ENDODERMAL DEVELOPMENT AND GERM CELL TUMORS – ROLE OF GATA TRANSCRIPTION FACTORS

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To Life

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Abstract

The endoderm, one of the three germ layers, forms the internal layer of the mammalian body. As a result of complex differentiation in interaction with underlying mesoderm, endoderm is transformed into various highly specific organs that facilitate nutrient, gas, and waste exchange, and participate in hormonal signaling as well as immunological defense. Structural and functional abnormalities of endodermal derivatives form a significant group of developmental abnormalities and malignant diseases.

Germ cell tumors (GCTs) are a heterogeneous group of tumors that arise from primordial germ cells, the precursors of gametes. Owing to the pluripotent nature of germ cells, these tumors may contain any embryonic or extraembryonic tissues. Benign GCTs, teratomas, are composed of normal tissues but are sometimes very harmful as a result of their location or strong proliferative activity. Germinomas are tumors in which the germ cells have retained their histology but adapted a malignant pattern of growth. Yolk sac tumor (YSTs), choriocarcinoma (CC) and embryonal carcinoma (EC) are malignant but rare GCTs. YSTs histologically resemble the yolk sac, i.e. the primitive endoderm. Like the normal yolk sac, these tumors produce α-fetoprotein, assay of which is a tool in diagnostics and patient follow-up. GCTs are rare entities, but since they are present during neonatal life or puberty, they have a long effect on patients' lives. A mixture of different tissue types in tumors is common and this creates diagnostic problems.

Two members of the GATA transcription factor family, GATA-4 and GATA-6, have been found to be essential for endoderm development. Genetically engineered mice devoid of these factors die *in utero* owing to impaired yolk sac development and function. If overexpressed, they promote embryonic stem cell differentiation towards yolk sac tissues, and up-regulate the expression of several genes essential for primitive endoderm development such as those for hepatocyte nuclear factor 4 (HNF-4), Indian hedgehog (Ihh) and bone morphogenetic protein 2 (BMP-2). Of these, the *HNF-4* gene has been shown to be a direct target of GATA-6 and depletion of the genes of either of these two factors in mice results in a similar phenotype.

Little is known about the factors influencing endodermal development and germ cell activation and differentiation. In this study we utilized mRNA *in situ* hybridization and immunohistochemistry to evaluate the expression patterns of GATA-4, GATA-6 and GATA cofactors, FOGs and their target genes during endodermal development and in GCTs. In addition, a transgenic mouse model with GATA-4 incapable of binding FOG proteins was studied to evaluate the role of GATA regulation during endodermal development.

The results presented herein show that GATA-4 and GATA-6, two factors needed for early differentiation of endoderm, continue to be strongly expressed in mouse extraembryonic

endoderm at least until E13.5. FOG proteins are also expressed in the endodermal tissues in mid-gestation mouse embryos. Specifically, FOG-1 is expressed in distal stomach epithelium, whereas *FOG-2* can be seen to be turned on in the yolk sac endoderm. The transgenic mice die at E12.5 owing to cardiac malformations and thus offer only a narrow window to study GATA-4:FOG interaction in endoderm. This interaction, however, seems to be needed for normal specification of distal stomach endoderm and proper signaling towards the underlying mesoderm. In the mutant mice, the morphogen Sonic hedgehog is not properly down-regulated and thus fibroblast growth factor 10 is not expressed by the underlying mesoderm.

We have also shown that GATA-4 and GATA-6 are abundantly expressed in YSTs along with their putative endodermal target genes, *HNF-4*, *Ihh* and *BMP-2*. Malignant, but undifferentiated germ cells in dysgerminomas are devoid of GATA-6 and HNF-4; a subset of these tumors does express GATA-4, Ihh and BMP-2. In teratomas, these factors are expressed according to their normal expression pattern. In addition, both GATA factors are expressed in blastematous immature neural cells and GATA-6 in dermoid cyst sebocytes. No GATA-4 or GATA-6 in these ectodermal derivatives has been shown prior to this study.

As GATA-6 and HNF-4 are expressed in malignant endoderm of yolk sac tumors but not in activated germ cells of dysgerminomas, it is conceivable that expression of these two factors in malignant germ cells is needed and sufficient to induce primitive endoderm differentiation. In addition, GATA-6 and HNF-4 could serve as clinical markers of malignant yolk sac tissue in GCTs. GATA-4 could also serve as a marker in the diagnostics of GCTs, both recognizing yolk sac differentiation and scattered foci of immature neural tissue.

Original Publications

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

I. Siltanen S., Anttonen M., Heikkila P., Narita N., Laitinen M., Ritvos O., Wilson D. B. and Heikinheimo M. (1999). Transcription factor GATA-4 is expressed in pediatric yolk sac tumors. Am J Pathol 155(6):1823-9.

II. Siltanen S., Heikkila P., Bielinska M., Wilson D. B. and Heikinheimo M. (2003). Transcription factor GATA-6 is expressed in malignant endoderm of pediatric yolk sac tumors and in teratomas. Pediatr Res 54(4):542-6.

III. Mannisto S., Butzow R., Salonen J., Leminen A., Heikinheimo O. and Heikinheimo M. (2005). Transcription factors GATA-4 and GATA-6 and their potential down-stream effectors in ovarian germ cell tumors. Tumor Biol 26(6):265-273.

IV. Jacobsen C. M., Mannisto S., Porter-Tinge S. B., Genova-Peeva E., Parviainen H., Heikinheimo M., Adameyko I. I., Tevosian S. G. and Wilson D. B. (2005). GATA-4:FOG interactions regulate gastric epithelial development in the mouse. Dev Dyn *in press*.

All original publications are reprinted with permission of the original publishers. In addition, some previously unpublished data is presented.

Abbreviations

A	Adenine
AFP	α-fetoprotein
AGM	Aorta-gonad-mesonephros region
AMKL	Acute megakaryoblastic leukemia
AVE	Anterior visceral endoderm
BMP	Bone morphogenetic protein
C	Cytosine
CC	Choriocarcinoma
CIS	Carcinoma *in situ*, a precursor of GCTs in the testis
Cys	Cysteine
DG	Dysgerminoma
DNA	Deoxyribonucleic acid
E	Embryonic day
EC	Embryonal carcinoma
ES cell	Embryonal stem cell
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FOG	Friend of GATA
G	Guanine
GCT	Germ cell tumor
hCG	Human chorionic gonadotropin
His	Histidine
HNF	Hepatocyte nuclear factor
ICM	Inner cell mass
Ihh	Indian hedgehog
mRNA	Messenger ribonucleic acid
NSAIDs	Non-steroidal anti-inflammatory drugs
PGC	Primordial germ cell
Shh	Sonic hedgehog
T	Thymidine
TGF-β	Transforming growth factor β
TMD	Transient myeloproliferative disorder
YST	Yolk sac tumor

Introduction

Early in development the fertilized egg divides to form all the cells that will give rise to the offspring. However, the first tissues to develop do not contribute to the final newborn. These are the extraembryonic membranes and placenta that are crucial for the early cell mass to be able to implant in the uterus. Primitive or extraembryonic endoderm forms the yolk sac that facilitates nutrition prior to formation of the placenta. In addition, primitive endoderm provides the early embryo with signals to guide the first differentiation process of the embryo proper: the formation of three germinal layers, gastrulation.

The three germinal layers formed in gastrulation are the ectoderm, mesoderm and endoderm. Ectoderm will mainly differentiate into neural tissues and skin. Mesoderm will form muscles, bones, blood and major parts of the intestinal organs. The definitive endoderm, the least studied of the germinal layers, forms the lining of the gastrointestinal tract and parts of the intestinal organs.

As the embryo folds, the definitive endoderm forms a tube on the inside of the developing body. This tube will undergo a set of morphogenetic changes that lead to formation of various organs involved in digestion, respiration, hormonal balance and waste disposal. Differentiation is influenced by interactions between endoderm and the underlying mesoderm. Recent advances in studies of endodermal differentiation have led to a deeper understanding of developmental mechanisms and their failures, some which may also lie behind human congenital malformations of the gastrointestinal tract.

At the beginning of gastrulation, a small number of cells migrate from the epiblast to the extraembryonic compartment. These are the primitive germ cells (PGCs), precursors of gametes, and the reason for their first migration may be the need to escape the differentiating signals present in the early embryo proper. Within a couple of days, the PGCs will again migrate to the developing gut wall and move towards the abdominal cavity where other components of the future gonads are developing. During this migration, however, the PGCs may go astray and end up in ectopic locations.

Germ cell tumors (GCTs) are a heterogeneous group of tumors that arise from PGCs. Owing to the pluripotent nature of germ cells, these tumors can contain any embryonic or extraembryonic tissues. Yolk sac tumor (YSTs), choriocarcinoma (CC) and embryonal carcinoma (EC) are malignant but rare GCTs. Germinomas are tumors where the germ cells have retained their histology but adapted a malignant pattern of growth. Benign GCTs, teratomas, are composed of normal tissues but are sometimes very harmful as a result of their location or strong proliferative activity. The age distribution of GCTs is bimodal, with a peak at less than 3 years of age and a second peak at puberty and young adulthood. The proportion

of malignant tumors increases with advancing age. As one tumor often is a mixture of several tissue types, diagnosis is difficult.

YSTs histologically resemble the extraembryonic yolk sac, a tissue vital for early embryonic development. Like the normal yolk sac, YSTs express α-fetoprotein (AFP), which is used as a tool in diagnostics and patient follow-up.

During development, the initially pluripotent cells differentiate into versatile tissues. A key event in determining cell fate is which genes are expressed and which are turned off in individual cells. Proteins controlling the initiation of gene expression, transcription factors, are known to play crucial roles in these events. Two members of a family of six zinc finger transcription factors, GATA-4 and GATA-6, have been found to be necessary for both primitive and definitive endodermal development. Deficiency of either of these two GATA factors in mice leads to early embryonic lethality as a result of impaired yolk sac development and function. If overexpressed, GATA-4 and GATA-6 promote embryonal stem cell differentiation towards yolk sac tissue and up-regulate several genes essential for yolk sac development.

This study was initiated to characterize the role of GATA factors, GATA cofactors, and their downstream effectors in normal endodermal development and GCT pathology. The aim is also to improve understanding of the molecular mechanisms involved in malignant transformation of germ cells, thus making way for better diagnostics and treatment. The results will add to our knowledge of embryonal development and pediatric malignancies and lead to understanding of the biological mechanisms behind the development of normal yolk sac and germ cell tumors.

Review of the Literature

1. Endoderm

During early human development, the inner cell mass (ICM) of the embryo forms into a disc of two layers: the hypoblast or the primitive endoderm, and the epiblast (Figure 1). The cells of the hypoblast migrate to line the blastocoelic cavity, thus forming the primary yolk sac. In the process known as gastrulation, cells from the epiblast migrate through the primitive streak to give rise to the three germ layers that will form the entire embryo proper: the ectoderm, the mesoderm and the endoderm (Figure 2). The position of the initiation of gastrulation will determine the anterior-posterior axis of the developing individual.

Endoderm forms the layer lining the inner surfaces of the mammalian body. It undergoes a complex series of changes during development, including elongation, budding and branching morphogenesis. The tissues of endodermal origin serve as the connection between the body and the outside environment: the lungs facilitate gas exchange, and the gastrointestinal system absorbs nutrients and disposes of waste. As these tissues come into contact with various foreign materials, they are also of great importance as the first line of the defense system. Portions of the endoderm, e.g. the tonsils, develop into immunologically active organs. Endodermal derivatives, such as the thymus and the pancreas, are also part of the endocrine system.

Primitive endoderm – the yolk sac

At the time of implantation, the human hypoblast has formed the primary yolk sac by covering the interior surface of the blastocoelic cavity (Figure 1E). Within a few days, the distal parts of the sac will pinch off, giving rise to the secondary yolk sac, which remains in direct contact with the forming primitive gut. As the embryo folds, the yolk sac will become more elongated and located between the amnion and the chorion, and the connection to the embryo proper is narrowed to the vitelline duct incorporated in a stalk with vessels. The yolk sac in humans continues to grow until the 10^{th} week of gestation, and thereafter regresses (Sadler 1990a; Gilbert 2003a).

Yolk sac formation in the mouse is different from that of other mammalian embryos. The mouse embryo at the two-layer stage forms a cylinder, where the hypoblast is facing outwards. The yolk sac is formed of two layers, parietal and visceral endoderm, the latter of which forms the structure corresponding to the human secondary yolk sac. As the mouse embryo folds, the yolk sac is left outside and surrounds the developing mouse throughout gestation (Jollie 1990).

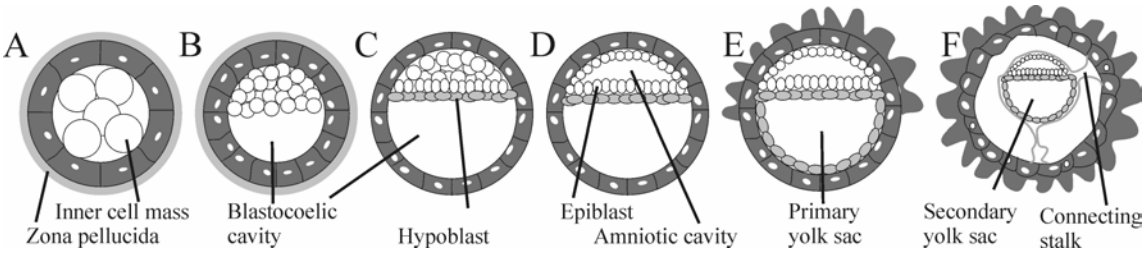


Figure 1. Primitive endoderm development.

At the 16-cell stage the human embryo has formed the morula (A) that will turn into the blastocyst, where the ICM (white) is clearly separate from the trophoblast (dark gray) (B). At the time of implantation, the blastocoelic side of the inner cell mass will differentiate into the primitive endoderm (gray) (C) that will induce cavitation and thus the formation of the epiblast and amniotic cavity in the rest of the inner cell mass (D). The primitive endoderm will then migrate to line the blastocoelic cavity and form the primary yolk sac (E). The distal parts of the primary yolk sac will pinch off, giving rise to the secondary yolk sac. Concomitantly, the connection between the developing embryo and the developing placenta is narrowed to a connecting stalk. *Adapted from Sadler 1990a*.

The primitive endoderm plays a vital role in the formation of a body plan in the embryo. In mice, active cell migration in the early visceral endoderm results in asymmetrical expression of transcription factors and growth factors. A specific area, the anterior visceral endoderm (AVE) is formed at the distal tip of the embryo, opposite the future gastrulation initiation site, before the node can be recognized. The cells of the AVE express specific genes such as *Lim1*, *goosecoid*, *Cerberus-like* (Belo *et al.* 1997) and the homeobox gene *Hex* (Thomas *et al.* 1998). Anterior visceral endoderm cells migrate anteriorly (Srinivas *et al.* 2004) and express inhibitors of the transforming growth factor beta (TGB-β) and Wnt signaling pathways, resulting in changes in the underlying epiblast and thus the initiation of gastrulation (Beddington and Robertson 1998; Beddington and Robertson 1999).

In mammals the main source of nutrition for the embryo is the mother, through the placental circulation. However, in human embryogenesis, maternal circulation to the placental intervillous space is not fully developed until 12 weeks of gestation (Jaffe *et al.* 1997). Instead, during the first trimester of human development, the uterine glands produce a clear secretion rich in nutrients into the intervillous space. The nutrients are then endocytosed by the trophoblast and either delivered to the embryo proper or secreted to the blastocoelic cavity (Exalto 1995; Burton *et al.* 2001; Burton *et al.* 2002). From there, the yolk sac filters nutrients for the embryo. The cells in the yolk sac have microvilli and contain phagocytic vesicles. In addition, they express nutrient transportation proteins, such as lipoproteins and transferrin (Bielinska *et al.* 1999).

Hematopoiesis, i.e. blood cell formation, is initiated in the yolk sac when the extraembryonic endoderm stimulates the cells in the extraembryonic mesoderm to differentiate towards hematopoietic and endothelial lineages (Belaoussoff *et al.* 1998; Yoder 2002). The yolk sac produces only nucleated primitive erythrocytes that soon are replaced when definitive

hematopoiesis begins in the embryo proper. The common stem cell of hematopoietic and endothelial lineages, however, is thought to originate in the yolk sac. From there, these cells migrate to the aorta-gonad-mesonephros (AGM) region (Dzierzak and Medvinsky 1995) or, according to recent findings, to the placenta, to mature (Gekas *et al.* 2005), and then to the liver and the bone marrow, the major hematopoietic sites in the embryo proper (Baron 2003).

Definitive endoderm derivatives

As the mammalian embryo folds, the three-layered disc is formed into a tube in which the definitive endoderm is the innermost layer, forming the primitive gut (Figure 2). This primitive gut is divided into three areas, the foregut, the midgut and the hindgut, from anterior to posterior. Closure of the abdominal wall is not complete until the 11th week of human development, so the connection between the primitive gut and the yolk sac is sustained and abdominal organs develop outside the embryo. Defects in abdominal wall closure, gastroschisis and omphalocele, complicate about one to two per 10 000 pregnancies and often are associated with other malformations (Weir 2003). As the result of continuous interaction between the endoderm and the underlying mesoderm, different parts of the digestive tube will form a variety of highly specific organs. All along the gastrointestinal tract the organs are formed by means of cooperation between all germinal layers: the endoderm forms the mucosa, the mesoderm the muscular layer underneath the mucosa, and the ectoderm provides the innervation of the gut system (Wells and Melton 1999; Gilbert 2003b).

Differentiation of the anterior endodermal tube begins with the formation of the pharyngeal pouches, four on both sides of the tube. The first of the pouches will form the auditory tubes and the endodermal lining of the middle ear. The ectodermal lining of the auditory channel is derived from the pharyngeal cleft opposite the first pharyngeal pouch. The second pouches give rise to the tonsils and the third pair will pinch off from the main endodermal tube and develop into the thymus, where the T cells mature later in development. The fourth pair forms the parathyroid glands, whereas the thyroid gland is formed from an individual anterior pouch of pharyngeal endoderm. Another, larger anterior pouch forms between the fourth pair of pharyngeal arches. This pouch elongates to the trachea, divides in two and then undergoes branching morphogenesis and finally differentiates into the lungs. The first pharyngeal arch is separated from the ectodermal stomodeum, the future mouth, by the pharyngeal membrane, which during the fourth week of human development breaks to create the anterior opening of the future digestive and respiratory systems.

Caudal to the forming pharyngeal pouches the tube dilates to form the future stomach. The esophagus elongates from a short section of the endodermal tube between the developing stomach and the pharynx as the lungs grow. In about one per 3500 term pregnancies, the separation of trachea and esophagus is incomplete and a fistula between the tube for food and the tube for air remains. This disorder is often accompanied by atresia in the trachea, esophagus, or both (Felix *et al.* 2004).

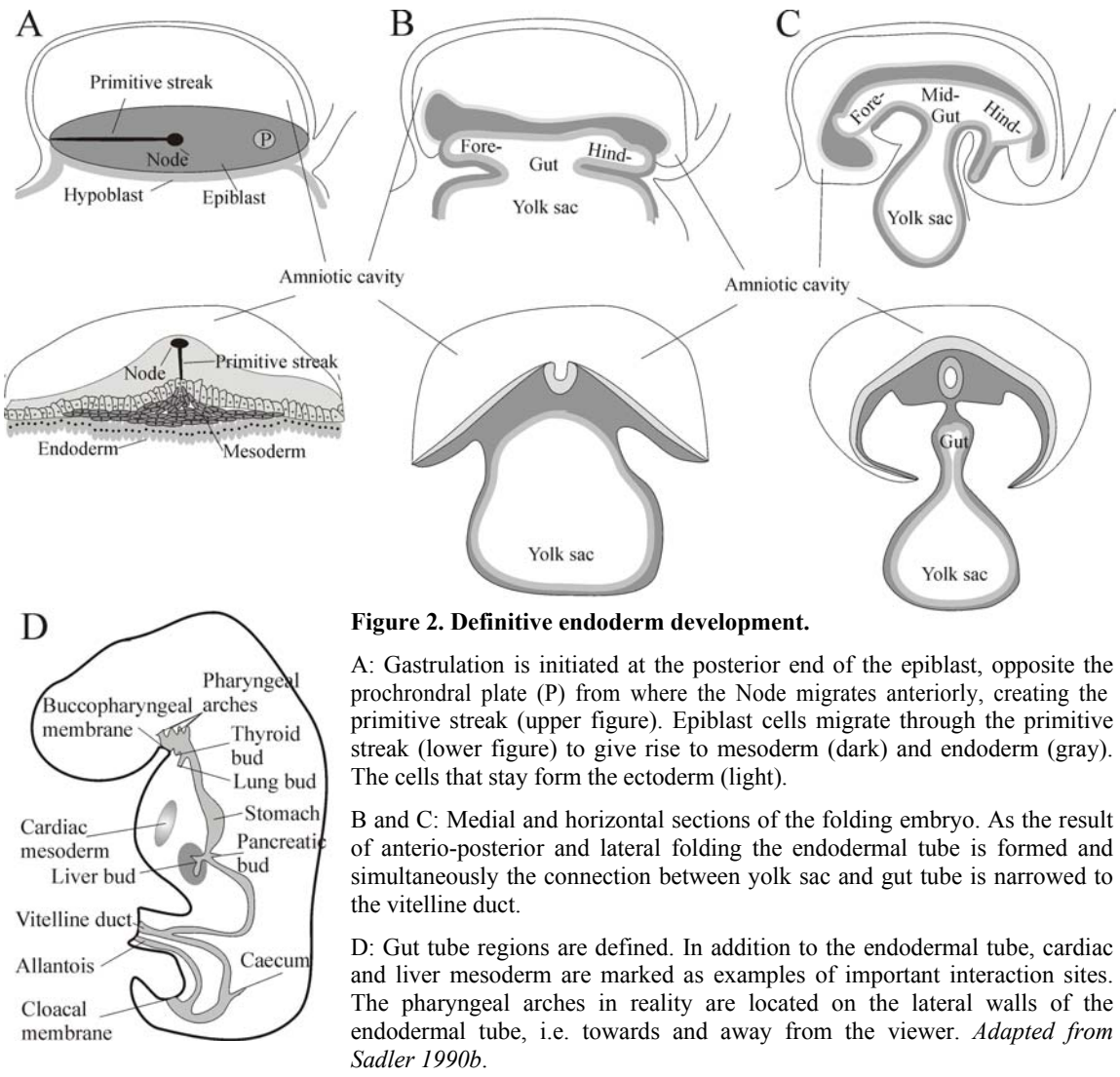


Figure 2. Definitive endoderm development.

A: Gastrulation is initiated at the posterior end of the epiblast, opposite the prochrondral plate (P) from where the Node migrates anteriorly, creating the primitive streak (upper figure). Epiblast cells migrate through the primitive streak (lower figure) to give rise to mesoderm (dark) and endoderm (gray). The cells that stay form the ectoderm (light).

B and C: Medial and horizontal sections of the folding embryo. As the result of anterio-posterior and lateral folding the endodermal tube is formed and simultaneously the connection between yolk sac and gut tube is narrowed to the vitelline duct.

D: Gut tube regions are defined. In addition to the endodermal tube, cardiac and liver mesoderm are marked as examples of important interaction sites. The pharyngeal arches in reality are located on the lateral walls of the endodermal tube, i.e. towards and away from the viewer. *Adapted from Sadler 1990b*.

At the junction of the esophagus and stomach, the surrounding musculature forms a sphincter to inhibit reflux. The most caudal region of the stomach, the fundus, is lined by stratified keratinized epithelium in mice and mucus-secreting epithelium in humans. The main part of the stomach, the corpus, contains glands, the gastric units, specialized to form gastric acids. The distal third, the pyloric region, secretes protective mucus and gastrin to stimulate acid secretion, and is continuous with the duodenum. At the junction of the stomach and the

duodenum, the muscular layer again forms a sphincter to regulate the passage of food (Wheather *et al.* 1987; Karam 1998). Hypertrophy of this muscular ring may obstruct the gastric outlet, hindering gastric emptying and thus causing vomiting in infants. This condition, known as pyloric stenosis, affects about two to five per 1000 newborns yearly and can be lethal if not treated (Hernanz-Schulman 2003).

The gastric units seen from the gastric lumen appear as pits in the mucosa. The pits are surrounded by cells that produce mucus that protects the gastric epithelium from the acidic secretion of the gland. Below the neck is the isthmus of the gland containing the self-renewing stem cells that continuously proliferate to maintain cell numbers in the gland. These cells in the human gastric gland are scattered along the whole gland below the neck area, and in the mouse these cells can even be seen in the pit (Wheather *et al.* 1987; Karam 1998).

The terminal part of the foregut and the midgut differentiate into the small intestine, which can be divided into three sections: the duodenum, the jejunum and the ileum. Induced by the cardiac mesoderm, the liver bud will emerge from the future duodenum (Figure 2D). This bud will interact with adjacent mesoderm and form the endodermal components of the liver, i.e. bile ducts and gallbladder. The molecules needed from the cardiac mesoderm for liver formation include members of the fibroblast growth factor (FGF) family (Zaret 2000; Serls *et al.* 2005). Also pancreatic development is initiated by formation of two buds that later fuse. Changes in the expression of some endodermal genes such as Sonic hedgehog (Shh) by signals from the notochord and mesoderm are essential for the pancreatic buds to form (Hebrok *et al.* 1998; Kumar *et al.* 2003). Both the liver and the pancreas develop as a result of continuous interaction of the endoderm and the surrounding mesoderm, forming complex organs that provide the body with elements needed for digestion, as well as numerous other functions. Pancreatic enzymes as well as the bile are secreted to the proximal duodenum to enhance digestion.

The most caudal part of the endodermal tube, the hindgut, differentiates to form the colon. The intersection of ileum and colon forms a flap to inhibit reflux of the intestinal contents. The proximal part of the colon forms the cecum, from which the appendix, a rudiment of the intestinal immunological system, hangs. As the most distal part of the hindgut comes in contact with the ectoderm, the cloacal membrane is formed. The urorectal septum then develops to separate the allantois and the hindgut, and thus the cloacal membrane is divided into the anal and urogenital membranes. The primitive urogenital sinus, formed anterior to the urorectal septum, will differentiate to the endodermal parts of the genito-urinary system. The anal membrane is surrounded by developing musculature from the mesoderm. At the 9th week of human development this membrane ruptures to form the distal contact between the endodermal tube and the outside world (Gilbert 2003b).

Regulators of endodermal differentiation

The differentiation of multipotent embryonic stem cells requires changes in cell behavior. These changes affect both the internal events of individual cells and their means of signaling towards other cells. Differentiation of endoderm has been, until recently, a less studied topic than differentiation of the two other germ layers. However, certain markers of endodermal development have been identified, and the number of factors known to affect endodermal differentiation is growing. It has been suggested that regional specification of the gut tube is guided by pre-patterned mesoderm. Indeed, migrating cells show regional differences in marker expression as early as during gastrulation (Robb and Tam 2004). Below are discussed some know endodermal regulators that are of importance to this study.

Transcription factors

Deoxyribonucleic acid (DNA) -encoded genes in eukaryotic cells contain the information needed for differentiation and function of an organism. Transcription factors are proteins that control the initial step of gene expression through recognizing specific DNA sequences in the promoter areas of target genes. Together with other transcription factors they trigger or restrain the activity of the basic transcription machinery that synthesizes messenger RNA (mRNA) to guide protein formation (Figure 4). As all the cells of multicellular organisms from plants to animals contain the same genetic data, the control of specific gene expression is needed for the differentiation of cell types. Thus, it is not surprising that a large proportion of human genes code for transcription factors (IHGMC 2001; Venter *et al.* 2001). Many of these factors form protein families and have been conserved in evolution, indicating their great importance in biology.

Several transcription factors have been implicated in endoderm differentiation. Hepatocyte nuclear factors (HNFs) 1, 3β and 4 have been shown to be essential for normal yolk sac differentiation. These factors were all first found to be expressed in hepatocytes, as their name indicates, but their structures and functions are remarkably different. Hepatocyte nuclear factor 1 is a homeodomain transcription factor (see below), that interacts with a multitude of endoderm and hepatocyte genes, such as *α-fetoprotein, albumin* and *CYP2E1* (Mendel and Crabtree 1991). HNF-3β is a winged-helix protein, the absence of which causes aberrant connections from the yolk sac to the embryo and embryonic lethality owing to failure in dorsal-ventral patterning (Ang and Rossant 1994). This factor, also known as Foxa2, later in development is expressed in the gut, stomach, liver and lung (Kaestner *et al.* 1994). HNF-4 is a steroid hormone family member necessary for visceral endoderm differentiation and future gastrulation of the embryo (Duncan *et al.* 1997). Later in development HNF-4 is expressed in several endodermal organs such as the gut and pancreas (Duncan *et al.* 1994; Eeckhoute *et al.*

2003). It has been shown to be involved in the transcription of insulin – mutations in either *HNF4* or *HNF1* that inhibit the interaction of these proteins lie behind maturity onset diabetes of the young (MODY) (Ryffel 2001).

Homeobox genes encode highly conserved transcription factors that are expressed in a spatially distinct pattern during development and guide the regional specification of the organism. In mammals, these genes can be found as clustered homeobox genes (*Hox*) as well as being dispersed along the genome (e.g. *Pdx, Gxh* and *Pax* families). In addition, a set of high-motility group transcription factors forms a structurally related but not clustered family of SRY-related homeobox (*Sox*) genes. Members of all these groups are expressed and are necessary during spatial differentiation of the gastrointestinal system. For example, Sox7 is needed for retinoic acid-induced visceral endoderm differentiation in the F9 mouse teratocarcinoma cell line (Futaki *et al.* 2004). Guided by signals from neighboring endodermal cells as well as the mesoderm, these factors drive specific gene expression in restricted gut segments that will then secrete signaling molecules that will again affect the differentiation of adjacent mesoderm (Bielinska *et al.* 1999; Wells and Melton 1999; Beck *et al.* 2000; de Santa Barbara *et al.* 2003).

Members of the GATA transcription factor family can be considered as master regulators of endoderm (Arceci *et al.* 1993; Soudais *et al.* 1995; Bossard and Zaret 1998; Morrisey *et al.* 1998; Koutsourakis *et al.* 1999; Reiter *et al.* 1999). Owing to their key role in this study, these factors are discussed in more detail below (Chapter 3).

Signaling molecules

Embryonic development from the earliest events is dependent on various cell-cell and tissue-tissue interactions. For endodermal development, signals from the mesoderm affect cell fate and organ formation, and this interaction apparently acts reciprocally through secreted signaling molecules. Members of the transforming growth factor (TGF)-β family such as nodal, activin and bone morphogenetic protein (BMP) play crucial roles in this signaling, as in most developmental processes in multicellular organisms (Kulkarni *et al.* 2002). These signaling molecules bind to TGF-β receptors on the cell surface, activating the intracellular serine/threonine kinase path leading to phosphorylation and nuclear translocation of effector molecules (Smad transcription factors). In the nucleus, Smads cooperate with specific transcription factors to alter gene expression. While there are 29 members of the TGF-β family in mammalian species, only twelve receptors, divided into type I and type II, and eight Smads have been recognized. However, the ability of the receptors to form dimers that bind to different ligands with varying affinities, activating specific combinations of Smads creates a high degree of complexity of signaling (Derynck and Zhang 2003). Smad4 is a modulating

protein common to all TGF-β pathways and requisite for both primitive and definitive endoderm development (Sirard *et al.* 1998).

Nodal, the mammalian member of the nodal family, is crucially needed as early as at the gastrulation stage. It binds to activin receptor ALK7-ActRIIB heterodimer that activates Smad2, leading to differentiation of cells towards endodermal and mesodermal lineages (Schier 2003). In the absence of Nodal in mice, no primitive streak is formed and thus no definitive germ layers are seen (Conlon *et al.* 1994). The expression of Smad2 in primitive endoderm is needed for formation of the primitive streak, and thus gastrulation (Heyer *et al.* 1999), as well as differentiation of definitive endoderm (Tremblay *et al.* 2000).

Endoderm formation is also dependent on activin signaling. In the complete absence of activin type II receptors, no primitive streak and thus no gastrulation is seen and mouse embryos die at embryonic day 8.5 (E8.5) (Song *et al.* 1999). In mice heterozygously deficient in both ActRIIA and ActRIIB, visceral endoderm formation and gastrulation are normal, but severe defects appear in the posterior foregut derivatives. Stomach epithelium in these mice is entirely of squamous epithelium that in the normal mice lines only the anterior stomach. Pancreatic islets are hypoblastic, specifically lacking β-cells, thus leading to a situation related to diabetes. As the phenotype resembles that of Sonic hedgehog (Shh, see below) -misexpressing mice (Apelqvist *et al.* 1997), it is probable that activin signaling to the endoderm is needed for specific down-regulation of Shh in posterior foregut differentiation (Kim *et al.* 2000). However, these studies were carried out on receptor-deficient mice, and some of the effects seen could be the results of impaired signaling by other TGF-β family members.

Signaling by BMPs has been shown to be essential for the formation and function of a normal yolk sac. BMP-4 is secreted by the pre-cavitation ICM cells and BMP-2 by the primitive endoderm. Blocking BMP signaling *in vitro* by means of a dominant negative receptor will inhibit differentiation of the yolk sac and ICM cavitation (Coucouvanis and Martin 1999). In addition, mice deficient of BMP-2, BMP-4 and BMP receptor I all show deficiencies in visceral endoderm development; the *Bmp4*$^{-/-}$ and *BmpRI*$^{-/-}$ phenotype is embryonically lethal as a result of gastrulation defects (Mishina *et al.* 1995; Winnier *et al.* 1995; Zhang and Bradley 1996). In addition, BMP signaling is involved in primordial germ cell genesis in the epiblast (Ying *et al.* 2001).

Members of the BMP family are co-expressed with Hedgehog family members – Indian hedgehog (Ihh) and Sonic hedgehog (Shh) – in several sites of endodermal-mesodermal interaction, such as limb development and endodermal organ branching (Bitgood and McMahon 1995). Hedgehog proteins bind to the cell membrane receptor Patched, which activates another transmembrane protein, Smoothened, leading to an intracellular signaling

cascade that is still partially unknown (Taipale and Beachy 2001). The *Ihh* gene is a direct target of BMP signaling (Seki and Hata 2004). It is expressed in the yolk sac and can induce expression of other markers of visceral endoderm development in cell culture assays (Becker *et al.* 1997). Indian hedgehog is needed for the induction and endothelial tube formation of the yolk sac vasculature (Byrd *et al.* 2002; Vokes *et al.* 2004). In embryoid bodies, inhibition of Ihh signaling hinders normal visceral endoderm maturation and inner cell mass cavity formation (Maye *et al.* 2000). The expression of Shh from the endoderm regulates the differentiation of adjacent mesoderm during gut development (Sukegawa *et al.* 2000). During later embryogenesis, Shh is involved in the differentiation of several endodermal and nonendodermal tissues (Hammerschmidt *et al.* 1997). Chemical inhibition of Shh signaling in gastrulating zebra fish inhibits development of pancreatic β-cells (DiIorio *et al.* 2002), but in later gut endoderm, repression of Shh is needed for early pancreatic development (Hebrok *et al.* 1998). Sonic hedgehog is also needed for proper separation of trachea and esophagus; the knockout phenotype recapitulates developmental disorders of the human foregut, gastroesophageal fistula and obstructions of the gastric and tracheal tubes (Litingtung *et al.* 1998; Ramalho-Santos *et al.* 2000).

Fibroblast growth factors (FGFs) in mammals constitute a 24-member family of extracellular signaling molecules. They bind to cell surface receptors (FGFRs), which triggers the cytoplasmic tyrosine kinase of the receptor, leading to activation of the intracellular signaling cascade (Ornitz and Itoh 2001; Alberts *et al.* 2002; Itoh and Ornitz 2004). Of the family members, FGF10 is expressed in the mesoderm underlying the developing endodermal tube and stimulates budding and branching of the developing lung (Bellusci *et al.* 1997). Mice devoid of FGF10 are born at term but lack limbs, lungs and thyroid and salivary glands. Owing to a lack of pulmonary development these mice are not viable. In addition, the stomach and pancreas are smaller in these animals than in wild-type littermates, and the amount of insulin-secreting cells in the pancreas is diminished (Ohuchi *et al.* 2000). In addition, in some of the mutant embryos duodenal or cecal atresia is seen (Fairbanks *et al.* 2004; Kanard *et al.* 2005). The phenotype is similar to that of mice lacking FGFR-2 isoform IIIb, indicating that FGF10 could act through this receptor (De Moerlooze *et al.* 2000).

2. Germ cell tumors

Normal germ cell development and tumor formation

Primordial germ cells (PGCs) originate in the epiblast very early in development. A cluster of cells positive for tissue nonspecific alkaline phosphatase, considered to be a marker of PGCs, can be seen posterior to the primitive streak as early as at E7 in the mouse. Bone morphogenetic protein 2 from the primitive endoderm, as well as BMP-4 and BMP-8b from the epiblast are needed for the formation of germ cells (Ying *et al.* 2001; Ying and Zhao 2001). As soon as the primitive streak appears during gastrulation, these cells migrate to the extraembryonic region, i.e. the yolk sac, to mature. From the yolk sac, they again migrate back to the embryo proper to the genital ridge in the abdominal wall. Mouse PGCs arrive at their destination at E11.0–11.5. In humans, PGCs can be seen in the extraembryonic endoderm during the fourth week of development. Early in the fifth developmental week these cells reach the urogenital ridge, and by the end of the sixth week of development, human PGCs have invaded the developing gonad (Sadler 1990c). Initial migration to the hindgut is thought to be passive, but from there on migration is active and directed by apparently genital ridge-secreted substrates (Buehr 1997). During migration PGCs express proteins such as β1 integrin, which recognize adhesion proteins like E cadherin secreted by the cells along their migration path. Germ cells maintain connection with each other during migration by physical cell-cell connections. The cells that lose their connection with others or lag behind die via apoptosis (Molyneaux and Wylie 2004). However, it is thought that occasionally PGCs that do not reach the developing gonads do survive and give rise to tumors. At least in the mouse the pro-apoptotic gene *Bax* is required for the normal death of ectopically located PGCs, indicating that disturbances of the apoptotic pathway may be needed for the survival of these cells (Stallock *et al.* 2003).

As the primitive gonad differentiates further to male testis or female ovary, germ cell fate is drastically different: in the ovary the majority of PGCs will undergo apoptosis at some stage of their differentiation and never form mature egg cells (Baker and Franchi 1967; Fulton *et al.* 2005). In the testis, the number of dividing sperm precursors is kept up by a constantly dividing stem cell population (Sutton 2000), whereas the absence of such stem cells in the ovary after birth is a dogma that only lately has encountered opposing evidence (Johnson *et al.* 2004).

Germ cell tumors (GCTs) are a histologically heterogeneous group of tumors thought to originate from PGCs. Germ cells can give rise to these tumors at any stage of their migration. Thus, the tumors can be located in the gonads, the abdominal cavity, or, in the case of

misplaced germ cells, in the mediastinum, maxillary cavities, the pineal region or other ectopic location. Extragonadal presentation is more common in children, accounting for about half of the cases (Gobel *et al.* 2000), whereas in adults only 2–5% of GCTs are located outside the gonads (Schmoll 2002; Dede *et al.* 2004; Mizushima 2004). In the adult testis GCTs are known to arise as carcinoma *in situ* (CIS), an accumulation of abnormal PGCs that express markers of both normal sperm line development and multipotent embryonic stem (ES) cells (Rorth *et al.* 2000; Almstrup *et al.* 2004; Hoei-Hansen *et al.* 2005). In rare cases, these premalignant lesions are found only after a more malignant metastasis has been found (Fossa *et al.* 2003). CIS-like precursors are not known to be present in the ovary. The cytogenetics of testicular GCTs has also been more extensively studied than that of the ovary. Isochromosome 12p is a characteristic of adult testicular GCTs but it has not been found in tumors of the infantile testis or ovary (van Echten *et al.* 2002; Veltman *et al.* 2003). Thus, the adult testicular teratoma can be considered as a tumor arising most commonly from malignant PGCs, whereas GCTs of the ovary and the infant testis are most often derived from benign germ cells (Ulbright 2004).

The different histological subtypes of GCTs, their alleged relationships to each other and prevalence in different age groups are depicted in Table 1 and Figure 3. The histological subtypes of GCTs most important in this study are discussed in more detail below.

Table 1. Proportions of different GCT histologies in different age groups and locations.

	Testicular	Ovarian	Extragonadal
Perinatal	Teratoma (50%) YST (50%) Mixed	Teratoma (50%) YST (50%) Mixed	Teratoma (50%) YST (50%) Mixed
Adolescent	Seminoma (50%) Teratoma (4%) YST (1%) EC (10%) Mixed (40%)	Dysgerminoma (2%) Teratoma (95%) YST (1%) EC (0.2%) Mixed	Germinoma Teratoma YST Mixed
Aged	Spermatocytic seminoma		

Adapted from Isaacs 2004; Ueno et al. 2004; Ulbright 2005.

Teratomas

Traditionally, teratomas have been thought to contain tissue types derived from all three germinal layers seen during normal development (Lahdenne *et al.* 1990; Ulbright 2004). The tissues may be fully differentiated, i.e. mature, or they may have become arrested at some stage of differentiation, giving rise to immature teratomas. This is most commonly seen in the neural and endodermal components. The immature tissues are highly proliferative, causing

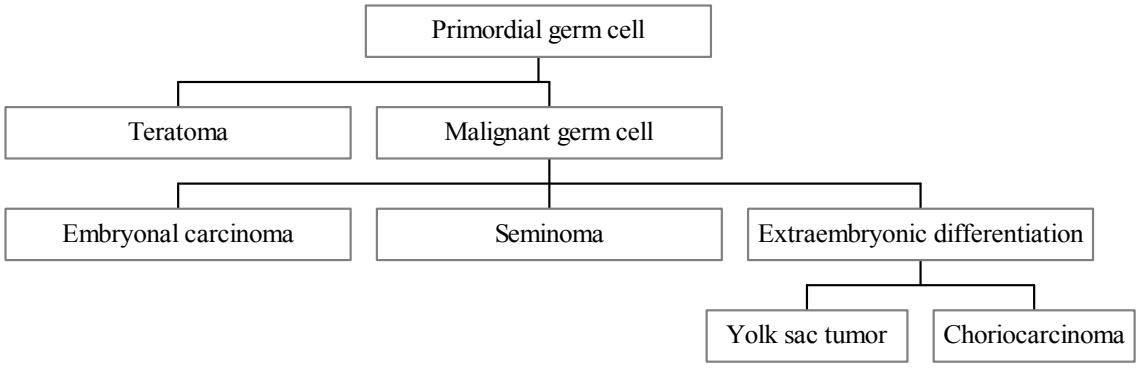


Figure 3. Germ cell tumor histologies.

Activated primordial germ cells (PGCs) give rise to tumors that may contain any embryonic or extraembryonic tissues. A teratoma may arise from either a benign activated PGC or PGCs that have already undergone malignant transformation. Malignant PGCs that retain their histological features give rise to germinomas, whereas in the case of dedifferentiation of a PGC an embryonal carcinoma is formed. Extraembryonic differentiation of PGCs is seen in yolk sac tumors and choriocarcinomas. All tumor types indicated can be found in a single tumor called a mixed germ cell tumor. *Adapted from Lahdenne 1991; Ulbright 2004*.

the tumor to grow rapidly. They are also more prone to malignant transformation, thus giving rise to teratomas with malignant transformation.

A mature teratoma is most often a benign tumor, if not located in an especially harmful place, such as the pineal region inside the skull or the mediastinum. Usually, surgical removal of the tumor is sufficient treatment for these cases. Immature and malignant components, however, complicate the treatment and prognosis. Small remnants of rapidly proliferating immature tissue may require additional surgery, and malignant components may send metastases that at the time of primary diagnosis may be too small to be recognized. Use of serum markers (Table 2) to detect small foci of malignant tissues within teratomas varies. In the Children's Hospital, Helsinki University Central Hospital, all GCT patients are studied for tumor markers for five years after the initial diagnosis, although final analysis of the benefits of the protocol is unfinished (M. Heikinheimo, personal communication).

In rare cases, especially in the testes of adolescent males, a teratoma can arise from PGCs that have already undergone malignant transformation. In these cases, the entire tumor is, of course, malignant and also often contains germinomatous, extraembryonic, or embryonal carcinoma components (Ulbright 2005).

Embryonal carcinoma

Embryonal carcinoma (EC) is a tumor of highly undifferentiated and malignant cells. In these tumors, the PGCs lose the markers normally associated with germ cell development and

express markers of ES cells such as *Nanog* (Korkola *et al.* 2005; Kuroda *et al.* 2005). The tumor cells thus retain the capability to differentiate, and occasionally differentiation resembling that seen in teratomas is evident (Ulbright 2005).

Germinomas

A germinoma is a moderately malignant tumor where the cells histologically resemble PGCs but have adopted malignant behavior (Reuter 2005). If located in the ovary, a germinoma is named a dysgerminoma (DG), whereas its testicular counterpart is the seminoma. Seminoma cells are indistinguishable from CIS cells but fill all the tubuli in the affected area (Reuter 2005). The third most common location for a germinoma is the mediastinum (Nakamura *et al.* 2004). The cells in these tumors express markers that are seen in primordial germ cells, such as OCT-3/4, a transcription factor that is needed for the maintenance of pluripotency (Looijenga *et al.* 2003). A distinct subgroup of seminomas is the spermatocytic seminomas occurring exclusively in undescended testis and at a more advanced age (Eble 1994).

Yolk sac tumor

Yolk sac tumors (YSTs) are the most prevalent malignant GCTs in the perinatal period and childhood (Isaacs 2004). In adult GCTs, malignant yolk sac tissue is much more rare and found in about 1% of the cases (Ulbright 2005). Yolk sac tumor tissue histologically resembles murine yolk sac and presents characteristic endoderm-lined cavities named Schiller-Duval bodies (Perlman and Hawkins 1998). These tumors also produce AFP, like the normal yolk sac (Teilum *et al.* 1975; Talerman *et al.* 1980), and this protein can be assayed in patient serum and in the tumor tissue as a method of diagnosis and follow-up (Norgaard-Pedersen *et al.* 1975; Schneider *et al.* 2001; Lahdenne and Heikinheimo 2002).

Clinical aspects

Although GCTs are rare, there are several features that highlight their importance. The prevalence of these tumors is highest during very early childhood, and puberty through early adulthood (Moller and Evans 2003). The youngest patients in the first age group are neonates; the tumors can start growing even during intrauterine life. In this age group, GCTs may be the most common neoplasm. In a recent study in two North American centers, the survival rate associated with perinatal GCTs was 63% for all cases but only 39% for cases where YST was present (Isaacs 2004). Thus the disease and its treatment can affect the entire life span or at least the reproductive age of an individual. In young males, testicular GCTs are the most

common malignant tumors. In females, however, the prevalence of other ovarian tumors is much higher (Koonings *et al.* 1989).

The prognosis of a patient with a GCT strongly depends on the histological subtype of the tumor; the more benign tumors usually are treatable by surgery alone, whereas the more malignant tissues require chemotherapy and occasionally radiation for treatment (Tewari *et al.* 2000; Dearnaley *et al.* 2001; Hussain 2005). The need for follow-up also differs, although the overall prognosis of GCT patients is good. Thus these rare tumors require rigorous diagnostics. Several markers are used to help determine the subtypes of these tumors (Table 2). Serum CA-125 is used for diagnostics and follow-up of EC. The serum level of this marker is elevated in several other conditions, such as endometriosis and epithelial cancer of the ovary (Rapkiewicz *et al.* 2004). Serum levels of α-fetoprotein (AFP) and human chorionic gonadotropin (hCG) are used in diagnostics and follow-up of tumors containing extraembryonic tissues; the detection of these proteins by immunohistochemistry is also used in diagnostics (Lahdenne and Heikinheimo 2002; Ulbright 2004). However, as all these markers are secretory proteins, they may not be detected by immunostaining even though the serum level may be high. In addition, cytoplasmic staining of a small number of cells in a mixed GCT may escape attention. Thus, new markers and methods in the diagnostics of GCTs are needed.

Table 2. Serum markers used in GCT diagnostics.

	Presumed diagnosis	Concerns
CA-125	Embryonal carcinoma	– Serum level also elevated in other ovarian malignancies, and endometriosis
AFP	Yolk sac tumor	– Serum level normally also high in neonates – Also elevated in hepatocellular carcinoma
hCG	Choriocarcinoma	– Serum level also high in normal pregnancy

Abbreviations: CA-125, cancer antigen 125; AFP, α-fetoprotein; hCG, human chorionic gonadotropin.

3. GATA transcription factors

Overview

The mammalian family of GATA transcription factors consists of six members that specifically bind to the DNA sequence (A/T)-G-A-T-A-(A/G). All members of the family are about 45–50 kD in size. The key elements of mammalian GATA-factors are the two Cys-X_2-Cys-X_{17}-Cys-X_2-Cys zinc fingers. The C-terminal finger binds to DNA, whereas the N-terminal finger stabilizes the binding and facilitates connection to cofactors (Figure 4) (Matthews and Sundem 2002). Other vertebrates and even invertebrates have their uni- or bifinger GATA-factors, and the DNA binding motive is conserved throughout phylogeny (Lowry and Atchley 2000). Expression patterns and mouse knockout phenotypes as regards GATA factors are summarized in Table 3.

Table 3. Mammalian GATA transcription factors.

	Expression	Mouse knockout phenotype	Reference
GATA-1	Hematopoietic lineage Testis	Lethal (E10.5–E11.5) – severe anemia (block in erythroid maturation)	(Evans *et al.* 1988) (Evans and Felsenfeld 1989) (Pevny *et al.* 1991) (Ito *et al.* 1993) (Fujiwara *et al.* 1996)
GATA-2	Hematopoietic lineage	Lethal (E11.5) – severe anemia (defect in proliferation of multipotential hematopoietic progenitors)	(Tsai *et al.* 1989) (Orkin 1992) (Tsai *et al.* 1994)
GATA-3	T-cells Central nervous system Adipocytes	Lethal (E11.5) – defects in central nervous system development, severe internal bleeding and anemia	(Yamamoto *et al.* 1990) (Pandolfi *et al.* 1995) (Hendriks *et al.* 1999)
GATA-4	Endoderm Heart Gonads Adrenal gland	Lethal (E7.0–E9.5) – defects in lateral folding and heart formation	(Arceci *et al.* 1993) (Soudais *et al.* 1995) (Kuo *et al.* 1997) (Molkentin *et al.* 1997)
GATA-5	Endoderm Heart Genitourinary tract	Female hypospadia	(Morrisey *et al.* 1997) (Molkentin *et al.* 2000)
GATA-6	Endoderm Heart Gonads Adrenal gland	Lethal (E6.5–7.5) – failure to gastrulate	(Morrisey *et al.* 1996) (Morrisey *et al.* 1998)
FOG	Hematopoietic lineage	Lethal (E10.5–E12.5) – severe anemia (block in erythroid maturation)	(Tsang *et al.* 1997) (Tsang *et al.* 1998)
FOG-2	Heart Brain Testis	Lethal (E12.5) – complex cardiac malformations	(Lu *et al.* 1999) (Svensson *et al.* 1999) (Tevosian *et al.* 1999) (Tevosian *et al.* 2000)

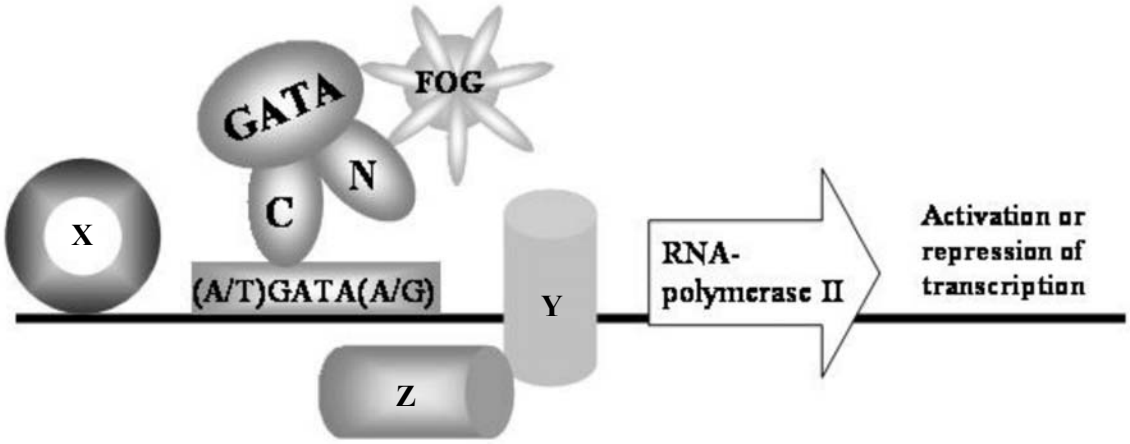


Figure 4. Schematic representation of GATA factor function.

The C-terminal zinc finger (C) binds to a GATA sequence in the target gene promoter area. The N-terminal zinc finger (N) binds to cofactors (FOG). Additional proteins (X, Y and Z) that bind either to GATA factors, DNA, or both, form a complex that either activates of represses RNA-polymerase II, which reads the gene to mRNA.

Hematopoietic GATA factors

The first nuclear protein binding to the GATA sequence was cloned by three groups in the late 1980s (Evans *et al.* 1988; Evans and Felsenfeld 1989; Plumb *et al.* 1989; Martin *et al.* 1990). In 1990, this factor was named GATA-1 by consensus of the Seventh Conference on Hemoglobin Switching (Orkin 1990). The two additional GATA factors expressed predominantly in the hematopoietic lineage were also cloned before the family name was decided and later named GATA-2 and GATA-3 (Orkin 1992). Even though these three factors are now defined as the hematopoietic subgroup of the GATA family, their expression patterns differ from each other, and they have specific roles in the differentiation of hematopoietic cells.

GATA-1 is expressed in developing erythrocytes, mast cells and eosinophils (Evans and Felsenfeld 1989). It has been shown to activate the transcription of numerous genes such as those for hemoglobins (Minie *et al.* 1992). GATA-1 is required for erythrocyte maturation both *in vitro* (Pevny *et al.* 1995) and *in vivo* (Pevny *et al.* 1991; Takahashi *et al.* 1997), and mice devoid of this factor die *in utero* at E12.5 and show accumulation of immature erythroblasts but no mature erythrocytes (Fujiwara *et al.* 1996). GATA-1 is also expressed in the testis (Ito *et al.* 1993; Ketola *et al.* 2002).

GATA-2 was the second family member found to be expressed in the hematopoietic lineage (Tsai *et al.* 1989; Dorfman *et al.* 1992; Orkin 1992) Knockout studies in mice have shown that it is needed for maintenance of the self-renewing stem cell population in the

hematopoietic compartment during early development (Tsai *et al.* 1994). As these mice die *in utero*, the role of GATA-2 during adult hematopoiesis has been unclear. However, adult *Gata2*$^{+/-}$ mice that are vital do show somewhat compromised early primitive hematopoietic cell proliferation (Ling *et al.* 2004).

GATA-3 was first cloned in chicken (Yamamoto *et al.* 1990) and soon after in mouse and human (Ho *et al.* 1991). In mammals, expression is strongest in the T-cell lineage, but this factor is also found in the placenta and in fetal brain, but not adult brain (Ko *et al.* 1991). GATA-3 is required for formation of the T-cell lineage and the brain, and mouse embryos devoid of GATA-3 die by E12 with severe defects of neural development (Pandolfi *et al.* 1995; Hendriks *et al.* 1999).

GATA factors expressed in endodermal tissues

GATA-4

The fourth member of the GATA family was cloned from a mouse E6.5 cDNA library (Arceci *et al.* 1993). It was the first non-hematopoietic GATA factor and was found to be expressed in both primitive and definitive endoderm, cardiac mesoderm (Arceci *et al.* 1993) and endocardium (Kelley *et al.* 1993), the gonads (Heikinheimo *et al.* 1997; Ketola *et al.* 1999; LaVoie 2003) and the adrenal gland (Kiiveri *et al.* 1999). When chicken GATA-4 was cloned, it showed a similar expression pattern (Laverriere *et al.* 1994). GATA-4 has now been implicated in the regulation of genes specific to the heart (*α-myosin heavy chain* (Molkentin *et al.* 1994), *Troponin C* (Ip *et al.* 1994; Murphy *et al.* 1997)), gonads (*MIS* (Tremblay and Viger 1999; Anttonen *et al.* 2003), *inhibin/activin β-subunit* (Feng *et al.* 2000)) and stomach (*H+/K+-ATPase, pepsinogen* (Nishi *et al.* 1997)), as well as more ubiquitously expressed genes such as the homeobox gene *Hex* (Denson *et al.* 2000; Molkentin 2000). In the heart GATA-4 was later also shown to regulate apoptosis (Suzuki and Evans 2004).

Mouse embryos devoid of GATA-4 die at E8.5–10.5. These embryos do not fold normally, and thus heart tube formation is not completed (Kuo *et al.* 1997; Molkentin *et al.* 1997). *Gata4*$^{-/-}$ ES cells are incapable of forming a normal visceral endoderm layer in embryoid bodies (Soudais *et al.* 1995). However, these ES cells are able to differentiate into beating cardiac myocytes, indicating that the effect of GATA-4 deficiency on cardiac morphogenesis is not direct (Narita *et al.* 1997b). Wild-type visceral endoderm cells abrogate the ventral folding defect of *Gata4*$^{-/-}$ mice (Narita *et al.* 1997a), suggesting that defective signaling between endoderm and mesoderm might be the cause of developmental problems in the GATA-4 deficient mice.

GATA-5

The next GATA factor was first cloned in *Xenopus*, and initially named GATA-4 (Kelley *et al.* 1993). This factor was soon named GATA-5 when a 74% identical factor was cloned in chicken along with another factor more closely resembling murine GATA-4 (Laverriere *et al.* 1994). In *Xenopus* and chicken this factor was shown to be expressed in the developing heart and gut. In mammals, GATA-5 was cloned only after GATA-6 (Morrisey *et al.* 1997). During mouse development, GATA-5 is found in the allantois, cardiac atria, mid- and hindgut, lung and later in pulmonary mesenchyme, especially in the airway smooth muscle, as well as the urogenital ridge. In the developing urinary system GATA-5 is also expressed in smooth muscle, whereas in the gut tube expression is restricted to epithelial cells. Unlike GATA-4 and GATA-6, GATA-5 is not expressed in the primitive endoderm. In the bladder and gastrointestinal system expression continues in the adult mouse (Morrisey *et al.* 1997).

Very different from all other GATA factors, GATA-5 does not appear to be critical for mouse development. Male *Gata5*$^{-/-}$ mice are vital and fertile, whereas the females exhibit gross morphological abnormalities in the genitourinary system and reduced fertility. Other organs that express GATA-5 during development are normal, indicating that other members of the family are able to compensate for the loss of one factor in these organs (Molkentin *et al.* 2000). However, *Gata5*$^{-/-}$ zebra fish die early in development, with a phenotype resembling that of *Gata4*$^{-/-}$ mice (Reiter *et al.* 1999).

GATA-6

Chicken GATA-6 (cGATA-6) was cloned simultaneously with GATA-5 (Laverriere *et al.* 1994). It was then shown to be expressed in the adult gastrointestinal system, heart, lung, liver and ovary. As with cGATA-4 and cGATA-5, cGATA-6 is expressed early in heart development. Developmental expression of cGATA-6 in the gastrointestinal system increases along with differentiation. During early mouse development, GATA-6 is expressed in the embryonic mesoderm of the future heart, allantois and the extraembryonic endoderm, although to a lesser amount than GATA-4 (Morrisey *et al.* 1996). As development progresses, mouse GATA-6 continues to be expressed in the heart and it appears in the gut tube with GATA-4. Mouse GATA-6 alone is expressed in the smooth muscle of the developing vasculature, lung buds and the urogenital ridge.

Of the GATA family members GATA-6 deficiency causes the earliest lethality: mouse embryos devoid of this factor die at E8.5 with greatly underdeveloped visceral endoderm (Koutsourakis *et al.* 1999). The phenotype is strikingly similar to that of HNF-4-deficient mice, and GATA-6 has been shown to be a direct transcriptional controller of the *Hnf4* gene

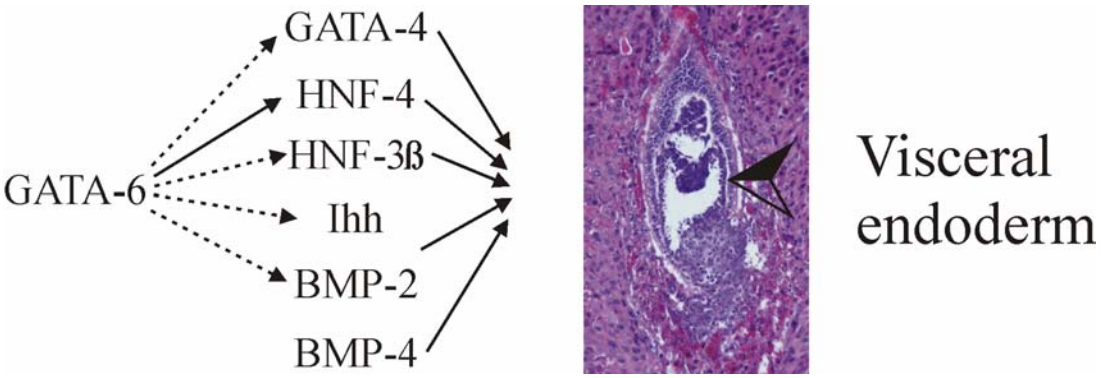


Figure 5. Proposed pathway of factors affecting the development of normal visceral endoderm

GATA-6 has been shown to directly trans-activate HNF-4 (solid arrow), whereas the up-regulation of other markers may be indirect (dashed lines). All factors indicated in the figure are needed for the formation of normal visceral endoderm indicated in E7.5 mouse embryo by arrow head.

(Morrisey *et al.* 1998). *Gata6*$^{-/-}$ embryos can be partially rescued with tetraploid embryo complementation. These embryos survive until E10.5 and undergo normal gastrulation, indicating that defective signaling from GATA-6-deficient primitive endoderm indeed is the cause of lethality in *Gata6*$^{-/-}$ mice (Zhao *et al.* 2005). If over-expressed in mouse embryonic stem cells, GATA-6 induces expression of a multitude of markers of primitive endoderm development. Among these markers are GATA-4, HNF-4, BMP-2 and Ihh (Fujikura *et al.* 2002) (Figure 5).

Friends of GATA

The two known Friends of GATA (FOG) cofactors are multitype zinc finger proteins approximately 110 kD in size (Tsang *et al.* 1997; Lu *et al.* 1999; Svensson *et al.* 1999; Tevosian *et al.* 1999). Both proteins contain four Cys-Cys:His-Cys zinc fingers that are able to bind the N-terminal zinc finger of GATA proteins (Fox *et al.* 1998). In addition, FOG-1 contains five and FOG-2 four additional zinc fingers of Cys-Cys:His-Cys configuration (Tsang *et al.* 1997; Holmes *et al.* 1999). FOG-1 is expressed in erythroid and megakaryocytic cells, where it cooperates with GATA-1 to promote cell differentiation (Tsang *et al.* 1997). In the absence of FOG-1 mice die at mid-gestation, presenting severe anemia owing to blockage in erythroid maturation, and failure of megakaryopoiesis (Tsang *et al.* 1998). FOG-2 is expressed in the developing heart, brain, testis and – to a lesser extent – lung and liver. It binds to GATA-4 and represses the transcription of cardiac genes (Svensson *et al.* 1999). *Fog2*$^{-/-}$ mice die between E13 and E14 as a result of congestive heart failure caused by tricuspid atresia syndrome (Tevosian *et al.* 2000). Mice with GATA-4 incapable of binding FOG zinc fingers present the same phenotype as *Fog2*$^{-/-}$ mice, showing that the interaction of these two factors is crucial in heart development (Crispino *et al.* 2001).

GATA factors of non-vertebrates

DNA binding proteins with GATA-type zinc fingers are present in organisms as low in the evolutionary scale as *Caenorhabditis elegans* and *Drosophila* (Patient and McGhee 2002). The *Drosophila* GATA factor *Serpent* and its FOG-related cofactor *U-shaped* are needed for hematopoiesis. Endodermal differentiation in this fly depends on GATA factors *pannier* and *tinman*. *Pannier* also plays a role in ectodermal differentiation. In *C. elegans*, GATA factors *elt-1* and *elt-3* are essential for ectoderm development (Gilleard *et al.* 1999; Gilleard and McGhee 2001). Further differentiation of the epidermal cells to seam cells requires two other GATA factors, *elt-5* and *elt-6* (Koh and Rothman 2001). Endodermal differentiation in this nematode needs GATA factors *med-1* and *med-2* as well as additional members of the *elt* and *end* GATA factor families. Altogether, the GATA-related protein family in *C. elegans* consists of eleven members. Confusingly, *C. elegans* has its own fog proteins crucial for sex determination but not related to vertebrate FOG factors (Schedl and Kimble 1988; Barton and Kimble 1990). In the sea urchin, GATA-E plays a role in a complex developmental signaling network (Davidson *et al.* 2002).

GATA factors in human disease

Acquired abnormalities in GATA factor expression or function have been implicated in numerous human diseases such as asthma (Ray and Cohn 1999), rheumatism (Kawashima and Miossec 2005), cardiac hypertrophy (Saadane *et al.* 1999; Pikkarainen *et al.* 2004), drug-induced cardiotoxicity (Aries *et al.* 2004), as well as in malignancies of the gastrointestinal tract (Akiyama *et al.* 2003), lungs (Guo *et al.* 2004), adrenal glands (Rahman *et al.* 2004; Kiiveri *et al.* 2005), gonads (Ketola *et al.* 2000; Laitinen *et al.* 2000; Lassus *et al.* 2001) and mammary gland (Parikh *et al.* 2005). Below are discussed the rare congenital mutations and diseases where GATA factor mutations play a fundamental role.

The risk of developing transient myelodysproliferative disorder (TMD) or acute leukemia is increased in children with trisomy 21, i.e. Down's syndrome. Approximately one fourth of the leukemias in these patients are acute megakaryoblastic leukemias (AMKLs)(Gurbuxani *et al.* 2004). The relationship between TMD and AMKL in Down's syndrome children is controversial. In TMD patients later diagnosed with AMKL, the leukemia cells contain the same chromosomal abnormalities as the initial TMD. On the other hand, not all AMKL patients have a previous diagnosis of TMD. TMD, however, is seen in Down's syndrome children at only a few weeks of age or even in the fetal period and it often has no symptoms, so this condition may be underdiagnosed. Mutations in the *GATA1* gene have been found in both TMD and AMKL cells but not in other Down's syndrome leukemias or non-Down's syndrome leukemia patients (Xu *et al.* 2003; Crispino 2005). The mutations in the *GATA1*

gene vary, but all of them are predicted to result in the expression of a shorter protein, GATA-1s, that lacks transcriptional activity (Calligaris *et al.* 1995). Thus, expression of GATA-1s apparently gives the cells the same proliferative advantage as the low level of GATA-1 in knock-down mice, but it is not capable of inducing differentiation in these cells. However, as not all patients with TDM or *GATA1* mutation without TDM diagnosis develop leukemia, the expression of GATA-1s alone is not sufficient to cause malignant disease.

The *GATA1* gene is located in the X chromosome and it is thus expected that mutations in the gene are more likely to cause problems for the male gender. Indeed, a germline mutation in the *GATA1* gene that inhibits cooperation between FOG-1 and GATA-1 has been found in one family where two male offspring suffered from severe anemia that was already present at the fetal period. Both boys also had cryptorchidism, whereas their three sisters were unaffected (Nichols *et al.* 2000).

HDR syndrome is a rare condition where the patients suffer from hypoparathyroidism, deafness and renal dysplasia, as first described by Barakat and colleagues in 1977 (Barakat *et al.* 1977). The disease locus was initially mapped near to the DiGeorge syndrome locus in chromosome 10p (Lichtner *et al.* 2000). It is an autosomal dominant disorder caused by mutations in the *GATA3* gene that either cause deletion of one *GATA3* allele or result in the production of a nonfunctional protein incapable of binding either DNA or FOG-2. However, the precise mechanism of how these mutations cause the defects in the affected organs (e.g. what target genes are not expressed normally) is still not clear. The patients do not suffer from the immunological problems that are seen in the *Gata3*$^{-/-}$ mice (Van Esch *et al.* 2000; Nesbit *et al.* 2004).

The chromosomal locus of *GATA4*, 8p23.1, has been found to be critical as regards congenital cardiac malformations (Johnson *et al.* 1997). The malformations found in these patients include atrioventricular canal, atrial septal and ventricular septal defects, double outlet right ventricle, dextrocardia, pulmonary stenosis and hypoplastic left heart (Pehlivan *et al.* 1999).

Considering the importance of GATA factors for mouse development, it is not surprising that defects in these proteins or their interactions with other factors are also found in human disease. Future studies will most likely show additional conditions such as failures in sex determination or cardiac malformations that are caused by defective GATA expression or function. The role of GATA factors in malignancies and immunological diseases is also under active investigation and this may result in new strategies as regards diagnostics and treatment.

Aims of the Study

The molecular events behind normal and abnormal endodermal differentiation as well as GCT formation are poorly known. However, these issues are worthy of study both as biological phenomena and clinically relevant problems. In order to better understand the development of endodermal tissues as well as GCT biology, this study was aimed at:

1. Defining the expression patterns of GATA-4, GATA-6 and Friends of GATA in different histological GCTs and in different stages of yolk sac and definitive endoderm development

2. Characterizing the expression patterns of endodermal downstream effectors of GATA-4 and GATA-6 in YSTs and other germ cell tumors

3. Defining the potential role of GATA factors and their downstream effectors as potential GCT diagnostic markers and therapy targets

4. Describing whether and how the loss of GATA:FOG interaction affects different stages of endodermal differentiation.

Materials and Methods

1. Human tumors (I–III)

Tumor samples used for this study were originally collected for diagnostic purposes in the Hospital for Children and Adolescents and the Department of Obstetrics and Gynecology of Helsinki University Central hospital. The pediatric material included nine YSTs (three gonadal and six extragonadal), one EC (vaginal), 16 benign teratomas (12 gonadal and four sacrococcygeal) and five immature teratomas (two gonadal and three extragonadal). The adult samples were all ovarian GCTs and included 6 YSTs, 5 immature teratomas and 8 DGs staged according to FIGO recommendations (FIGO 1987). The tumor material is summarized in Tables 4a and 4b.

Table 4a. Pediatric GCTs.

Malignant tumors						
Pt.			S-AFP at	Outcome	Tumor	
no.	Localization	Age and sex	diagnosis (mg/l)	(yrs after dg)	Histology	Metastasis
1	Testis	1.0 M	430	NED (7)	YST	
2	Testis	4.0 M	2173	NED (2)	YST	
3	Pelvis	1.3 M	48500	Died (5)	YST	Lung
4	Pelvis	1.5 F	16500	NED (2)	YST	
5	Pelvis	1.6 F	19300	NED (16)	YST	
6	Pelvis	1.6 M	53200	NED (8)	YST	
7	Pelvis	2.1 F	19954	NED (1)	YST	Lung
8	Pelvis	2.5 F	92700	NED (7)	YST	
9	Ovary	14.5 F		NED (14)	YST	Omentum
10	Vagina	11 F	25500	NED (1)	EC	

Benign tumors			Tissue presentation (number of tumors indicated)						
		Age		Bone or		Resp.		Neural	
n	Localization	(average)	Skin	cartilage	Muscle	epith.	Gut	Mature	Immature
	Mature teratomas								
3	Testis	4.2 y	2	1		1			
8	Ovary	8.5 y	7	4	1	2	1	7	
4*	SCT	24.8 d	2	2	1	2		3	
	Immature teratomas								
1	Testis	66 d				1		1	1
1	Ovary	11.6 y	1	1				1	1
2	SCT	2 d	2	1			1	1	1
1	Abd. cavity	1 d		1				1	1

Abbreviations: NED, no evidence of disease; EC, embryonal carcinoma; YST, yolk sac tumor.

SCT, sacrococcygeal teratoma; Abd. cavity, abdominal cavity; Skin, skin and appendages; Resp. epith., tissue reminiscent of respiratory epithelium; Gut, tissue reminiscent of mature gut epithelium.

* Three patients, one residual tumor.

Table 4b. Adult ovarian GCTs.

		Yolk sac tumor	Dysgerminoma	Immature teratoma
n (19)		6	8	5
Mean age (range)		39.0 (29–57)	26.9 (12–58)	28.0 (16–40)
Stage n (%)	I	4 (67%)	4 (50%)	4 (80%)
	II–IV	2 (33%)	4 (50%)	1 (20%)
Status at the end of follow-up				
	Complete response	4 (67%)	7 (88%)	2 (40%)
	Partial response			1 (20%)
	Died	2 (33%)	1 (12%)	2 (40%)
Serum tumor markers elevated				
	AFP	6		1
	hCG	1	1	2
	CA-125	3	1	2

Abbreviations: CA-125, cancer antigen 125; AFP, α-fetoprotein; hCG, human chorionic gonadotropin.

2. Human germ cell tumor cell lines (I)

Samples of mRNA from four human germ cell tumor lines, originally derived from testicular YSTs and kindly provided by Dr. Pera, Institute of Cancer Research, Surrey, UK, were used for Northern blot analysis of GATA-4 expression (I). The GCT 72 cell line exhibits properties of visceral endoderm, whereas the GCT 44, GCT 46 and GCT 85 lines resemble parietal endoderm (Pera *et al.* 1987).

The NCC-IT cell line (Tesima *et al.* 1988) was obtained from ATCC (Manassas, VA). This cell line expresses markers of both pluripotent germ cells and embryonal stem cells (Damjanov *et al.* 1993). The cells were cultured in RPMI-1640 medium supplemented (10%) with fetal bovine serum and penicillin-streptomycin. For immunocytochemistry, the cells were grown on Lab-Tek™ Chamber Slides (Nunc International, Rochester, NY).

3. Normal and transgenic mouse tissue (I, II, IV)

Normal mouse embryos were obtained by mating male and female B6DJLF1/J (I) or NMRI (II) mice. For estimating embryonal age, noon of the day on which the copulation plug was found was considered as 0.5 days p.c. Precise staging of dissected embryos was performed using The Atlas of Mouse Development, Academic Press, London. The yolk sacs and placentas from 6 to 14 days p.c. were collected together with the embryos and frozen or fixed in 4% paraformaldehyde and embedded in paraffin.

Mice with mutated GATA-4 unable to bind FOG cofactors (IV) were prepared as described previously (Crispino *et al.* 2001). In short, an 8.2-kb fragment of the murine *Gata4* gene containing the N finger of GATA-4 was subcloned into pBluescript plasmid (Stratagene). By means of site-directed mutagenesis, valine 217 was changed to glycine (codon GGC was

changed to GTC; GeneEditor, Promega). A floxed neomycin expression cassette was introduced and HSV-tk was cloned into a SalI site 5' of the homology region. The targeting construct was linearized and electroporated into ES cells. The *Gata4* gene from targeted ES clones was amplified by PCR and sequenced. A correctly mutated clone was injected into C57BL/6 blastocysts to generate chimeras. Genotyping of the embryos was carried out thereafter by Southern blot analysis. Heterozygous littermates were used as controls.

4. mRNA in situ hybridization (I, II, IV)

Gene expression at mRNA level was studied by radioactive *in situ* hybridization. Tumor tissue sections from paraffin-embedded patient samples were fixed in 4% paraformaldehyde. Mouse embryos along with the extraembryonic membranes and placenta were frozen or fixed in 4% paraformaldehyde. Radioactive riboprobes were generated using previously described cDNA templates for mouse GATA-4, GATA-6 (Heikinheimo *et al.* 1994; Heikinheimo *et al.* 1997), Shh (Bitgood and McMahon 1995) and FGF-10 (Pauley *et al.* 2003), and human GATA-6 (Kiiveri *et al.* 2002). Tissue sections (8 µm) were permeabilized with proteinase K (10 µg/ml) in 0.05 M EDTA/0.1 M Tris-HCl (pH 8) at 37 ^{o}C for 90 minutes. Autoradiography background was minimized by immersion in 0.1 M triethanolamine (pH 8) for 3 minutes and in a solution containing 0.25% acetic anhydride (Applied Biosystems, Foster City, CA, USA) in 0.1 M triethanolamine (pH 8) for 10 minutes. Tissue sections were incubated with 1×10^6 cpm of [^{33}P]-labeled (1000–3000 Ci/mmol, Amersham, Arlington Heights, IL) antisense or sense riboprobe diluted in a buffer containing 0.5 vol deionized formamide, 0.2 vol 50% dextran sulfate, and 5 × Denhardt's solution in 10 mM Tris-HCl/1 mM EDTA/0.3 M NaCl in a total volume of 80 µl at 60 ^{o}C for 16–24 hours. After hybridization the slides were subjected to high-stringency washing by immersion in 4 × standard saline citrate (SSC). Non-hybridized stranded riboprobe was removed by treatment with a solution of RNase A (20 µg/ml) in 0.5 M NaCl/10 mM Tris-HCl/1 mM EDTA (pH 8) followed by SSC. The sections were dehydrated through graded concentrations of ethanol and air-dried. Autoradiography was performed for 7–14 days with Kodak Autoradiography Emulsion NTB2 (Kodak, Rochester, NY). The slides were then developed in Kodak D-19 developer (Kodak), fixed with Kodak Fixer (Kodak) according to the manufacturer's instructions, and counterstained with Harris hematoxylin.

5. Semi-quantitative RT-PCR (VI)

Anterior and posterior foregut were dissected from E11.5 wild-type, *Gata4*$^{ki/+}$ and *Gata4*$^{ki/ki}$ mice and then homogenized in TRIzol (Invitrogen, Carlsbad, CA). RT-PCR was done from purified RNA (20 ng) using a TITANIUM™ one-step kit (BD Biosciences, Palo Alto, CA), oligo(dT) primers for the reverse transcriptase reaction, and previously described PCR

primers for *Gata4* (Bielinska *et al.* 2004), *Shh* (Madison *et al.* 2005) and *Fgf10* (Bellusci *et al.* 1997). *β-actin* primers were provided in the kit. After a hot start (94 °C for 4 min) the polymerase reactions were conducted for 30 cycles at the thermal conditions indicated in Table 5 (*β-actin* was cycled as *Shh*). Agarose gel electrophoresis (1.4%) in the presence of ethidium bromide demonstrated a single band of the expected size for each of the PCR primer pairs. Threshold cycles were normalized to threshold cycles for *β-actin*.

Table 5. RT-PCR primers

	Primers	Thermal conditions
GATA-4	5' – TTCTCGAGCTAGCCAAACATACATCTCAGGTACACTGAT – 3' 5' – TTGCGGCCGCAATGCTTCTAACAATTGCTTCAGGGTCCT – 3'	94 °C (1 min) 58 °C (1 min) 72 °C (3 min)
Shh	5' – GCTGTGGAAGCAGGTTTCG – 3' 5' – GGAAGGTGAGGAAGTCGCTG – 3'	94 °C (1 min) 55 °C (1 min) 72 °C (3 min)
FGF-10	5' – GGATACTGACACATTGTGCCTCAG – 3' 5' – TGTTTTTTGTCCTCTCCTGGGAG – 3'	94 °C (1 min) 60 °C (1 min) 72 °C (3 min)

6. Northern blotting (I)

Poly-A RNA from lysed cells was analyzed for expression of GATA-4 mRNA using Northern hybridization as described previously (Arceci *et al.* 1993). Cyclophilin was used as a loading control. One microgram of Poly-A RNA was subjected to electrophoresis on a 1% denaturing agarose gel and then transferred onto nylon membranes (Hybond N, Amersham). Partial length human *GATA4* cDNA was used to prepare the GATA-4 probe (Heikinheimo *et al.* 1994). After baking and prehybridization, the membranes were subjected to hybridization with cDNA inserts labeled with [^{32}P]deoxy-CTP (3000 Ci/mmol; Amersham), using a Prime-a-gene kit (Promega, Madison, WI). A Fujifilm IP-Reader Bio-Imaging Analyzer (BAS 1500, Fuji Photo Co. Ltd., Tokyo, Japan) was used to analyze hybridization signals, with the MacBas software supplied by the manufacturer and a Macintosh personal computer (Apple Computer Inc., Cupertino, CA).

7. Immunohistochemistry (I–IV)

Protein expression in normal and tumor tissue was studied by immunohistochemistry. Tissue sections from paraffin-embedded tumor or mouse samples were deparaffinized and rehydrated by using descending ethanol concentrations. Antigen retrieval was performed by incubating the slides in 0.1M citric acid (pH 8.0) at ca. 100 °C for 10 minutes (cytoplasmic antigens) or 20 minutes (nuclear antigens). Endogenous peroxidase activity was blocked with 3% H_2O_2 in water for 5 minutes. The slides were then subjected to immunohistochemistry using

commercially available primary antibodies (Table 6) and an avidin-biotin immunoperoxidase system to visualize bound antibody (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA), with 3-amino-9-ethylcarbazole (Sigma Chemicals, St. Louis, MO) or 3,3´-diaminobenzidine (Sigma) as chromogen.

Table 6. Antibodies used for immunohistochemistry.

Antigen	cat #	Dilution	Host
BMP-2(N-14)	sc-6895	1:100	goat
FOG(M-20)	sc-9361	1:200	goat
FOG2(L-20)	sc-9365	1:100	goat
FOG2(M-247)	sc-10755	1:100	rabbit
GATA-4(C-20)	sc-1237	1:200	goat
GATA-6(H-92)	sc-9055	1:50–1:250	rabbit
HNF-4(C-19)	sc-6556	1:200	goat
Ihh(I-19)	sc-1782	1:100	goat
Ki-67	M7240	1:75	mouse

Antibody for detecting Ki-67 antigen was from DakoCytomation, Glostrup, Denmark. All other antibodies were from Santa Cruz Biotechnology, Santa Cruz, CA

Staging the staining

MIB-1 for Ki-67 antigen was scored as follows: + (<5% of cells positive), ++ (5–30% positive) and +++ (>30% positive). Scoring for the other antigens was based on the staining intensity of at least 10% of tumor cells: -, no staining; +, weak staining; ++, moderate staining; +++, strong staining.

8. Ethical considerations (I–IV)

The study on human tumors was approved by the ethics committees of the departments involved. Moreover, permission from the National Authority for Medicolegal Affairs was obtained to use the paraffin-embedded tissue samples of ovarian tumor material for research purposes, as required by current legislation.

Transgenic animals were from the laboratory of Dr. S. Tevosian. Normal mouse embryos were obtained from Washington University Department of Pediatrics Mouse Core (I) or Helsinki University, Meilahti Laboratory Animal Facility (II). All experiments on animals were accepted by local authorities and performed according to guidelines of the National Research Council.

Results and Discussion

1. GATA factors and endodermal development (I, II, IV)

Expression of GATA-4, GATA-6 and FOG proteins in the primitive endoderm (I, II, IV)

Early expression of GATA-4 and GATA-6 in the developing embryo is crucial for formation of the primitive endoderm as well as for the survival and future development of the embryo (Bielinska *et al.* 1996; Bielinska and Wilson 1997; Molkentin *et al.* 1997; Morrisey *et al.* 1998). However, the expression or role of GATA factors in later development of the primitive endoderm has not been previously studied. The yolk sac plays important developmental roles even after gastrulation. It transports nutrients, produces important proteins for the developing embryo, and nurses hematopoietic stem cells and primordial germ cell. Thus it is possible that the factors crucial for differentiation of extraembryonic endoderm are also needed for later yolk sac function.

We studied the expression of GATA factors in later murine extraembryonic tissues to reveal whether these factors play a role in the function as well as establishment of the yolk sac. Messenger RNA *in situ* hybridization and immunohistochemistry showed that GATA-4 and GATA-6 continue to be strongly expressed in mouse extraembryonic endoderm at least until E13.5 (Figure 7) (II and present data). This finding suggests that GATA proteins necessary for the initial differentiation of extraembryonic endoderm are also likely to play a role in the maintenance of yolk sac phenotype and may thus be needed for the continuation of pregnancy. However, further studies are needed for a more precise understanding of the role of these factors in early development as well as their importance in later yolk sac function.

Friends of GATA have not been found in early primitive endoderm, but FOG-1 is expressed in hematopoietic cells as early as in the first yolk sac blood island (Tsang *et al.* 1997; Svensson *et al.* 1999). However, prior studies on older embryos have not included extraembryonic membranes. In E11.5 mouse yolk sac, we found (in immunohistochemistry) very faint FOG-2 expression that grew stronger towards E12.5 (Figure 7) (IV and present data). This may suggest a potential role for this factor in the yolk sac endoderm later in gestation. It is conceivable that as the extraembryonic endoderm becomes unnecessary for the developing embryo, it is programmed to regress rather than merely dying out as a result of a lack of trophic signals. During development, many other tissues are modified by apoptosis (programmed cell death) (Abud 2004). The mouse embryo developing inside the yolk sac may need regression of this extraembryonic membrane in order to weaken it before delivery. However, as the mouse yolk sac structure is so very different from that of other mammals, it

is difficult to interpret the ultimate role of GATA factors in, for example, human extraembryonic endoderm development.

FOG-1 is expressed in mid-gestation gastric endoderm (IV)

The expression pattern of FOG proteins in the definitive endoderm is also still poorly understood. Here we studied by immunohistochemistry and *in situ* hybridization how these GATA cofactors are expressed in mouse stomach epithelium during development. We found that in the pyloric region of the mouse stomach, FOG-1 expression becomes evident at E11.5 and continues to E18.5. In adult mouse gastric endoderm FOG-1 was not expressed. No FOG-2 mRNA or protein, on the other hand, was found in the gastric epithelium during fetal or postnatal development (IV).

The expression of GATA factors in definitive endoderm derivatives is tightly timely and spatially regulated. GATA-4, GATA-5 and GATA-6 are all expressed in early post-gastrulation definitive endoderm, but soon their expression patterns diverge. GATA-6 is specifically needed for lung morphogenesis (Keijzer *et al.* 2001), and cells devoid of GATA-4 cannot contribute to the formation of gastric epithelium (Jacobsen *et al.* 2002). In the mature mouse gastrointestinal tract, these three GATA proteins are expressed in a specific pattern. In the stomach, all three proteins are found, but in different parts of the gastric units; GATA-4 in the neck region, GATA-5 in a subpopulation of neck cells at the transitional region between the fundus and glandular stomach, and GATA-6 in the basal 2/3 of the pits. In the intestine, GATA-4 and GATA-6 are expressed, but in the colon, only GATA-6 can be found (Divine *et al.* 2004).

FOG-1 has traditionally been thought to be the cofactor of GATA-1 that is not expressed in endodermal derivatives (Evans and Felsenfeld 1989). However, the interacting zinc fingers of GATA and FOG proteins are highly conserved in all family members, enabling interaction between either of the FOG proteins and any GATA factor. Correspondingly, *in vitro* studies have shown that FOG-1 is capable of repressing GATA-4- or GATA-6-induced activation of gonadal promoters (Robert *et al.* 2002). Thus it is likely that interaction would also be possible between FOG-1 and other GATA family members, e.g. GATA-4 or GATA-6, in the developing stomach epithelium. The fact that FOG-1 is no longer expressed in gastric epithelium during postnatal life suggests that regulation of GATA activity by FOG-1 is specifically needed during late embryonic development.

GATA-4:FOG interaction is needed for normal gastric development (IV)

In addition to spatially and temporally regulated expression of GATA factors, their specificity is increased by regulation of transcriptional activity by FOG proteins (Svensson *et al.* 1999; Deconinck *et al.* 2000). Previous studies on mice with GATA-4 N-terminal zinc fingers incapable of binding to FOG proteins (*Gata4*$^{ki/ki}$ mice) have shown that cooperation between FOG-2 and GATA-4 is crucial for normal development of the cardiac outlet channel and vasculature formation as well as for sex determination (Tevosian *et al.* 2000; Tevosian *et al.* 2002). However, the developmental problems of *Gata4*$^{ki/ki}$ mice are not as severe as those of *Gata4*$^{-/-}$ mice, indicating that anterior folding of the embryo does not depend on interaction between GATA-4 and FOG proteins. Whether FOG regulation of GATA factors is needed for later endodermal development has not been studied.

We analyzed embryos from *Gata4*$^{ki/ki}$ mice to study how hindering GATA-4:FOG interaction affects the development of definitive endoderm. As *Gata4*$^{ki/ki}$ mice, like *Fog1*$^{-/-}$ and *Fog2*$^{-/-}$ mice, die by E12.5 they offer no possibilities to study the potential effects of GATA:FOG interaction on development after mid-gestation. Thus is it not presumable that changes could be seen in the yolk sac of these mutant mice, since FOG-2 expression in the extraembryonic endodermal cells was found in this study only from E12.5 onwards. However, in the gastrointestinal tract of *Gata4*$^{ki/ki}$ mice, subtle changes in the expression of signaling molecules were already seen at this stage, although the histology appeared normal (IV).

We showed that in *Gata4*$^{ki/ki}$ mice, Sonic hedgehog (Shh), a molecule participating in endodermal-mesenchymal signaling, was abnormally expressed in the distal stomach epithelium (IV). Sonic hedgehog should normally be down-regulated in the hind-stomach by activin-dependent mechanisms, and concomitantly FGF-10 is up-regulated in the mesenchyme adjacent to the distal stomach (Kim *et al.* 2000). Accordingly, we found that FGF-10 expression in the gastric mesenchyme of *Gata4*$^{ki/ki}$ mice was negligible, whereas in the heterozygous littermates the expression patterns were normal. Epithelial-mesenchymal interaction involving Shh and FGF-10 is needed for appropriate differentiation of the glandular epithelium as well as formation of the spleen and pancreas (Apelqvist *et al.* 1997; Ohuchi *et al.* 2000; Aubin *et al.* 2002). As FOG-2 is not expressed in the stomach epithelium, it is likely that the cofactor for GATA-4 in hind-stomach development is FOG-1 (Figure 6).

The inappropriate expression of Shh in the hind-stomach epithelium of *Gata4*$^{ki/ki}$ mice recapitulates that of *Gata4*$^{-/-}$ chimera mice (Jacobsen *et al.* 2002). However, what remains unexplored is whether or not GATA-4 and FOG-1 directly regulate the Shh gene or whether additional factors are required in this pathway. In the first case, the *Shh* gene in the absence of GATA-4 might be regulated by another GATA factor, e.g. GATA-6, the effect of which would not be controlled by FOG-1. However, it can be concluded that GATA-4:FOG-1

interaction lies in the activin-induced TGF-β signaling pathway during the differentiation of gastric epithelium. Problems in this interaction may lie behind some of the developmental disorders of the human gastrointestinal tract.

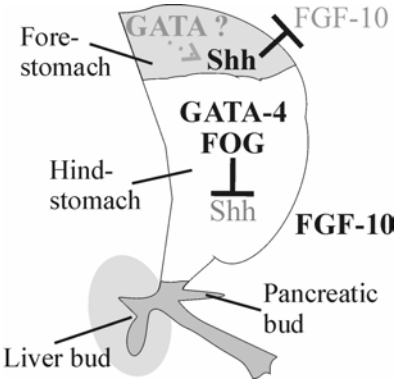


Figure 6. Schematic presentation of GATA-4 and FOG-1 function on gastric development.

GATA-4 is expressed in the hindstomach (white) throughout development, whereas FOG-1 is expression in the hindstomach becomes evident at E11.5. Neither of these factors are expressed in the forestomach (light gray). However, other GATA factors (e.g. GATA-5 or GATA-6) may affect Shh expression in this area.

Sonic hedgehog (Shh) is expressed in the forestomach but in the presence of FOG-1 in hindstomach Shh is down-regulated. Thus Fibroblas growth factor 10 (FGF-10) that cannot be found in the mesencyme of forestomach is expressed in the mesenchyme underlying the hindstomach.

2. GATA factors in germ cell tumors (I–III)

Disturbances in the normal expression of GATA-4 and GATA-6 have been linked to various human tumors. For example, frequent methylation, inhibiting normal transcription of the *GATA4* gene, is seen in colorectal and pulmonary carcinomas (Bai *et al.* 2000; Akiyama *et al.* 2003). GATA-6, on the other hand, has been connected to the regulation of cell proliferation and apoptosis. It induces expression of the anti-proliferation factor p21 and thus cell cycle arrest (Perlman *et al.* 1998; Morrisey 2000; Nagata *et al.* 2000). In vascular smooth muscle cells GATA-6 is down-regulated in response to mitotic signals (Suzuki *et al.* 1996; Walsh and Takahashi 2001). In the primitive endoderm layer of GATA-6-deficient embryos, an elevated amount of apoptosis can be observed (Morrisey *et al.* 1998). However, in colorectal cancer cells the apoptotic pathway induced by non-steroidal anti-inflammatory drugs (NSAIDs) includes down-regulation of GATA-6, indicating that in this endodermal malignancy GATA-6 may protect cells from programmed cell death (Shureiqi *et al.* 2002).

In GCTs, the variable differentiation pattern of activated germ cells is an additional interesting aspect for research. It is presumable that the same factors that determine cell fate during normal development are involved in the diverse differentiation in GCTs. However, little is known about the factors that influence the tissue composition of these tumors. The composition of a tumor strongly influences the prognosis of the disease and thus the differences in molecular pathways in different tumors may offer new strategies for diagnostics and treatment.

Pediatric germ cell tumors (I, II)

The expression of GATA-4 and GATA-6 proteins in pediatric tumors was studied by immunohistochemistry. Other studies have shown that the antibodies used give congruent results with mRNA expression (Ketola *et al.* 2003). In addition, GATA-6 mRNA expression was studied by *in situ* hybridization. Pediatric GCTs in this study are summarized in Table 4a.

Expression of GATA factors in pediatric germ cell tumors (I, II)

Of the nine YSTs included, seven expressed both GATA-4 and GATA-6, one tumor was positive only for GATA-4 and one for GATA-6, as studied by immunohistochemistry. GATA-6 mRNA *in situ* hybridization confirmed the immunohistochemical results. The one EC included (Table 3a, patient 10) expressed GATA-6 but not GATA-4; the serum AFP level at diagnosis was also high in this patient (Table 7). In teratomas, GATA-6 expression was found in normal endodermal-like structures (gut- or lung-type epithelium), whereas GATA-4

was only expressed in immature teratomas by blastematous neural tissue and occasional Goblet cells within columnar epithelium (I, II).

GATA-6 expression in the pediatric teratomas followed the same pattern seen during normal development (Brewer *et al.* 2002). Surprisingly, we also found GATA-6 in immature and mature neural tissue, where no expression has been shown during normal development. This expression was seen on both mRNA and protein levels and it is thus unlikely that it could be a result of antibody crossreactivity (II).

The low level of GATA-4 seen in the endoderm of the teratomas is surprising. It suggests that either the epithelium is more of mature colon rather than intestine type or that other GATA factors are able to induce gut-type differentiation in these tumors. GATA-6 is expressed more widely in mature definitive endoderm, i.e. the lungs and gastrointestinal tract, and its wider expression in teratomas was also expected. The expression of GATA-4 and GATA-6 in neural tissue was surprising, as no role for these factors in neuronal development has been found. One possibility is that the antibody used may also bind to GATA-3. However, crossreactivity has not been seen in any prior study with this antibody, and the protein expression pattern for GATA-6 was congruent with mRNA expression.

We also found that sebocytes in teratomas expressed GATA-6. Normal mouse skin was studied to confirm the result. Indeed, GATA-6-positive nuclei were also found in mouse skin appendages (II). No prior study has revealed GATA-6 expression in vertebrate ectodermal derivatives. However, in the nematode *Caenorhabditis elegans*, the GATA factors ELT-1 and ELT-2 are expressed in the ectoderm (Gilleard *et al.* 1999; Gilleard and McGhee 2001).

Table 7. GATA-4 and GATA-6 expression in pediatric germ cell tumors.

Of the nine yolk sac tumors studied, one was devoid of GATA-4 (pt. no. 4) and one was devoid of GATA-6 (pt. no. 8). For comparison, AFP immunohistochemistry is shown. The one embryonal carcinoma (EC) included in the studies expressed GATA-6 but no GATA-4 or AFP immunoreactivity was detected.

Pt. no.	Age at diagnosis (y) and sex	Tumor			Marker expression		
		Localization	Histology	Metastasis	GATA-6	GATA-4	AFP
1	1.0 M	Testis	YST		+	+	+
2	4.0 M	Testis	YST		+++	+	+
3	1.3 M	Pelvis	YST	Lung	++	+	-
4	1.5 F	Pelvis	YST		+++	-	+
5	1.6 F	Pelvis	YST		+++	+++	+
6	1.6 M	Pelvis	YST		+++	+++	+
7	2.1 F	Pelvis	YST	Lung	++	++	+
8	2.5 F	Pelvis	YST		-	+++	++
9	14.5 F	Ovary	YST	Omentum	+++	+	+
10	11 F	Vagina	EC		+++	-	-

Abbreviations: M, male; F, female; YST, yolk sac tumor; EC, embryonal carcinoma; AFP, α-fetoprotein.

Role of GATA factors in pediatric germ cell tumors (I, II)

This study showed that GATA-4 and GATA-6 are both expressed in pediatric YSTs, as expected on the basis of the crucial role of these factors in normal extraembryonic endoderm development (Soudais *et al.* 1995; Morrisey *et al.* 1998). These results add to molecular similarities between murine yolk sac and malignant YST endoderm. In addition, it can be speculated that these factors also play a role in abnormal differentiation towards a yolk sac phenotype in activated germ cells. GATA-4 and GATA-6 are both capable of inducing expression of markers of extraembryonic endoderm differentiation if over-expressed in mouse ES cells (Fujikura *et al.* 2002). As the number of tumors studied was limited and GATA expression was relatively constant, no differences in the clinical progress of the patients (Table 4a) were observed (I, II).

In teratomas, GATA-4 was detected in endodermal components at a very low level whereas GATA-6 was more widely expressed in these tissues (I, II). In mice, GATA-4 seems to be needed for differentiation of gastric-type epithelium (Jacobsen *et al.* 2002), but it is not known whether GATA-5 or GATA-6 could overcome the loss of GATA-4 in intestinal differentiation. Although teratomas have traditionally been used as models of embryonic differentiation, they are neoplasms, and future studies on normal tissues are needed to clarify whether this finding possibly reflects a normal developmental back-up system.

Neural expression of GATA-4 and GATA-6 must also be considered in the context of potentially underlying, unknown aberrations of cellular function. In abnormal cells, it is possible that GATA-4 or GATA-6, with other factors that normally interact with GATA-3, can induce neuronal development. On the other hand, aberrant GATA-4 expression in cells programmed to a neural fate may hinder full differentiation of these cells. GATA-4 is expressed in granulosa cells and gastric epithelial cells in their proliferative but not fully differentiated stage (Anttonen *et al.* 2003; Divine *et al.* 2004).

Our finding that GATA-6 is expressed by sebocytes in teratomas (II) suggests a previously unknown role for this transcription factor. Sebocytes in mammals regulate androgen homeostasis in the skin (Fritsch *et al.* 2001), and thus it could be that GATA-6, a regulator of other hormonal events, also has a function in these cells. Recently, new data has emerged showing that GATA-6 is indeed expressed in normal human pilosebacous units and can induce the transcription of steroidogenic enzymes expressed in these cells. Thus, manipulating GATA-6 expression or function may offer a new means of treatment of androgen-dependent dermopathies such as acne, hirsutism and alopecia (Ho *et al.* 2004).

Although the expression of neither GATA-4 nor GATA-6 was pathognomonic to malignant yolk sac tissue, they can still offer additional tools for diagnostics. Gut- and lung-type tissue

in teratomas is easy to recognize morphologically. Immature neural tissue is also morphologically distinct from YST endoderm, but differentiation between mature and immature neural tissues may be difficult. GATA-4 and GATA-6 immunohistochemistry may assist in the recognition of potentially more harmful tissue in mixed GCTs.

Adult ovarian germ cell tumors (III)

Expression of GATA factors (III)

The adult ovarian GCTs in this study included six YSTs, eight DGs and five immature teratomas. Two of the teratomas also contained CC, EC or DG components. The expression of GATA-4 and GATA-6 in these tumors was studied by immunohistochemistry, using the same antibodies as in the studies of the pediatric material.

Both GATA-4 and GATA-6 were expressed in the malignant tumor cells of all six YSTs. Surprisingly, five of the eight DGs also expressed GATA-4, but no GATA-6 was detected in this tumor type. We found no correlation between any tumor characteristic and GATA-4 staining in DGs, although there was a tendency towards lower GATA-4 expression in higher grade tumors. Teratomas expressed GATA-4 and GATA-6 according to the normal expression pattern of these factors. Both factors were found in endodermal structures. Embryonal carcinoma and CC components in mixed GCTs were devoid of GATA-4 or GATA-6 immunoreactivity (Figure 8 and Table 8) (III).

None of the YSTs or DGs expressed the GATA cofactor FOG-2 in the same tissues as GATA-4 or GATA-6. However, FOG-2 was expressed with GATA-4 and GATA-6 by immature neural cells in one teratoma, as well as in another teratoma devoid of GATA expression. The tumors studied did not contain any skin-type differentiation where sebocytes would be present and thus the GATA-6 expression seen in the sebocytes of the pediatric GCT could not be confirmed.

Role of GATA factors in germ cell tumor development (III)

The expression of GATA-6 exclusively in endodermal components of GCTs is not surprising considering the normal expression pattern of this transcription factor. However, as GATA-6 during normal development is crucial for cells to adopt an endodermal fate, and no GATA-6 was observed in any non-endodermal tissue type in GCTs, it can be speculated that GATA-6 in activated germ cells is needed for the induction of endoderm differentiation. In particular, the strictly different expression of GATA-6 in YSTs compared with DGs implies that this

factor may play a role in the differentiation pathway of activated PGCs. However, more extensive functional studies are needed to verify this observation.

Interestingly, fetal rat ovarian germ cells as well as neonatal rat oocytes express GATA-6 (LaVoie *et al.* 2004). Expression of GATA-6 has been connected with proliferation and in many tissues it is down-regulated concomitantly with differentiation (Perlman *et al.* 1998; Morrisey 2000; Nagata *et al.* 2000). However, no GATA-6 expression has thus far been found in normal human oocytes (Laitinen *et al.* 2000; Vaskivuo *et al.* 2001), although no studies of GATA-6 expression in fetal human ovaries have been published. If GATA-6 is present in human PGCs or fetal oocytes, analogously to rat oocytes, it could be that loss of GATA-6 in these cells contributes to malignant transformation and germinoma formation.

Potential GATA downstream effectors in adult ovarian GCTs (III)

To clarify the roles of GATA-4 and GATA-6 in the development of GCTs we analyzed how ovarian GCTs express putative GATA target genes involved in normal extraembryonic endoderm development. The factors studied included hepatocyte nuclear factor 4 (HNF-4), bone morphogenetic factor 2 (BMP-2) and Indian hedgehog (Ihh), which are all expressed in the yolk sac and play essential roles in endoderm formation. Specifically, the phenotype of *Hnf4*$^{-/-}$ mice is identical to that of *Gata6*$^{-/-}$ mice, and GATA-6 has been shown to be able to bind to and activate the *Hnf4* gene promoter (Duncan *et al.* 1997; Morrisey *et al.* 1998). Bone morphogenetic factor 2, again, is expressed by the primitive endoderm (Coucouvanis and Martin 1999), and BMP signaling is essential for normal development of the yolk sac. The expression patterns of these factors in ovarian GCTs are summarized in Table 8 and representative images of immunohistochemical staining are shown in Figure 8.

We found that like GATA-6, HNF-4 expression was restricted to endodermal tissues in teratomas and YSTs. This suggests that HNF-4 also plays a role in determining the differentiation pathway of activated PGCs and that functional HNF-4 protein may be needed for malignant yolk sac-type differentiation. The expression patterns of BMP-2 and Ihh were more divergent. We detected BMP-2 in all YSTs and endodermal as well as cartilage/bone structures in teratomas. Indian hedgehog was detected in malignant endodermal cells of all but one YST and in benign endoderm in teratomas. We found that BMP-2 was also expressed in three of the eight DGs included, and Ihh in two of these tumors.

All GCTs in this study, apart from teratomas, were pure tumors of one cell type. It can thus be stated that the secreted signaling molecules BMP-2 and Ihh were indeed expressed by the tumor cells themselves, and not by supporting tissue. In teratomas, however, it is possible that the immunohistochemical staining of BMP-2 or Ihh around endodermal cells reveals the

presence of secreted protein from nearby mesoderm, thus reflecting the normal tissue-tissue interaction seen during endodermal differentiation.

Table 8. Summary of marker expression in adult ovarian GCTs.

Pat. no.	Dg.	Stage	Status (yrs after dg.)	Immunohistochemistry					
				GATA-4	GATA-6	FOG-2	HNF-4	BMP-2	Ihh
1	YST	IIIC	Died (0.17)	++	++	-	+	+	+
2	YST	IC	CR (10)	+++	++	-	++	+	+
3	YST	IC	CR (3.4)	+++	++	-	++	++	-
4	YST	IIIA	CR (9.2)	+++	++	-	++	+	+
5	YST	IA	CR (7.0)	+++	++	-	++	++	+
6	YST	IC	Died (0.17)	+++	+++	-	++	+	+
7	DG	IIB	CR (13.8)	-	-	-	-	++	-
8	DG	IIIC	Died (0.5)	-	-	+	-	-	-
9	DG	IIIC	CR (0.6)	+/-	-	-	-	-	-
10	DG	IB	CR (10.2)	+	-	-	-	-	-
11	DG	IIA	CR (9.4)	+	-	-	-	-	-
12	DG	IC	CR (9.75)	++	-	-	-	++	-
13	DG	IC	CR (9.3)	++	-	-	-	-	+/-
14	DG	IC	CR (7.6)	+++	-	-	-	++	+
15	Mixed	IA	Died (0.67)	-	-	-	+	+	-
16	Mixed	IIIC	PR (0.58)	-	-	-	-	-	-
17	TI	IA	CR (11)	-	-	++	*	-	-
18	TI	IC	Died (1.4)	++	++	++	++	++	++
19	TI	IC	CR (2.8)	++	++	-	-	-	+

Abbreviations: YST, yolk sac tumor; DG, dysgerminoma; TI, immature teratoma; yrs, years; Dg./dg., diagnosis; CR, complete response; PR, Partial response; *, cytoplasmic HNF-4 staining.

As GCTs are extremely rare, acquiring fresh tumor samples and creating cell lines for functional studies is difficult. However, some conclusions can be drawn from the study of factors that are members of known signaling pathways. The expression of these putative GATA target genes in YSTs suggests that the GATA proteins (especially GATA-6) in the tumors are functional. As no FOG-2 immunoreactivity was detected, GATA modulation by this factor was not evident. Whether the lack of this regulation plays a role in YST patophysiology remains to be studied.

The expression of factors essential for normal development in endodermal tissues suggests that the differentiation of germ cells into various endodermal types follows the normal molecular pathways. However, as BMP-2 and GATA-4 are to some extent expressed in

Figure 8 Expression of endodermal markers in ovarian germ cell tumors and seminoma cell line.

All factors but FOG-2 are expressed in yolk sac tumors (YST, left collumn). Five of eight dysgerminomas (DG) (second collumn from left) express GATA-4, three BMP-2 and two Ihh – rest of these tumors showed now immunoreactivity for the studied factors (third collumn from left). Teratomas (TE) expressed the studied factors accordin to their normal expression pattern. Endodermal components (fourth collumn frmon left) and cartilage (fifth collumn from left) are shown. Seminoma cell line NCC-IT (right collumn) was negative for all factors in immunocytochemistry.

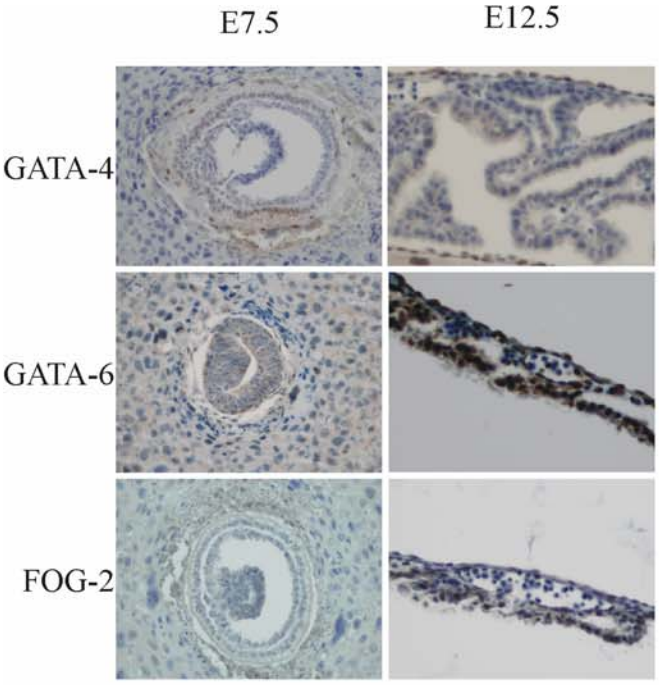
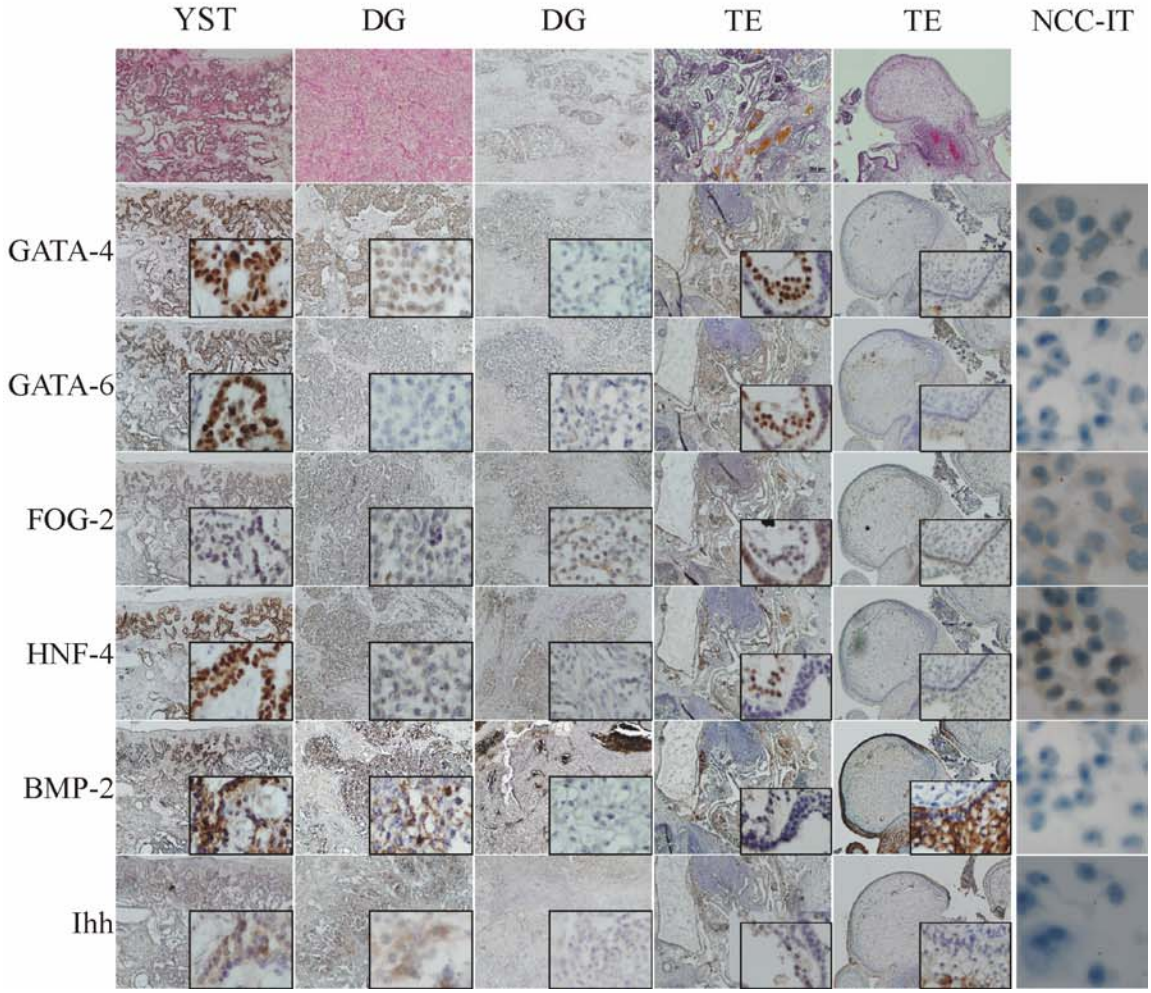


Figure 7 GATA expression in extraembryonic endoderm.

Immunohistochemsitry on mouse embryos at E7.5 and E12,5 are shown.

GATA-4 (upper row) and GATA-6 (middle row) are expressed in both early and midgestation extraembryonic endoderm.

FOG-2 (lowest row) is not expressed in early embryo but expression is turned on in midgestation.



germinomatous components of GCTs, it is apparent that these factors are not sufficient to induce endoderm differentiation in activated germ cells. In particular, differentiation towards the YST phenotype possibly requires expression of GATA-6, HNF-4, and probably additional factors. All in all, it can be speculated that normal pathways of endodermal differentiation are in action and potentially needed for the malignant endoderm differentiation that is seen in YSTs (Figure 9). Additionally, similar to GATA-6, HNF-4 may serve as a diagnostic tissue marker of endodermal differentiation and this offers new possibilities for distinguishing malignant yolk sac endoderm in mixed GCTs.

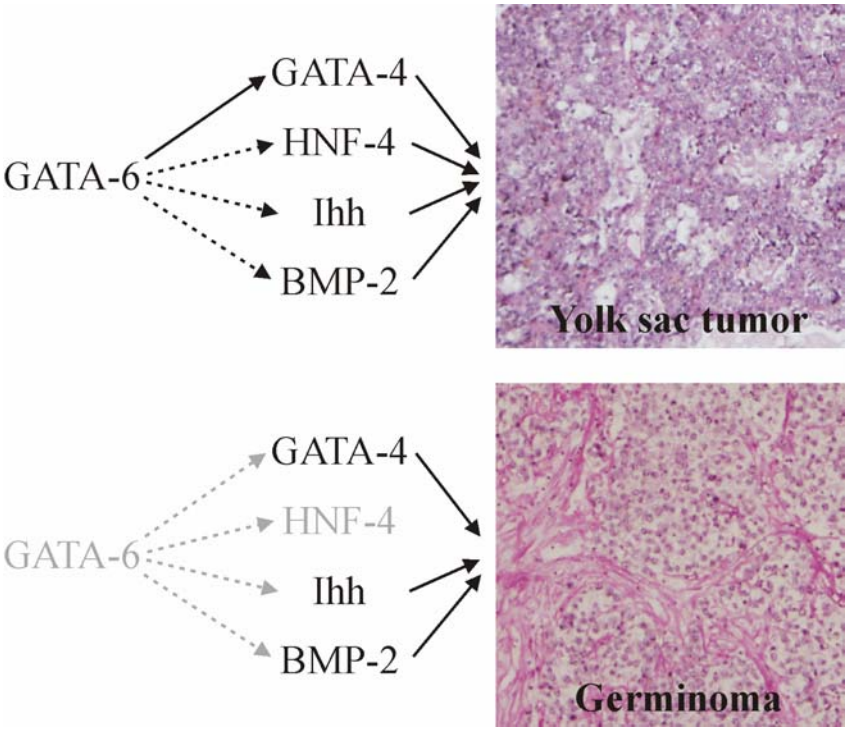


Figure 9. Schematic representation of GATAs and their downstream targets in the differentiation of malignant germ cells.

In the presence of GATA-6, additional factors promoting visceral endoderm development are expressed and the tumor differentiates to malignant yolk sac endoderm. If no GATA-6 is present, the cells maintain their pluripotency, giving rise to a germinoma.

Germ cell tumor cell lines (I)

In addition to tumor samples, GATA expression was studied in five human GCT cell lines. Messenger RNA from four YST cell lines was used for Northern hybridization and a human seminoma cell line was used for immunocytochemistry. GATA-4 mRNA was expressed in all YST cell lines, confirming the results of immunohistochemistry in YST samples (I). The NCC-IT seminoma cell line was studied by immunocytochemistry for the same markers as in

the adult ovarian GCTs, i.e. GATA-4, GATA-6, FOG-2, HNF-4, BMP-2 and Ihh. No immunoreactivity as regards any of these factors was detected (present data, Figure 8).

Potential markers for germ cell tumor diagnostics (I–III)

Germ cell tumors are heterogeneous and complex. Along with other aspects, the composition of the tumors drastically affects the prognosis and need for chemotherapy. However, histological differentiation between different tissue types may be difficult. In particular, mixed tumors containing malignant tissues within otherwise less harmful tumors cause problems. The traditionally used oncodevelopmental markers AFP and hCG are secreted proteins and thus play an important role in serological diagnostics and follow-up of patients. However, as regards immunohistochemical diagnostics, these factors are not highly reliable and easy to work with. Thus, new markers are needed.

In this study, three transcription factors, i.e. nuclear proteins, were differentially expressed according to the composition of the GCTs. GATA-4 was expressed in all but one of the YSTs studied, in malignant germ cells of DGs (five of eight cases) and, surprisingly, in immature neural cells of pediatric teratomas. Thus, GATA-4 might serve as a marker of malignant cells in mixed germ cell tumors, although it is not pathognomonic to any selected tumor type. In addition, GATA-4 immunohistochemistry might aid in differentiating immature neural cells among more mature teratoma components. GATA-6 and HNF-4, factors crucial for early endodermal development, were also expressed in all YSTs studied. However, pluripotent cells in DGs and the seminoma cell line did not express any GATA-6 or HNF-4. Recently it has been shown that a marker of pluripotency, Oct-3/4, is expressed in germinomatous tissues and EC components in GCTs but not in malignant yolk sac endoderm (Cheng *et al.* 2004). Together, these factors (Table 9) may serve as immunohistochemical markers of malignant yolk sac differentiation in GCTs.

Table 9. Differential expression of nuclear antigens in various GCTs.

	YST	DG	EC	Teratoma
GATA-4	+++	++	- *	Endoderm Immature neural tissue
GATA-6	+++	-	+/- *	Endoderm Neural tissue Sebocytes
HNF-4	+++	-	-	Endoderm
Oct-3/4	-	+++	+++	-

Abbreviations: YST, yolk sac tumor; DG, dysgerminoma; EC, embryonal carcinoma.

*Expression data in EC is based on only one pediatric pure EC and one adult ovarian mixed GCT containing EC components.

Conclusions and Future Prospects

1. The results presented herein demonstrate that the transcription factors GATA-4 and GATA-6, which are indispensable for differentiation of extraembryonic endoderm, are expressed in the yolk sac throughout embryonic development. Thus these factors may be needed not only for establishment but also for maintenance of visceral endoderm phenotype. GATA cofactors, FOG proteins, are expressed in a timely and spatially specific manner in the developing endoderm. The studies on germ cell tumors show that GATA-4 and GATA-6 are expressed in the malignant endoderm of yolk sac tumors and in endodermal components of teratomas.

2. Other markers of endodermal differentiation, which can be considered to be downstream effectors of GATA-4 and GATA-6, are also expressed in GCTs according to the normal developmental pattern. It can thus be concluded that the two GATA factors indeed are functional and active proteins. Furthermore, since all YSTs expressed all endodermal markers studied, it can be postulated that normal pathways present in yolk sac development may be active in yolk sac tumors. Since some DGs expressed GATA-4 and (less commonly) other markers, but no GATA-6 or HNF-4, normal pathways of yolk sac development may be needed for yolk sac differentiation of primordial germ cells. Most interestingly, HNF-4, a direct and crucial target of GATA-6 in extraembryonic endoderm development, is expressed in the endodermal components of GCTs, indicating that this factor also plays a role in determining the endodermal fate of activated germ cells.

3. Since GATA-6 and HNF-4 were not expressed in malignant germ cells of dysgerminomas, these two factors could serve as clinical markers of malignant yolk sac tissue in GCTs. GATA-4 could also serve as a marker in the diagnostics of GCTs, recognizing both yolk sac differentiation and immature neural tissue. However, future studies on larger tumor materials are needed to confirm this concept.

4. Interaction between GATA-4 and a FOG protein, presumably FOG-1, is needed for normal differentiation of gastric epithelium and interaction with the underlying mesoderm.

These studies are mainly based on expression data. Clarifying the expression of GATA-4 and GATA-6 in GCTs has confirmed the alleged similarity between malignant YSTs and normal primitive endoderm. The results offer new diagnostic opportunities as regards the complex group of GCTs. In addition, it is tempting to speculate that expression of GATA-6 and HNF-4 in YSTs and the absence of these factors in germinomas would account for the higher malignancy of YSTs. Thus pharmacological manipulation of GATA factors in GCT patients is an interesting concept as regards better treatment strategies.

Well described data on expression patterns of different factors, combined with existing functional data, create a firm basis for speculation concerning the molecular pathways involved in normal and malignant differentiation. However, future studies are needed to verify the role of GATA factors in endodermal differentiation. Cell studies need to be utilized to clarify more profoundly how GATA-4:FOG interaction affects the differentiation of gastric epithelium at a molecular level. The available GCT cell lines are also of interest as regards elucidation of how the differential expression of GATA-6 and HNF-4 affects the fate of malignant germ cells. The role of GATA factors and their interaction with FOG proteins in developmental malformations of the gastrointestinal tract should also be examined.

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