-dependent genes also in the testis.

The basal promoter activity of inhibin α is repressed by FOG-1 and FOG-2 in MSC-1 Sertoli cells that endogenously express GATA-1 and GATA-4 (Robert et al. 2002). Furthermore, the transcriptional activity of GATA-4 on the inhibin α promoter is restored when FOG proteins are unable to interact with the N-terminal zinc-finger of GATA-4 in which the FOG binding site is mutated. These in vitro experiments link FOG proteins to the regulation of the inhibin α gene, together with GATA-1 and GATA-4.

Based on the expression patterns of GATA-1, GATA-4, FOG-1, and FOG-2 as well as of inhibin α, it is plausible that FOG-1 interacts with GATA-4 during fetal testicular development, and it may well suppress the activation of GATA-4 on the inhibin α gene. Inhibin α is first detected in fetal mouse testis at E16, thus coinciding with the appearance of FOG-1 expression (Tone et al. 1990). In postnatal Sertoli cells, FOG-1 expression coincides with that of GATA-1 from the appearance of the first wave of spermatogenesis. Later, the number of FOG-1- and GATA-1-positive Sertoli cells declines, and in adult seminiferous tubules, these factors are predominantly expressed in stage VII-XII tubules. Thus, FOG-1 may well repress GATA-1 activity during postnatal testicular development.

Mice deficient in inhibin α develop testicular tumors with nearly 100% penetrance, suggesting a role for inhibin α as a tumor suppressor (Matzuk et al. 1992). Inhibin α and inhibin β-B chains form inhibin B that is endocrinologically the most important form of inhibin in men (Anawalt et al. 1996, Illingworth et al. 1996). Interestingly, in gonadal cell lines, not only inhibin α, but also the inhibin β-B-subunit is regulated by GATA-1 and GATA-4 (Feng et al. 2000). These findings further underscore the importance of GATA-4 in the regulation of the inhibin pathway essential for normal gonadal development and function.

8.3.3 GATA-4 and FOG-2 required for early testicular development

In vivo studies provide the most convincing evidence for the essential nature of GATA-4 and FOG-2 in testicular development. In mice, abrogation of GATA-4/FOG-2 interaction or abolition of the FOG-2 gene (with FOG-2 function partially rescued) results in abnormalities in gonadogenesis (Tevosian et al. 2002). Testes fail to develop probably due to insufficient SRY expression, and as a consequence, Sertoli cell differentiation is blocked, which in turn results in the absence of testicular cords. Leydig cells are present, but the steroidogenic program is not initiated, due to ectopic WNT-4 expression. These results do not directly indicate that GATA-4 and/or FOG-2

regulate SRY expression. In the absence of Sertoli and Leydig cells and their gene products, it is impossible to assess their role in subsequent events in testicular development and spermatogenesis. However, their presence and physical interaction in the genital ridge are required for normal testicular differentiation. In these mice, the rescue of SRY function may be a valuable tool to better reveal the role of GATA-4 and FOG-2 in testis development and function.

In the present animal model, testicular development was blocked before Sertoli cell differentiation, and as a consequence such genes as MIS and SOX-9 were not expressed. It was impossible, therefore, to evaluate the impact of GATA-4/FOG-2 interactions on those genes expressed after SRY expression. Taken that, after testicular differentiation, FOG-2 is downregulated and FOG-1 is upregulated the latter would be the gene of interest "as a friend of GATA-4" in the development of the late fetal testis. In the ovary, GATA-4 and FOG-2 are expressed throughout the fetal period, but the ovarian phenotype of these mutant mice is to be described on later (Tevosian et al. 2002).

8.3.4 Several gonadal genes regulated by GATA-4 and FOG proteins

Besides inhibin α, several other gonadal genes, such as MIS, SF-1, StAR (Steroidogenic acute regulatory protein), and aromatase contain GATA motifs in their promoters (Tremblay and Viger 2001a). StAR and aromatase are enzymes essential for normal steroidogenesis, which in turn is required for gonadal development and adult spermatogenesis (Carreau et al. 2001, Conley and Hinshelwood 2001, Stocco 2001, Stocco 2002). GATA-4 activates all of these genes in in vitro assays (Figure 10). The role of GATA-4 in the regulation of MIS has received the most intensive study. GATA-4 activates the MIS promoter on its own (Viger et al. 1998) and interacts with SF-1 through complex mechanisms to activate the MIS transcription (Tremblay and Viger 1999, Watanabe et al. 2000, Tremblay et al. 2001, Tremblay and Viger 2001a). Furthermore, on the MIS promoter, FOG-1 and FOG-2 repress GATA-4 activity and GATA-4/SF-1 synergism (Tremblay et al. 2001, Robert et al. 2002, Anttonen et al. 2003). Dax-1 also disrupts transcriptional synergism between GATA-4 and SF-1, resulting in repression of MIS transcription (Tremblay and Viger 2001b). The role of GATA-4 in MIS gene activation is growing more complex, taken that GATA-4 activates SF-1 also (Tremblay and Viger 2001a).

This activation or repression of MIS occurs through a complex interplay between transcription factors and accessory proteins. The interactions of GATA-4, SF-1, FOG-1, and FOG-2 do not limit the MIS gene only. Results from recent studies have shown that GATA-4 interacts with SF-1 and FOG proteins to regulate not only MIS but also StAR, aromatase, and inhibin α genes. SF-1 interacts with GATA-4 to promote StAR, aromatase, and inhibin α transcription (Tremblay and Viger 2001a, Robert et al. 2002), and FOG proteins repress transcriptional activity (Robert et al. 2002, Tremblay et al. 2002). These congruent results in in vitro studies suggest that GATA-4 regulates gonadal development and function at various levels, together with other factors such as FOG proteins.

Human studies indirectly support the in vitro data on GATA-4-induced gene activation. Leydig cells are the major source of testosterone, and the StAR gene is expressed in fetal and adult Leydig cells. GATA-4 expression in fetal Leydig cells was most intensive before midgestation (II, IV) when fetal testosterone production peaks (Tapanainen et al. 1981). In addition, the initiation of testosterone production at puberty coincides with upregulated GATA-4 expression in Leydig cells. Overlapping expression patterns of GATA-4 and StAR in Leydig cells and the results from transactivation studies strongly suggest that GATA-4 regulates testosterone biosynthesis also in vivo.

Taken together, the results from in vitro and in vivo studies clearly demonstrate that GATA-4 plays an important role in the testis by participating in regulation of several key molecules during testicular development (Figures 11 and 12). Not only GATA-4, but also GATA-1 and GATA-6 are most likely to play a role in the regulation of gene expression of the testis. They were capable of activating multiple gonadal genes such as MIS, inhibin α, and StAR. This activity was enhanced by simultaneous expression of SF-1 and repressed by FOG-1 and FOG-2 (Robert et al. 2002). Cell-specific temporal and spatial expression patterns of GATA and FOG proteins will ultimately identify their target genes expressed during testicular development. Furthermore, their individual affinities for various promoters are likely to modify the expression of their target genes (section 5.4.1).

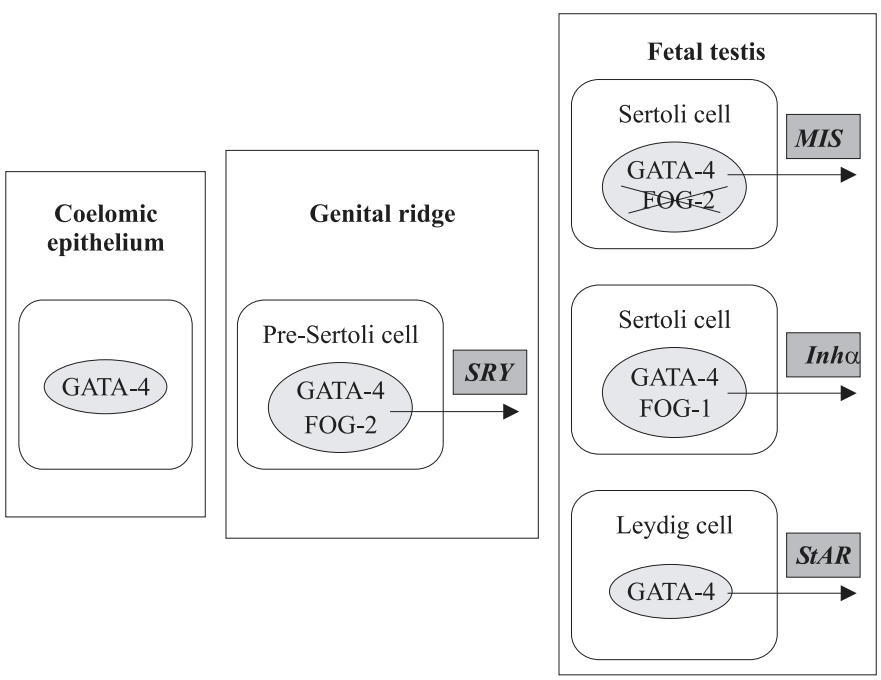

Figure 11. Genes (grey boxes) regulated by GATA-4 and FOG-1 or FOG-2 during fetal testicular development. Crossed-out FOG-2 means disappearing Sertoli cell expression. Inhα, Inhibin α.

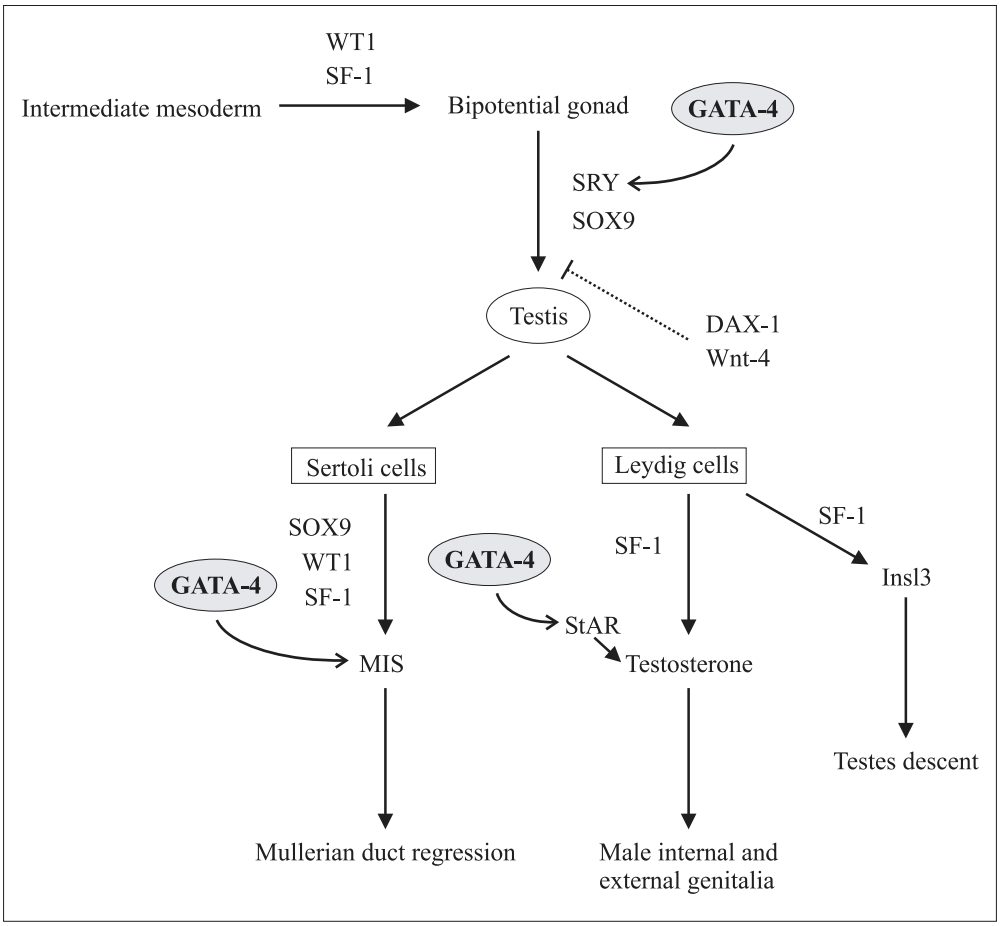

Figure 12. Summary of the essential sites of GATA-4 action in the fetal testis.

8.3.5 The role of GATA-4 in hypothalamus and pituitary

Within the **hypothalamic-pituitary-gonadal axis**, the expression of GATA and FOG proteins is not restricted to the gonads. GATA-4 is expressed in the mouse pituitary (Heikinheimo and Wilson, unpublished results) and in migrating GnRH neurons (Lawson and Mellon 1998). In the pituitary-derived cell line T3-1, GATA-binding protein regulates the gonadotropin α-subunit gene (Steger et al. 1994), a common subunit both of FSH and of LH (Griffin and Wilson 1998). In the GnRH-secreting hypothalamic neuronal cell line GT1, GATA-4 regulates the activity of the GnRH gene (Lawson et al. 1996, Lawson et al. 1998, Lawson and Mellon 1998). These findings strongly suggest that GATA-4 plays a functional role also in the hypothalamus and pituitary. Furthermore, FOG proteins may act as GATA-4 cofactors, since both FOG-1 and FOG-2 are expressed in the brain (Tsang et al. 1997, Lu et al. 1999, Svensson et al. 1999, Tevosian et al. 1999). Their cell-specific expression has not been addressed in detail, but based on previous studies one might expect that

FOGs share an overlapping expression pattern with GATA-4. The interaction of FOG-2 with GATA-4 is necessary for the development of the heart and gonads (Crispino et al. 2001, Tevosian et al. 2002). It is therefore plausible that FOG-2 has a role, along with GATA-4, also in the development and function of pituitary and/or hypothalamus. Thus, GATA and FOG proteins are likely to act at various sites of the hypothalamic-pituitatary-gonadal axis.

8.3.6 Apoptosis and cell proliferation in fetal human testis (IV)

Apoptotic cell death plays a major role in the regulation of testicular function (Griswold 1998, Matsui 1998, Beumer et al. 2000, Kierszenbaum and Tres 2001). GATA-4 and GATA-6 are abundantly expressed in the testis and are involved in apoptotic processes of various tissues from the fetal period onwards. It is thus plausible that they are involved in apoptotic cell death also in fetal testis. Proper tissue development requires a balance between cell death and proliferation. Both GATA-4 and GATA-6 have also been linked to cell proliferation (Grepin et al. 1997, Perlman et al. 1998, Nagata et al. 2000). We therefore sought any spatial and temporal relationships between GATA-4 and GATA-6 expression, apoptosis, and the apoptosis-related proteins bcl-2 and bax during human fetal testicular development (IV). Cell proliferation was assessed in the same samples by means of anti-Ki-67 antibody and immunohistochemical analyses (IV).

GATA-1, GATA-4, and GATA-6, respectively, are linked to the regulation of apoptosis in the red blood cells, in the ovary, and in colorectal carcinoma cells. Caspase-mediated degradation of GATA-1 results in apoptotic death of maturing red blood cells, and it may represent a negative control mechanism in erythropoiesis (De Maria et al. 1999, Orkin and Weiss 1999). In the human ovary, the expression pattern of GATA-4 suggests that, from fetal to adult life, it is involved in the mechanisms protecting granulosa cells from apoptosis (Vaskivuo et al. 2001). GATA-4 expression was at its highest level in the youngest fetal ovaries and decreased towards term, parallel with apoptotic oocyte death. Similarly, GATA-4 downregulation precedes apoptotic cell death of granulosa cells in the mouse ovary (Heikinheimo et al. 1997). In colorectal cancer cells, nonsteroidal anti-inflammatory drugs down-regulate GATA-6, which results in up-regulation of 15-lipoxygenase-1 and induction of the apoptosis of these cancer cells (Shureiqi et al. 2002).

In fetal testis, a TUNEL assay revealed apoptotic cell death in both the testicular cords and interstitium from the second trimester to term (IV). The overall number of apoptotic cells was low, and these cells were distributed equally between somatic and germ cell lineages. After midgestation, there occurred a temporary peak in Sertoli cell apoptosis. The number of apoptotic Sertoli cells rose markedly, but then returned to a low level during the third trimester. Proliferative activity was the most intense at the beginning of the second trimester. The cells with the most active proliferation rate as measured by Ki-67 immunohistochemistry were predominantly Leydig cells. Beginning from midgestation, proliferation diminished gradually with advancing gestational weeks and ceased totally at term.

Bcl-2 protein was expressed in the Leydig and peritubular myoid cells between fetal weeks 16 and 23 (IV). Bcl-2 was no longer detectable in any testicular cell compartment by week 27. The expression of bax differed markedly from that of bcl-2; abundant cytoplasmic bax expression was detected both in testicular cords and in the interstitium from weeks 16 until term. Peritubular myoid cells expressed no bax protein at any fetal age.

The overall rate of apoptosis and proliferation in the fetal testis was low throughout all gestational weeks (weeks 16 to 40). These results indicate that the major portion of testicular cell proliferation occurs before the second trimester. This is in agreement with previous findings implying that Sertoli, Leydig, and germ cell proliferation is most intensive during the first trimester (Wartenberg 1978, Codesal et al. 1990). Thus, before midgestation, human fetal testis seems to reach a quiescent balance between cell proliferation and apoptosis.

Sertoli cell apoptosis was at its highest level at the end of the second trimester (IV). This may reflect the effect of declining serum FSH levels on Sertoli cells (Kaplan et al. 1976). It has been proposed that the number of Sertoli cells decreases rather than increases from the second trimester to term (Helal et al. 2002). The temporarily enhanced apoptosis of the Sertoli cells after midgestation provides a possible mechanism for declining Sertoli cell number. Leydig cell proliferation was observed before midgestation (IV) when testicular androgen production peaks (Tapanainen et al. 1981). Since GATA-6 activates steroidogenic enzyme genes (Robert et al. 2002), and the hormonally active Leydig cells do not proliferate (Murray et al. 2000), GATA-6 expression may be linked to testosterone synthesis rather than to cell proliferation.

Expression patterns of GATA-4 and GATA-6 failed to match the patterns of apoptosis or cell proliferation (II, IV), meaning that in human fetal testis, these GATA factors are unlikely to be involved in either bcl-2-/bax-mediated pro-/anti-apoptotic processes or in mechanisms regulating cell proliferation. However, that the expression of GATA-4 is downregulated prior to granulosa cell apoptosis in fetal mouse ovary (Heikinheimo et al. 1997) indicates that GATA-4 may play a role in regulating apoptosis in the ovary.

In human fetal testis, Bcl-2 expression changes in a cell-specific manner along with testicular development (IV) (Quenby et al. 1999, Murray et al. 2000) (Table 11). During the first trimester, this anti-apoptotic protein is expressed in the Sertoli and Leydig cells. At the beginning of the second trimester, bcl-2 is detected in the Leydig and myoid cells. Thus, bcl-2 expression coincides with major testicular morphogenetic and developmental changes, and bcl-2 may protect somatic cell types from apoptosis during their proliferation and maturation. During the second trimester, testicular development reaches a quiescent steady state level, and bcl-2 is no longer needed or, alternatively, other factors fulfill its function.

The number of apoptotic cells was low even in the presence of abundant bax protein (IV). Because bax protein activity is dependent on its intracellular localization (Hsu et al. 1997, Wolter et al. 1997,

Zamzami et al. 1998, Putcha et al. 1999), intense bax expression does not necessarily reflect the amount of biologically active bax protein. Alternatively, in fetal testis, anti-apoptotic factors such as bcl-x counterbalance bax (Rucker et al. 2000). Loss of bcl-x function induced germ-cell loss through enhanced apoptosis even during the fetal perioid, and spermatogonia were not detectable postnatally. Loss of bcl-x function was corrected by deletion of the bax gene, which resulted in a restoration of germ cell survival. In contrast, bax-deficient mice have an excessive number of spermatogonia in the maturing postnatal testis (Russell et al. 2002). Overproduction of spermatogonia blocks the normal maturation of spermatocytes that eventually results in bax-independent germ-cell death and in testicular atrophy in adults. Bax-dependent apoptosis therefore probably serves a major purpose during postnatal testis development and is required for functional spermatogenesis (Rodriguez et al. 1997, Yan et al. 2000b, Russell et al. 2002).

Table 11. Summary of bcl-2 and bax expression in human fetal testis. Bax expression not addressed before gestational week 13. (Data from IV, Quenby et al. 1999, and Murray et al. 2000).

Cell type	Bcl-2	Bax
Sertoli cell	6-8 weeks	13-40 weeks
Germ cell	Not present	Not present
Myoid cell	13-23 weeks	Not present
Leydig cell	8-23 weeks	13-40 weeks

8.4 GATA-4 and diseases of human testis

8.4.1 Developmental disorders (II)

Developmental testicular diseases represent a heterologous group of disorders. Defects range from testicular agenesis to normal virilization but reduced fertility. Given the emerging role of GATA-4 in the development and gene regulation of mammalian testis, we wanted to elucidate the role of GATA-4 in human developmental diseases of the testis. Expression of GATA-4 protein was explored by immunohistochemistry in testis samples from patients with androgen resistance, cryptorchidism, Kallman's syndrome, or Sertoli-cell only syndrome (II, unpublished results).

Expression of GATA-4 was studied in testis samples from patients who underwent orchidectomy due to **androgen resistance** (II). Staining intensity in Sertoli cells and spermatogonia was either very weak or totally absent and decreased along with advancing age from 1 to 14 years. Leydig cells were mainly negative for GATA-4. The overall expression of GATA-4 was lower than in normal testes (Table 12). No difference existed in GATA-4 expression between testicular samples from patients with partial or with complete androgen resistance.

In patients with **androgen resistance**, the impact of androgens is reduced due to defects in the androgen receptor or to lack of the 5αreductase enzyme which metabolizes testosterone to dihydrotestosterone (DHT). This disorder of androgen action is the main cause of male pseudohermaphroditism (Sultan et al. 2002). Testes of these patients may develop normally, whereas

differentiation of the Wolffian ducts, urogenital sinus, and external genitalia is androgen dependent. Abnormalities in these derivatives therefore occur in androgen resistance, and it may result in sexual ambiguity, i.e., genitalia typical of neither male or female. Testicular morphology also varies considerably between androgen-resistant patients; testes may develop normally or resemble "testes-like structures" with clusters of Sertoli and Leydig cells.

Testes of androgen-resistant patients secrete testosterone normally. The reduced GATA-4 expression in their Sertoli cells is thus likely to be related to their defective response mechanism for androgen stimulus. In other words, testosterone and DHT have no direct effect on GATA-4 expression; their effects are mediated through indirect mechanisms. The actions of testosterone and DHT are mediated via the AR that is coexpressed with GATA-4 in Sertoli, Leydig, and myoid cells (Suarez-Quian et al. 1999, Zhou et al. 2002). The gene for AR also has putative GATA-binding sites on its promoter (Song et al. 1993, Mizokami et al. 1994). Thus, GATA-4 may well regulate the expression of androgen receptors in the testis. In the Sertoli cells of androgen-resistant patients, reduced GATA-4 expression may diminish the expression of its downstream target genes such as MIS, which in turn may have effects on the development of diverse sexual phenotypes.

Patients with **cryptorchidism** underwent surgery on their unilateral or bilateral undescended testes with or without preceding hCG treatment (II). In cryptorchid testes, GATA-4 immunoreactivity was present in Sertoli cells and spermatogonia but was very weak in or absent from Leydig cells (Table 12). These findings are comparable to the expression pattern in normal testes. GATA-4 is therefore unlikely to play a role in the pathogenesis of cryptorchidism. Furthermore, the treatment used, the localization of the testis, and the period from treatment to biopsy had no effect on GATA-4 expression in any of the testicular cell types.

In the adult patient with **Kallman's syndrome**, Sertoli and Leydig cells were positive but spermatogonia were negative for GATA-4 (II). This expression pattern was similar to that of normal testes, but the staining intensities in Sertoli and Leydig cells appeared weaker than normal (Table 12). In testicular samples from the patient with **Sertoli-cell-only syndrome** (SCOS), GATA-4 was expressed in Sertoli and Leydig cells as in the controls or the expression was slightly reduced (unpublished results). SCOS is a syndrome of unknown etiology in which spermatogenesis is impaired, and it results in azoospermia and infertility (Anniballo et al. 2000). Due to the low number of testicular samples of Kallman's syndrome and SCOS, it is impossible to reach definitive conclusions as to GATA-4 expression and pathogenesis in these diseases.

Table 12. Intensity of GATA-4 expression in samples from human testicular diseases compared to normal age-matched control samples. Number of samples in parentheses.

Disease		Sertoli cells	Leydig cells	Germ cells
Androgen resistance	(9)	reduced	reduced or normal	reduced
Cryptorchidism	(33)	normal	normal	normal
Kallman's syndrome	(1)	reduced	reduced	normal
SCOS	(1)	normal/reduced	normal/reduced	absent

8.4.2 Tumors (II)

Enhanced or persistent expression of GATA-4 has been reported in non-gonadal tumors, including adrenocortical carcinomas, esophageal/gastric adenocarcinomas, and yolk sac tumors (Kiiveri et al. 1999, Siltanen et al. 1999, Lin et al. 2000), suggesting a role for GATA-4 in the progression of these neoplasms. Furthermore, GATA-4 was expressed in tumors of gonadal origin, including ovarian sex-cord and testicular yolk sac tumors (Siltanen et al. 1999, Laitinen et al. 2000). Given that GATA-4 is abundantly expressed in the testis from the fetal period to adulthood, the expression of GATA-4 in testicular tumors was analyzed by immunohistochemical staining (II).

Human Sertoli tumor cells exhibited very intense expression of GATA-4 in testis samples from a 12-year-old boy with a **Sertoli cell tumor** (Large-cell calcifying Sertoli cell tumor). The staining intensity of the tumor cells was clearly stronger than of Sertoli cells of the adjacent healthy tubules. This finding was consistent in all affected tubuli. Upregulation of GATA-4 expression was also evident in all five patients' **Leydig cell tumors**, in which GATA-4 was abundantly expressed in tumor cells. Unaffected Leydig and Sertoli cells in these tumors stained like the control samples. In contrast to the findings for Sertoli and Leydig cell tumors, no GATA-4 was detected in any of the six germ-cell-derived **seminomas** analyzed (unpublished results) (Table 13).

High GATA-4 expression may be due either to the processes leading to tumor formation or be induced after tumor transformation. Within the endocrine system, GATA-4 may play a role in tumorigenesis of the adrenal gland (Kiiveri et al. 1999). Transgenic mice that develop adrenocortical tumors exhibit abundant GATA-4 expression in their malignant adrenocortical cells, whereas normal adrenocortical cells are devoid of GATA-4. Such a difference, accompanied by tumorigenesis and the expression of GATA-4 has not, however, been detected in gonads. Besides testicular tumors, GATA-4 is expressed in ovarian granulosa and theca cell tumors, i.e., tumors arising from ovarian counterparts for testicular Sertoli and Leydig cells, respectively (Laitinen et al. 2000). Given that GATA-4 is abundantly expressed in granulosa, theca, Sertoli, and Leydig cells during normal development, it is unlikely that GATA-4 alone initiates tumor growth in gonadal somatic cells. On the other hand, GATA-4 may promote the growth of testicular and ovarian tumors. In the ovary, downregulation of GATA-4 precedes the granulosa cell atresia that occurs via apoptotic cell death (Heikinheimo et al. 1997). Therefore, their abundant GATA-4 expression may inhibit apoptosis in gonadal tumor cells and thus promote tumor growth.

Given the intense GATA-4 expression during the proliferative phase of Sertoli cells (I, II, III) (Viger et al. 1998), GATA-4 may influence cell proliferation also in Sertoli cell tumors. Alternatively, GATA-4 may participate in enhanced hormone production by the tumor cells. Inhibin subunits shown to be regulated by GATA-1 and GATA-4 (Feng et al. 1998, Feng et al. 2000) (I), are abundantly expressed in Sertoli tumor cells, and serum inhibin levels are elevated (Toppari et al. 1998). Thus, GATA-4 may regulate the production of inhibin α and βB-subunits in Sertoli cell tumors. Leydig cell tumors are hormonally active and produce testosterone or estradiol (Griffin and Wilson 1998).

The biosynthesis of these hormones is controlled by StAR and aromatase, which in turn are both regulated by GATA-4 (Silverman et al. 1999, Tremblay and Viger 2001a). It can therefore be proposed that GATA-4 expression is linked to hormone secretion also in Leydig cell tumors.

No direct evidence allows us to draw conclusions as to causality between GATA-4 expression and tumorigenesis. However, GATA-4 regulates putative tumor suppressor genes such as MIS and inhibin α (section 8.3) (Matzuk et al. 1992, Matzuk et al. 1995). Loss of the activity of these genes results in testicular tumor growth. Interestingly, one of the allelles of the GATA-4 gene appeared to be lost in serous ovarian carcinomas (Lassus et al. 2001). These tumors arise from the surface epithelium of the ovary where GATA-4 is normally expressed. Loss of the GATA-4 gene suggests that GATA-4 possesses either direct or indirect tumor suppressor activity in specific gonadal tumors.

Table 13. Expression of GATA-4 in gonadal tumors. Number of GATA-4 positive tumors of all tumors studied in parenthesis.

Testicular tumors		Ovarian tumors	
Sertoli cell	(1/1)	Granulosa cell	(15/15)
Leydig cell	(5/5)	Theca cell	(8/9)
Seminomas	(0/6)	Surface epithelium	
		Serous	(3/41)
		Mucinous	(9/10)

9 CONCLUSIONS

Gene expression data, as presented herein, provide an important resource for defining gene function and for identifying hierarchies and networks of genes that regulate specific developmental programs. It does not allow us to reach definitive conclusions as to the interactions of various developmental factors. However, it gives us a physiological backbone for evaluating the results of in vitro studies. Moreover, it helps us to focus further studies on understanding and clarifying the role of GATA and FOG proteins during testicular development.

Testicular development requires temporally and spatially controlled expression of a number of genes. Some of these genes are expressed for only a limited period of time, whereas others form a network that regulates not only fetal but also postnatal development and function. This study elucidated the expression of transcription factors GATA-1, GATA-4, and GATA-6. These are expressed in a distinct but partially overlapping manner in the developing testis, and their expression patterns indicate that they may be important regulators of testicular development and function. Their cofactors, FOG-1 and FOG-2, are likely to modulate their actions depending on the cellular context of any given factor(s). FOG-2 may act with GATA-4 during the earliest stages of testicular development; later in the fetal testis, FOG-1 functions as a cofactor for GATA-4. In adult testis, GATA-1 and FOG-1 are likely to be the interacting partners.

This study revealed that GATA-4 is hormonally regulated, but neither gonadotropin nor androgen action is required for its basal expression. Furthermore, in gonadal cell lines, GATA-4 upregulates the inhibin α gene. Others have shown that GATA-4 regulates a number of other genes such as MIS and StAR that are important for testicular function. GATA-4 is also essential for proper expression of the SRY gene, implying a role for this transcription factor in the earliest steps of testiculogenesis. All these findings together indicate that GATA-4 may be one of the master regulators of testicular development and function.

In addition to the gonads, members of the GATA family of transcription factors are expressed at various sites within the endocrine system. They regulate reproductive functions along the hypothalamic-pituitary-gonadal axis. Furthermore, GATA proteins are already being expressed at the earliest stages of development in the adrenals and pancreas; both organs are essential for normal hormonal homeostasis and eventually for the survival of the individual. Therefore, based on the results of previous and current studies, the GATA family may play a non-redundant and essential role in a number of different endocrine organs.

10 ACKNOWLEDGEMENTS

I wish to express my warmest gratitude to all those who have participated and helped me in my thesis work. Without a number of enthusiastic, talented, and skillful co-workers, this study could never have been accomplished.

This study was carried out at the Research Laboratory of the Hospital for Children and Adolescents at the University of Helsinki between 1997 and 2002. I express my sincere gratitude to the Heads of the Hospital for Children and Adolescents, University of Helsinki: Professor Jaakko Perheentupa, Professor Kari Raivio, Professor Mikael Knip, Professor Martti Siimes, and the Head of the Research Laboratory, Professor Erkki Savilahti, for providing excellent research facilities and supportive research atmosphere.

I acknowledge the Heads of the Hospital for Children and Adolescents, Helsinki University Central Hospital, Dr. Veli Ylitalo and Professor Christer Holmberg.

I am most grateful to my supervisor, Professor Markku Heikinheimo, for offering me the opportunity to enter the world of GATA factors - and science in general. I admire his exemplary diligence and scientific enthusiasm. Markku's continuous support and encouragement have been invaluable. I also wish to express my gratitude to my second supervisor, Professor Juha Tapanainen. His insightful comments and vision were essential for me to focus on issues relevant to this study.

Professor Raimo Voutilainen and Docent Jorma Palvimo are gratefully acknowledged for reviewing the thesis manuscript and for their constructive comments. The tutors of my thesis work, Docent Päivi Miettinen and Dr. Vesa Ilvesmäki, are warmly thanked for their help. The Pediatric Graduate School is also acknowledged for its support.

I wish to express my sincerest gratitude to my collaborators and co-authors with whom I have been privileged to work: Professor David B. Wilson at the University of Washington in St. Louis and the members of his research group: Dr. Malgorzata Bielinska and Susan B. Porter-Tinge; Professor Ilpo Huhtaniemi and Dr. Nafis Rahman at the University of Turku; Docent Riitta Herva and Dr. Tommi Vaskivuo at the University of Oulu; Docent Leo Dunkel and Dr. Virve Pentikäinen at the University of Helsinki. In particular, I owe my gratitude to Professor Jorma Toppari, whose expertise and contributions to my study have been irreplaceable.

Docent Timo Otonkoski, Docent Olli Ritvos, Drs. Mika Laitinen, Jianqi Liu, Krista Erkkilä, Markku Pentikäinen, and Mikko Huhtanen recieve my gratitude for their help.

Dr. Annikki Sarnesto and Erja Karjalainen, Sinikka Heikkilä, Ritva Löfman, Sari Lindén, Terttu Louhio, Sirkku Kristiansen, Virpi Ahokas, Kaisa Alasalmi, Ulla Kiiski, and Merja Haukka are thanked for help and technical assistance.

I thank Dr. Carol Norris for author-editing of the language of my thesis (and for excellent food), and Dr. Nick Bolton for the revision of the language of my original publications III and IV.

It has been a great pleasure to work in our research group with my dear colleagues Sanne, Susanna, Mikko, Helka, and Hanna. I have enjoyed the relaxed and frank atmosphere of our group, and I am grateful for all their help.

I wish to warmly thank my colleagues at the Research Laboratory and Lastenlinnantie office for their friendship and help. Specifically, I wish to mention Eeva, Tiina, Anna-Liisa, Henrikka, Mika, Nina, Petra, Terhi, Laura, Marjut, Mia, Minna, Sanna and Tuula.

My parents-in-law, Kaarina and Kalevi Äijö, have helped me and my growing family in so many ways. Thank you.

I am grateful to my parents Tuula and Sakari, for bringing me up with care and love, and yet giving me considerable freedom. Your positive attitude towards studying has been of great importance to me. My brother Antti, thank you for your help, especially for your time with my children.

My sons, Oskari, Valtteri, and Konsta, you are the most valuable assets I have; I am so proud of you. I devote my deepest gratitude to my dear wife Mari, with whom I have shared the most important moments in life.

This study was financially supported by the Foundation for Pediatric Research, the Helsinki University Research Foundation, the Finnish Medical Foundation, the Finnish Cultural Foundation, the Maud Kuistila Foundation, and the Research Funds of the Hospital for Children and Adolescents and Helsinki University Central Hospital.

Helsinki, January 2003

Ilkka Ketola

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