Biology and Molecular Markers of Malignant Gonadal Germ Cell Tumors

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ACADEMIC DISSERTATION

To be publicly discussed with permission of the Medical Faculty of the University of Helsinki, in the Seth Wichmann auditorium at the Department of Obstetrics and Gynaecology, Helsinki University Hospital, Haartmaninkatu 2, Helsinki on October 16th, 2009, at 12 noon

Helsinki 2009
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ISBN 978-952-10-5745-8 (PDF)

http://ethesis.helsinki.fi

Helsinki University Print 2009
Accept the failures as opportunities for growth, 
get excited by the successes. 
Enjoy the journey.

- Raymond Floyd -
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Abstract

Germ cell tumors occur both in the gonads of both sexes and in extra-gonadal sites during adolescence and early adulthood. Malignant ovarian germ cell tumors are rare neoplasms accounting for less than 5% of all cases of ovarian malignancy. Testicular cancer, in contrast, is the most common malignancy among young males. Most patients survive the disease. Prognostic factors of gonadal germ cell tumors include histological classification, clinical stage, size of the primary tumor and residua, and levels of tumor markers.

Germ cell tumors most likely develop from early primordial germ cells; oocytes or gonocytes. In the testis a common tumor precursor, the carcinoma in situ (CIS) cell, was recognized in 1972 by Skakkebaek. Despite their common origin, germ cell tumors include heterogeneous histological subgroups. The most common subgroup includes germinomas (ovarian dysgerminoma and testicular seminoma); other subgroups are yolk sac tumors, embryonal carcinomas, immature teratomas and mixed tumors. Factors behind their differential development are still poorly known.

Pathohistological diagnosis of germ cell tumors is based on histological evaluation and distinctive immunohistological markers. However, differential diagnosis is challenging, as some of the tumors may be confused with other tumor types. Correct knowledge of the histological type of the tumor is essential, as prognosis and treatment are dependent on the tumor type.

The serum tumor markers α-fetoprotein (AFP) and the β-subunit of human chorionic gonadotropin (hCGβ) are used in diagnosis and follow-up. Concentrations of AFP are elevated in yolk sac tumors whereas those of hCGβ are elevated in choriocarcinomas. However, only a subset of gonadal germ cell tumors express these serum markers. Measurements of carcinoma antigen 125 (CA 125) is used in diagnosis, prognosis and follow-up of ovarian epithelial tumors. However, its value in the prognosis of ovarian germ cell tumors is unknown.

The peak incidence of malignant ovarian germ cell tumors occurs soon after puberty. Thus, gonadal steroids have been speculated to play a role in tumor development and progression. Estrogen signalling is mediated via estrogen receptors (ERs) α and β. These belong to the nuclear receptor superfamily, and various co-regulators modify their actions. The co-activator small nuclear ring finger protein 4 (SNURF/RNF4) is one of these co-regulators. In testicular germ cell tumors ERβ and SNURF are down-regulated. However, the role of ERs in ovarian germ cell tumors is unknown.

Given that the origin of germ cell tumors is most likely primordial germ cells, pluripotent factors are of interest in the development of these tumors. The transcription factor Oct-3/4 (POU5F1) is expressed in embryonic stem cells and primordial germ cells and it is involved in maintenance of pluripotency. Activator protein-2 gamma (AP-2γ) is required for early post-implantation development. Both Oct-3/4 and AP-2γ are expressed in fetal germ cells as well as in testicular CIS cells. Estradiol induces AP-2γ expression in prostate cancer cells. Thus, estradiol signalling might regulate AP-2γ expression in germ cell tumors.
Many genes are involved in germ cell and gonadal development and may thus play a role in germ cell tumorigenesis. Of the six GATA transcription factors, GATA-4 and GATA-6 are involved in gonadal differentiation and function. In addition, GATA-4 and -6 as well as their downstream target genes (HNF-4, BMP-2, Ihh) are essential for normal yolk sac development. GATA-4 and its interaction with cofactor FOG-2 are essential for appropriate GATA-4 function in testis development. In addition to GATA-4, its target genes, including SF-1, AMH and INH-α, are involved in normal testicular differentiation, development and function.

The purpose of this study was to define novel diagnostic and prognostic factors associated with malignant gonadal germ cell tumors. Another aim was to shed further light on the molecular mechanisms regulating gonadal germ cell tumorigenesis and differentiation by studying the roles of GATA transcription factors, pluripotent factors Oct-3/4 and AP-2γ, and estrogen receptors.

In the present study, elevated preoperative levels of serum CA 125 were associated with poor outcome in malignant ovarian germ cell tumor patients. In addition, age above 30 years and residual tumor were associated with more adverse outcomes.

In the fetal testis, GATA-4 was expressed in a subpopulation of early fetal gonocytes at the 15th gestational week. However, GATA-4 expression was down-regulated during further embryogenesis and absent in adult testicular germ cells. In testicular CIS cells GATA-4 was heterogeneously expressed. Thus, GATA-4 expression in early fetal germ cells and in testicular tumor precursors provides evidence for the fetal origin of testicular germ cell tumors. The essential endodermal gene GATA-4 was expressed in ovarian dysgerminomas and in testicular seminomas. In addition, their endodermal (HNF-4, BMP-2, Ihh) and gonadal (SF-1) target genes were expressed in these tumors. Thus, expression of GATA-4 and its target genes displays evidence for a functional role of GATA-4 in the germinoma subgroup of germ cell tumors.

In the ovary, expression of AP-2γ and Oct-3/4 was positive in most of the dysgerminomas, whereas the majority of other subtypes were negative. Thus, AP-2γ and Oct-3/4 are of value in differential diagnosis of ovarian germ cell tumors. In addition, their dysgerminoma-specific expression provides evidence for the primordial nature of this subgroup of germ cell tumors.

All evaluated malignant ovarian germ cell tumor subtypes (dysgerminomas, yolk sac tumors, immature teratomas) expressed both ERα and ERβ, and their co-activator SNURF. The role of estrogen regulation of dysgerminomas was studied by using a human germinoma-derived cell line (NCC-IT). Stimulation with estradiol significantly increased the expression of both ERs and SNURF in a dose- and time-related manner. In addition, the effect of estradiol was counteracted by an anti-estrogen (ICI 182,780).

In conclusion, this study revealed the prognostic value of CA-125 in malignant ovarian germ cell tumors. In addition, several novel markers for histological diagnosis were defined (GATA-4, GATA-6, AP-2γ, Oct-3/4). Moreover, these factors may be involved in gonadal germ cell tumorigenesis and differentiation. In early fetal development the transcription fac-
tor GATA-4 was expressed in gonocytes in addition to CIS cells, and may thus have a role in fetal testicular CIS development. In addition to testicular CIS cells, GATA-4 was expressed in both types of gonadal germinoma: thus it may play a role in the differentiation of these tumor subtypes. In addition to GATA-4, the pluripotent factors Oct-3/4 and AP-2γ were expressed in dysgerminomas: thus they could be used in differential diagnosis. This study provided evidence of steroid hormone regulation in ovarian germ cell tumors. MOGCTs expressed estrogen receptors and their co-receptor SNURF. In addition, ER expression was up-regulated by estradiol stimulation. Thus, the gonadal steroid hormone burst in puberty may play a role in germ cell tumor development in the ovary.
Original Publications


In addition, some unpublished data are presented (referred to in the text by V).

Study I has been used in the Doctoral Thesis by Susanna Mannisto, University of Helsinki, 2005.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AFP</td>
<td>Alpha fetoprotein</td>
</tr>
<tr>
<td>AMH/MIS</td>
<td>Antimüllerian Hormone/Müllerian-Inhibiting Substance</td>
</tr>
<tr>
<td>AP-2γ</td>
<td>Activator protein 2 gamma</td>
</tr>
<tr>
<td>BMP</td>
<td>Bone morphogenic protein</td>
</tr>
<tr>
<td>ERα</td>
<td>Estrogen Receptor alpha</td>
</tr>
<tr>
<td>ERβ</td>
<td>Estrogen Receptor beta</td>
</tr>
<tr>
<td>ES cell</td>
<td>Embryonic stem cell</td>
</tr>
<tr>
<td>CA 125</td>
<td>Carcinoma antigen 125</td>
</tr>
<tr>
<td>CIS</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>DAX</td>
<td>Dosage-dependent sex reversal; Adrenal hypoplasia congenital; X-chromosome</td>
</tr>
<tr>
<td>FOG-2</td>
<td>Friend of GATA-2</td>
</tr>
<tr>
<td>FOXL2</td>
<td>Forkhead box L2</td>
</tr>
<tr>
<td>hCGβ</td>
<td>β-subunit of human chorionic gonadotropin</td>
</tr>
<tr>
<td>HNF-4</td>
<td>Hepatocyte nuclear factor 4</td>
</tr>
<tr>
<td>Ihh</td>
<td>Indian hedgehog</td>
</tr>
<tr>
<td>INHα</td>
<td>Inhibin-α</td>
</tr>
<tr>
<td>iPS cell</td>
<td>Induced pluripotent stem cells</td>
</tr>
<tr>
<td>MOGCT</td>
<td>Malignant ovarian germ cell tumor</td>
</tr>
<tr>
<td>Oct-3/4</td>
<td>Octamer binding protein</td>
</tr>
<tr>
<td>PGC</td>
<td>Primordial germ cell</td>
</tr>
<tr>
<td>PLAP</td>
<td>Placental-like alkaline phosphatase</td>
</tr>
<tr>
<td>RSPO1</td>
<td>R-spondin 1</td>
</tr>
<tr>
<td>SF-1</td>
<td>Steroidogenic factor 1</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SNURF</td>
<td>Small nuclear ring finger protein</td>
</tr>
<tr>
<td>SRY</td>
<td>Sex determining region of Y-chromosome</td>
</tr>
<tr>
<td>TDS</td>
<td>Testicular dysgenesis syndrome</td>
</tr>
<tr>
<td>TGCT</td>
<td>Testicular germ cell tumor</td>
</tr>
<tr>
<td>WT1</td>
<td>Wilm’s tumor factor 1</td>
</tr>
</tbody>
</table>
Introduction

At the beginning of a new life a sperm cell fuses with an oocyte. This fusion of germ cells creates a population of dividing cells. These dividing embryonic cells form a population – the inner cell mass of the blastocyst. These cells are pluripotent, with the capability of forming all the cell types within the human body. As embryogenesis progresses these undifferentiated cells lose their pluripotency and differentiate into specific cells forming the various tissues and organs. However, germ cells maintain their pluripotency, and form new sperm cells and oocytes with the potential to create new life once again.

The primordial gonad, in which the germ cells develop, is unique among all organs because of its bi-potential nature during the first weeks of embryogenesis. Both the male and female gonads develop from the same uniform primordial gonad. In humans sex determination is genetically regulated by the presence of the Y chromosome in the male. The secondary sex characteristics develop through orchestrated complex cascades leading to gonadal differentiation either into a testis or an ovary. Initiation of the human male pathway depends on gonadal expression of the Y-linked gene, \textit{SRY}. Ten percent of people showing partial or complete sex reversal carry mutations in \textit{SRY} (Hawkins et al. 1992b).

Tumors arising from a germ cell lineage are called germ cell tumors. While these tumors develop during early embryogenesis from primordial germ cells, pluripotent factors play a key role in their formation. Prolonged expression of pluripotent factors (Oct-3/4, AP-2) in germ cells may lead to maturation defects of these cells and activation of growth factors, which in turn promote cell proliferation.

The aim of the present work was to elucidate the molecular mechanisms behind germ cell tumor formation and differentiation. Identification of the factors responsible for the survival of primordial germ cells may also be a crucial for understanding ovarian folliculogenesis and testicular spermatogenesis. Work in the field of germ cell tumors is challenging, as these tumors are rare, but at the same time they are fascinating because of their close relationship with pluripotent stem cells.
Review of the Literature

1. Germ cells

1.1 Inner cell mass and embryonic stem cells

Pluripotency refers to the ability to generate all differentiation lineages, whereas self-renewal is described as infinite cell division maintaining stem cell characteristics. Self-renewal and pluripotency are characteristics of embryonic cells. Dividing embryonic cells form a morula at the 16-cell stage. This further develops into a blastocyst, where the inner cell mass (ICM) is surrounded by trophoectoderm cells (Figure 1). Embryonic stem cells (ES cells) are derived from the ICM population, with pluripotent and self-renewing characteristics. They are able to generate all differentiated cell lineages; endodermal, mesodermal and ectodermal. Recently even germ cells have been derived from mouse and human ES cells (Hubner et al. 2003, Toyooka et al. 2003, Clark et al. 2004).

1.2 Factors essential for maintenance of pluripotency and self-renewal

Pluripotency is maintained in embryonic stem cells by the actions of various genes. OCT-3/4 (POU5F1), NANOG and SOX2 are factors regulating the maintenance of pluripotency in ES cells (Niwa et al. 2000, Mitsui et al. 2003). Moreover, OCT-3/4, in addition to KLF4, C-MYC, and SOX2, is essential in generating induced pluripotent stem cells (iPS cells) from mouse and human terminally differentiated cells (Takahashi et al. 2007, Lowry et al. 2008, Nakagawa et al. 2008). iPS cells are similar to ES cells in morphology, proliferation and pluripotency. They show enormous promise for stem cell research and clinical therapeutics without the ethical and legal issues associated with embryonic stem cells. These cells have the potential to generate patient-specific cell types for cell replacement therapies. Potential target cells include pancreatic beta cells in cases of diabetes, myocardial cells after myocardial infarction and motor neurons in cases of spinal cord injuries. In addition to various cell types even adult mice have been generated from iPS cells (Boland et al. 2009).
Figure 1. A–G. Development and migration of primordial germ cells. **A.** A fertilized embryo has formed a 16-cell morula by day 4 after fertilization. **B.** By the 5th day after fertilization the inner cell mass is visible, surrounded by trophoblasts. **C.** - **D.** On the 9th day the bi-laminar germ disc is composed of epiblast and hypoblast (gray cells) and the amniotic cavity is forming within the epiblast (white cells). **E.** Primordial germ cells (black circles) originate within the epiblast during the 2nd week of development. **F.** Primordial germ cells migrate into the yolk sac at 4 to 6 weeks of development and **(G)** into the dorsal body wall at 6 to 12 weeks, inducing the formation of genital ridges. Modified from Larsen (1998).
The protein OCT-3/4 belongs to the subgroup of octamer-binding proteins, which bind by the POU domain to the promoter and enhancer regions of various genes. Human OCT-3/4 is encoded by a homeobox-containing gene, POU5F, which is located on chromosome 6. During early embryogenesis OCT-3/4 is highly expressed in the ICM (Hansis et al. 2000), being essential for ES cell self-renewal. Loss of Oct-3/4 is lethal in mice because of lack of ICM formation (Nichols et al. 1998). Later in development OCT-3/4 is present in early primordial germ cells (PGCs), gonocytes and oogonia (Scholer et al. 1989, Rajpert-De Meyts et al. 2004). One of the putative target genes of OCT-3/4 is the stem cell-specific growth factor FGF-4, which is associated with human non-seminomatous testicular tumors (Suzuki et al. 2001, Wang et al. 2003). In ovarian germ cell tumors, OCT-3/4 is expressed in dysgerminomas, gonadoblastomas and in some yolk sac tumors (Hoei-Hansen et al. 2007). In addition, testicular seminomas, embryonal carcinomas, teratomas, yolk sac tumors and carcinoma in situ (CIS) also express OCT-3/4 (Hoei-Hansen et al. 2007).

Members of the DNA-binding transcription factor AP-2 family (AP-2α -β, -γ) play important roles in the development and differentiation of the neural tube, neural crest, skin, heart and urogenital tissues (Hilger-Eversheim et al. 2000). Activator protein-2γ is coded by the TFAP2C gene, located on chromosome 20, and it is required for early post-implantation development (Auman et al. 2002). In mice, loss of Ap-2γ is lethal as a result of malformation of extraembryonic tissues (Auman et al. 2002).

In human embryogenesis AP-2γ has a role in germ cell development (Pauls et al. 2005). In fetal ovary, AP-2γ is expressed in oogonia from the 9th week, but it is not expressed later in oocytes (Hoei-Hansen et al. 2004) (Table 1). In fetal testis AP-2γ is expressed in gonocytes from the 10th until the 22nd week of gestation (Hoei-Hansen et al. 2004) (Table 2). However, it is not detected in spermatogonia of adult testes. In germ cell tumors AP-2γ has been detected in ovarian dysgerminomas and gonadoblastomas (Hoei-Hansen et al. 2007). Moreover, testicular CIS and seminomas (Pauls et al. 2005) as well as embryonal carcinomas, gonadoblastomas and to lesser extent teratomas express AP-2γ (Hoei-Hansen et al. 2004). In addition, AP-2γ is associated with other forms of neoplasia, particularly breast cancer but also with advanced stage epithelial ovarian cancer (Odegaard et al. 2006). One of the gonadal target genes for AP-2γ is c-KIT, a tyrosine kinase receptor for stem cell factor. C-KIT is involved in the migration of PGCs (Mo-lyneaux et al. 2004). Mutations of c-KIT are involved in ovarian dysgerminomas as well as in bilateral testicular seminomas (Looijenga et al. 2003, Hoei-Hansen et al. 2007).

Activator protein-2γ is regulated by the estrogen signalling pathway, as shown by induction of AP-2γ expression in breast tumor cells by estrogens (Orso et al. 2004). In addition, AP-2γ regulates the transcription of ERβ in prostate cancer cells by transactivating the ERβ promoter (Zhang et al. 2007). Thus, AP-2γ expression may also be hypothesized to be regulated by estrogens in germ cell tumors.
Table 1. Expression of selected proteins involved in female germ cell development

<table>
<thead>
<tr>
<th></th>
<th>Primordial germ cell</th>
<th>Oogonia</th>
<th>Oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLAP</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>c-Kit</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Oct-3/4</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AP-2(\gamma)</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>ER</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

PLAP; placental-like alkaline phosphatase, c-KIT; stem cell factor, ER; estrogen receptor.

Table 2. Expression of selected proteins in male germ cell development

<table>
<thead>
<tr>
<th></th>
<th>Primordial germ cell</th>
<th>Gonocyte</th>
<th>Spermatocyte</th>
<th>Spermatid</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLAP</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>c-Kit</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Oct-3/4</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>AP-2(\gamma)</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

PLAP; placental-like alkaline phosphatase, c-KIT; stem cell factor.

In addition to pluripotent factors, self-renewal factors may also contribute to the development of germ cell tumors. The polycomb group (PcG) member BMI-1 (B cell-specific Moloney murine leukaemia virus integration site 1) is involved in cell proliferation, stem cell renewal and human oncogenesis (Park et al. 2003). BMI-1 acts as proto-oncogene by down-regulating tumor suppressor genes and it is found in various malignancies including neuroblastomas, breast cancer, lymphomas and epithelial tumors (Raaphorst 2005). Moreover, BMI-1 is expressed in epithelial ovarian cancer (Zhang et al. 2008a). BMI-1 cooperates with c-MYC and regulates self-renewal of stem cells and may thus be involved in tumorigenesis in germ cell tumors.

1.3 Development and migration of germ cells

Primordial germ cells give rise to both types of gametes, oocytes and sperm cells. In mice PGCs originate from the proximal epiblast (adjacent to the extra-embryonic ectoderm) (Figure 1E) (Lawson et al. 1994). The formation of PGCs is influenced by bone morphogenetic proteins (BMPs). BMP-4 and BMP-8b are secreted by the extra-embryonic ectoderm and BMP-2 by the extra-embryonic endoderm (Lawson et al. 1999, McLaren 2000, Ying et al. 2000). Thus,
mouse embryos lacking \textit{bmp-4} or \textit{bmp-8b} do not have any germ cells.

In human development, between the 3\textsuperscript{rd} and 4\textsuperscript{th} week, a cluster of approximately 100 cells appears in the endoderm of the dorsal wall of the yolk sac near the allantois (Figure 1F). During the 4\textsuperscript{th} and 5\textsuperscript{th} weeks of development PGCs migrate from the extra-embryonic site via the yolk sac and the hindgut endoderm at the caudal end of the embryo, and via the dorsal mesentery of the hindgut to the genital ridges at the ventral sides of the mesonephros (Figure 1G). The nature of this migration is under debate (Freeman 2003); it has been proposed to be active (amoeboid movements of PGCs) or passive (induced by growth-movements of the adjacent tissues). During the migration primordial germ cells proliferate and finally by the end of the 5\textsuperscript{th} week or early in the 6\textsuperscript{th} developmental week approximately 1000 PGCs reach the gonadal ridges. By the 7\textsuperscript{th} week of development the gonads have differentiated into testes or ovaries, and the migration of PGCs is completed. During the migration some PGCs might be stranded along the migration pathway. Thus, germ cell tumors might also appear outside of the gonads.

Survival of the migrating PGCs is dependent on the interactions between these cells and the somatic cells along the migratory pathway. This interaction is mediated via the tyrosine kinase receptor c-KIT, expressed on the surface of PGCs, and its ligand, Kit ligand (stem cell factor), which is expressed in somatic cells (Fleischman 1993). In mammals, germ cells differentiate according to their somatic cell environment: XY germ cells can develop as oocytes in female embryos (Burgoyne et al. 1988). When germ cells enter ectopic sites such as the adrenal gland, they develop as oocytes even in male embryos (Zamboni et al. 1983).

Dysgenetic gonads display an increased risk of germ cell tumors as a result of improper gonadal and germ cell maturation. Turner’s syndrome (45, X) patients are mainly infertile as a result of lack of oocytes, caused by massive loss of germ cells during childhood (Speed 1986). Oogonia are normally detected from the 18\textsuperscript{th} gestational week and primordial follicles from the 20\textsuperscript{th} week. In contrast, in Turner’s syndrome embryos, only some oogonia and no primordial follicles are detected in fetal ovaries (Reynaud et al. 2004). Turner’s syndrome is also associated with an increased risk of germ cell tumors (Schoemaker et al. 2008).

Primordial germ cells in the ovary divide mitotically and transform into oogonia at the 9\textsuperscript{th} week of development. The oogonia become surrounded by mesonephros-derived somatic cells, thus forming germ cell clusters. They undergo further DNA replication, thereafter entering meiosis and becoming oocytes. The oocytes develop into leptotene, zygotene and pachytene stages of meiotic prophase I before becoming arrested at the diplotene stage of meiosis I. This stage may be retained for up to 40–50 years until a follicle becomes atretic or develops into a full-grown follicle. In contrast to the ovary, primordial germ cells arriving at the testis are arrested in mitosis and further male gametogenesis is delayed until puberty.

Oocytes reach their peak number of approximately 7 million during midgestation. The number of oocytes is already dramatically reduced by approximately 90 % at the time of birth. Whether or not a female will lose the capacity for oocyte production during embryogenesis or will retain the ability to produce oocytes later in life from potential PGCs within the ovary or at extrago-
Gonadal development

In humans, bi-potential gonads arise as a paired structure within the intermediate mesoderm during the 4th week of development. This urogenital ridge region is located between the limb buds, filling most of the coelomic cavity. The bi-potential gonad is similar in female and male embryos until the 7th week of gestation. Thus, it can give rise to both testis and ovary. The urogenital ridge develops into three different sections: the pronephros, giving rise to the adrenals, the mesonephros, giving rise to the gonads, and the metanephros, giving rise to the kidneys. During the 6th week of development, cells from the mesonephros and coelomic epithelium invade the mesenchyme in the region of the presumptive gonads to form aggregates of supporting cells, the primitive sex cords.

Intermediate mesoderm expresses various genes, including that for Wilms’ tumor suppressor 1 (WT1) when developing into the bi-potential gonad (Figure 2). Later, WT1 is expressed in the developing Sertoli and granulosa cells (Hanley et al. 1999). WT1 activates other genes including those for steroidogenic factor 1 (SF-1) and Antimüllerian hormone/Müllerian-inhibiting substance (AMH/MIS) (Nachtigal et al. 1998, Hossain et al. 2003). SF-1 regulates many genes including that for AMH/MIS (Shen et al. 1994, Ingraham et al. 2000). In addition, transcription of AMH/MIS is regulated by GATA-4 and its interaction with SF-1 (Viger et al. 1998, Tremblay et al. 1999). WT1 regulates Sex determining Region of the Y-chromosome (SRY) and AMH/MIS by binding and activating their gene promoters (Hossain et al. 2001, Hossain et al. 2003, Matsuzawa-Watanabe et al. 2003).

Steroidogenic factor 1 forms a link between SRY and the male developmental pathway and it is essential in the developing bi-potential gonad. Its expression is maintained in differentiating testis and it has a role in Sertoli and Leydig cell development (Hanley et al. 1999). In Sertoli cells, SF1 acts in concert with Sox9, elevating AMH/MIS expression (Shen et al. 1994, Arango et al. 1999). In addition, SF-1 activates genes encoding the synthesis of testosterone in the Leydig cells. In humans, mutations in the SF-1 gene cause XY sex reversal, presenting with malformed fibrous gonads and fully developed Müllerian duct structures (Achermann et al. 1999).
Figure 2. Essential genes involved in gonadal differentiation. Solid line, activating effect; dashed line, inhibiting effect. Based on literature cited in section 2.

2.1 Ovarian differentiation

Ovarian differentiation is less well understood than testicular differentiation. The discovery that gonads develop as ovaries in the absence of the Y-chromosome (or SRY gene) supports the view that the testicular pathway is the active pathway in gonadal development. Thus, ovarian differentiation has been thought to be a passive transformation in the absence of SRY. However, some genes are now known to be essential for ovarian development (Figure 2). Mutations in genes including Wnt-4, rspo1, DAX1 and FOXL2, present complete or partial male sex reversal in mice and humans.

In XX mice lacking Wnt-4 the ovary fails to form properly and its cells express testis-specific markers, including AMH- and testosterone-producing enzymes (Vainio et al. 1999). In Wnt-4-deficient XX mice the Müllerian duct fails to develop and the Wolffian duct continues to develop; thus the gonad has the appearance of a testis. However, the gonads do not form testicular cord structures or express Sertoli cell-specific markers. In humans, those with heterozygous Wnt-4 defects show Müllerian duct agenesis and signs of ovarian hyperandrogenism.

Wnt-4 acts through the β-catenin pathway by causing stabilization of β-catenin and its entry into the nucleus. In the presence of Wnt, rspo1 also stabilizes β-catenin. Mice lacking rspo1 are characterized by a male phenotype similar to that in XX mice lacking Wnt-4 (Maatouk et al. 2008). Humans with mutation in the R-SPONDIN1 (rspo1) gene display sex reversal (Parma et al. 2006).
In ovarian development Wnt-4 activates DAX1 (Dosage-dependent sex reversal; Adrenal hypoplasia congenital; X-chromosome) (Mizusaki et al. 2003). DAX1 plays a role in female gonadal development, as it appears to antagonize the functions of SRY and Sox9 and it down-regulates SF-1 expression (Nachtigal et al. 1998, Swain et al. 1998). In addition, DAX1 reduces the synergy between GATA-4 and SF-1 (Nachtigal et al. 1998, Tremblay et al. 2001b) as well that between WT1 and SF-1 (Nachtigal et al. 1998). In the XX gonad, two ligands, Wnt-4 and rspo1, are capable of activating the β-catenin signalling pathway, and loss of either Wnt-4 or rspo1 in mice results in a partial sex-reversal (Vainio et al. 1999, Chassot et al. 2008, Tomizuka et al. 2008). Moreover, human XY subjects carrying duplication of both Wnt-4 and rspo1 show male-to-female sex reversal (Jordan et al. 2001).

Another repressor of the male developmental pathway is FOXL2 (forkhead box L2). In humans, mutations in the FOXL2 gene display abnormal eye functions and premature ovarian failure, causing blepharophimosis/ptosis/epicanthus inversus syndrome (BPES) (Uhlenhaut et al. 2006). This syndrome is the only known autosomal dominant disorder to cause premature ovarian failure. In foxl2 -/- mice the ovaries transform after birth into seminiferous tubule-like structures with testosterone-producing Leydig cells (Ottolenghi et al. 2005).

Migrating PGCs seem to play a more active role in ovarian development than they do in testis development. In the absence of PGCs, supporting cells in the ovary differentiate into prefollicle cells that aggregate into mesenchymal condensations, but these eventually degenerate, leaving only stromal tissue (Merchant-Larios et al. 1981).

### 2.2 Testicular differentiation

The formation of testicular cords is initiated between the 6th and 8th weeks of development. Once the PGCs have started their migration and entered the genital ridge during the 5th week they become enclosed by the differentiating Sertoli cells. Testis differentiation is induced by the expression of SRY (Sekido et al. 2009). SRY initiates the male pathway by triggering the differentiation of Sertoli cells in the genital ridge. The Sertoli cells become organized into cord structures that encircle immature germ cells (gonocytes). At the same time, in the interstitium of the testicular cords, Leydig cells differentiate and begin to secrete testosterone. Deregulation of SRY expression can result in partial or complete failure of testis determination and the formation of ovotestes or ovaries within XY individuals. In humans, SRY mutations are found in 15 % of XY females (Hawkins et al. 1992a).

The transcription factors WT1 and SF-1 are able to bind and transactivate human SRY promoters (Merchant-Larios et al. 1981) (Figure 2). Müllerian duct regression and Wolffian duct development is influenced by testosterone and by AMH produced by Sertoli cells. Production of AMH by fetal testicular Sertoli cells leads to initiation of irreversible Müllerian duct regression in the male fetus. Several transcription factors including SF-1, WT1, Sox-9 and GATA-4, regulate expression of AMH (Viger et al. 1998, Ingraham et al. 2000, Tremblay et al. 2001a, Hossain et al. 2003, Manuylov et al. 2007) (Figure 2).
Bi-potential genital ridges will develop into the female phenotype unless influenced by testosterone and AMH. Absence of AMH in humans causes persistent Müllerian duct syndrome, a form of pseudohermaphroditism characterized by the retention of Müllerian duct structures (Josso et al. 2005).

3. GATA transcription factors in gonadal development and physiology

GATA factors are a family of six zinc-finger transcription factors that recognize the consensus nucleotide sequence WGATAR (the GATA motif) in the promoter regions of target genes. Of the six GATA factors, GATA-4 and GATA-6 are essential in gonadal differentiation and in reproductive functions. GATA-4 and -6 are expressed in fetal developing and adult gonads. Gata4 -/- mice die as a result of defects in lateral folding and heart formation. Gata6 -/- mice die as a result of a gastrulation defect and the presence of underdeveloped visceral endoderm. Both of these GATA factors are essential for normal development of embryonal endoderm and extraembryonal endoderm forming visceral yolk sac (Soudais et al. 1995, Morrisey et al. 1998).

GATA-4 is involved in testicular development, as it enhances AMH expression by directly binding to DNA and cooperatively interacting with SF-1 (Viger et al. 1998) (Figure 2). GATA-4 cooperates with SF-1 to activate AMH promoter-driven reporter gene activity in Sertoli cells in utero (Tremblay et al. 1999). In mice GATA-4, with its ability to interact with FOG-2, is essential for normal determination and differentiation of the gonad (Tevosian et al. 2002, Manuylov et al. 2008). In XY GATA-4-mutated homozygous mice, GATA-4 is not able to interact with FOG-2, and Fog-2-/- mice show similar defects in gonadal differentiation. In these mice, testicular cords do not develop, WT1 and SF-1 are not up-regulated, SRY expression is reduced, and Sox-9, AMH and steroidogenic enzymes are absent (Tevosian et al. 2002). Moreover, Wnt-4, a mediator of ovarian development, is not down-regulated.
In the human fetal ovary GATA-4 is expressed in pregranulosa/stromal cells (Figure 3). At weeks 14 to 33, GATA-4 is localized in the ovarian granulosa cells, but it is not expressed in fetal oocytes (Vaskivuo et al. 2001). GATA-4 is possibly also involved in the survival of ovarian granulosa cells (Heikinheimo et al. 1997). Similarly as in the testis, GATA-4/FOG-2 interaction is essential in the ovary (Manuylov et al. 2008). Gata-4/Fog-2 mutants show a compromised female program in XX gonads. In the absence of GATA-4 interaction with FOG-2, expression of Wnt-4 and FOXL2 is lost, but rspo1 expression remains normal. However, the loss of Gata-4/Fog-2 interaction does not affect germ cells, as they enter meiotic prophase normally (Manuylov et al. 2008).

In human fetal testis the expression patterns of GATA-4 and GATA-6 partially overlap and they are localized mainly to the Sertoli and Leydig cells (Ketola et al. 2000). Expression of GATA-4 in Sertoli cells is strongest during the period of proliferation of these cells (weeks 19 to 22). In Leydig cells the expression is strongest during the periods associated with testosterone production (gestational week 15 and after puberty). In fetal and prepubertal germ cells GATA-4 expression is detected, but it is absent in germ cells after puberty (Ketola et al. 2000). In addition, GATA-4 is expressed in Sertoli and Leydig cell tumors (Ketola et al. 2000).

GATA-6 is expressed in fetal testis during gestational weeks 16 to 40 (in the testicular cords and interstitium), with an expression peak during the second trimester (Ketola et al. 2003). It is localized to Sertoli and Leydig cells, with the strongest expression between gestational weeks 16 to 23. Human fetal germ cells are devoid of GATA-6 (Ketola et al. 2003).

The actions of GATA factors are modified by a number of co-activators and co-repressors. FOG-1 is expressed in hematopoietic cells, cooperating with GATA-1 in promoting cell dif-
ferentiation (Tsang et al. 1997). FOG-2 was originally described as a cofactor of GATA-4 (Tevosian et al. 1999). FOG-2 is expressed in developing mouse heart, the urogenital ridge, neuroepithelium, adult heart, brain and testis, and it interacts with all GATA factors, at least in vitro, either by activating or repressing, depending on cell type (Robert et al. 2002). FOG-2 and its interactions with GATA-4 are essential for heart morphogenesis and testicular development (Tevosian et al. 2000, Robert et al. 2002).

In mouse testis FOG-1 and FOG-2 are co-expressed with GATA-1, -4 and -6 in Sertoli and Leydig cells (Ketola et al. 2002, Robert et al. 2002). GATA-4/FOG-2 interaction is required for SRY expression, leading to gonadal differentiation and sex determination (Tevosian et al. 2002). Thus, testicular development is hampered if their interaction is unable to occur as a result of gene manipulation leading to inability of FOG-2 to bind GATA-4.

4. Estrogen receptors in gonadal development and physiology

Estrogen receptors are members of a large nuclear receptor superfamily of ligand-regulated DNA-binding transcription factors. In humans, a total of 48 nuclear receptors have been characterized, including estrogen, androgen, glucocorticoid, progesterone and thyroid hormone receptors. Lipophilic steroid hormones, including estrogens, bind to intracellular steroid receptors. Ligand binding induces a conformational change in the steroid receptor and facilitates receptor dimerization, nuclear transport and interaction with target DNA motifs. Many synthetic ligands have been designed to target these receptors pharmacologically, offering widespread clinical use.

Estrogen actions are mediated via two estrogen receptors, ERα and ERβ (Green et al. 1986, Kuiper et al. 1996). The main ER ligand is 17β-estradiol and it binds equally well to both ERs. Estrogen action in a cell is the result of a balance between these two receptors. ERα often plays an activating role whereas ERβ plays a suppressive role.

The genes for these two receptors are located on different chromosomes. However, ERs show close similarity in their DNA-binding sites (97 % homology in amino acids) and less homology in their ligand-binding sites (55 % homology) (Kuiper et al. 1996, Mosselman et al. 1996). Estrogen receptors mediate their actions by ligand-dependent binding to the estrogen responsive elements (EREs) of target genes.

Recent studies have revealed a novel rapid non-genomic pathway of estrogen action. One of the suggested receptors involved is GPR30. However, GPR30 knockout mice do not show any histological or functional variation in the main estrogen target tissues (ovary, breast and uterus) (Otto et al. 2009).

Work on transgenic mice has provided insight into the role of ERs in the development and function of the reproductive tract. These receptors are not vital in the embryonic period, given
that ERα, ERβ and ERαβ knockout (ERKO) mice develop beyond the fetal stage. αERKO mice are infertile, with a hypoplastic uterus, ovaries with only a few granulosa cells and cystic hemorrhagic follicles (Lubahn et al. 1993, Couse et al. 1998). Thus, in αERKO mice corpora lutea do not form. βERKO female mice have reduced fertility, and anatomical findings include normal uterus, and ovaries with many atretic follicles and fewer corpora lutea than normal (Krege et al. 1998). αβERKO female mice are also infertile, with hypoplastic uteri, and ovaries with seminiferous tubule-like structures similar to those in male mice (Couse et al. 1999). Moreover, mice lacking both ERs display elevated levels of Sox9 and AMH and undergo postnatal sex reversal (Couse et al. 1999).

Several human tissues including breast, brain, cardiovascular system, bone and urogenital tract express ERs. ERα is the main subtype in the liver, mammary gland, kidney, heart, skeletal muscle and pituitary, whereas ERβ is the major type in the prostate and colon. The distribution of the two ERs in the reproductive organs is different according to tissue and cell type. Thus, ERα is dominantly expressed in the uterus and in the ovarian thecal and interstitial cells, whereas ERβ is the predominant form in the granulosa cells (Kuiper et al. 1996, Enmark et al. 1997). In fetal ovaries expression of ERs is localized to the granulosa cells and oocytes from the 20th week of gestation onward, with ERβ being the predominant form (Vaskivuo et al. 2005) (Figure 4).

In addition to normal tissues, differential expression of the two ERs is seen in various neoplasms. Decreased expression of ERβ mRNA and protein, and an increased ERα/ERβ ratio has been reported in various malignancies, including breast, ovary, colon and prostate cancer (Rutherford et al. 2000, Campbell-Thompson et al. 2001, Roger et al. 2001, Fixemer et al.)

Figure 4. Expression of estrogen receptors (ERs) α and β in fetal and adult gonads. Based on the literature review in section 3 and the results of the present study. Black circles: primordial germ cells, white circles: Sertoli cells/granulosa cells, triangles: Leydig cells, squares: theca cells.
Most (90%) malignant ovarian tumors are of epithelial origin. Of these epithelial ovarian cancers, two thirds express estrogen receptors (Lindgren et al. 2004). In addition, granulosa cell tumors express ERs (Chu et al. 2000). Anti-estrogen therapy has resulted in only modest responses in cases of recurrent ovarian cancer, possibly because of the low levels of ERs in these cancers (Makar 2000).

Estrogen receptors regulate transcriptional activation by binding to EREs of target genes. In addition, co-regulators modify steroid receptor actions, acting as co-activators or co-repressors in nuclear receptor-mediated transcription. Co-activators regulate nuclear receptor function in a variety of ways. Their role may change depending on the tissue and cell type. There are up to 300 co-regulators and they have many roles in various diseases, e.g. cancer, metabolic syndromes and inheritable syndromes (Lonard et al. 2007).

Small nuclear ring finger protein 4 (SNURF/RNF4) can co-activate both androgen- and estrogen-dependent transcription by interacting directly with these receptors (Moilanen et al. 1998). SNURF is expressed in fetal murine male and female germ cells, Sertoli cells and granulosa cells (Hirvonen-Santti et al. 2003, Hirvonen-Santti et al. 2004). In addition, the expression of SNURF has been reported to be increased in murine ovaries treated with estradiol (Hirvonen-Santti et al. 2004).

5. Gonadal Germ Cell Tumors

Gonadal germ cell tumors are found in both sexes. In addition, germ cell tumors may be located in extra-gonadal sites (e.g. mediastinum, hypothalamic/supracellular region) due to improper migration of primordial germ cells during embryogenesis. In the ovary malignant germ cell tumors are rare, accounting for 3–5% of all ovarian malignancies. In contrast, more than 95% of testicular tumors are of germ cell origin. The incidence of testicular cancer has doubled during the past 40 years (Huyghe et al. 2003). An annual 3–6% increase is seen among Caucasian males. The highest incidence worldwide is in Denmark (9.2/100 000) (Huyghe et al. 2003). Moreover, there is an approximately fivefold difference in testicular cancer incidence between white and black males in the US and in southern England (McGlynn et al. 2005). This varying incidence among ethnic groups provides evidence for a genetic etiology in testicular cancer. There is strong evidence of an inherited risk of testicular cancer, as shown by the increased risk of the disease among first-degree relatives (Hemminki et al. 2004). Along with genetic factors, environmental factors play a role in testicular germ cell tumor development, as shown among the offspring of Finnish immigrants in Sweden. The immigrants maintained their lower risk, but their offspring showed an incidence similar to that in the Swedish population (Montgomerie et al. 2005). Environmental factors including endocrine-disrupting chemicals (xenoestrogens and antiandrogens) are hypothesized to be involved in testicular tumorigenesis. Organochlorines, e.g. polychlorinated biphenyls (PCBs) show estrogenic and antiandrogenic activity. Moreover, elevated PCB levels have been associated with mothers of TGCT patients (Hardell et al. 2003).
The only characterized chromosomal abnormality in TGCTs is relative gain of the short arm of chromosome 12 (Looijenga et al. 1999). Overrepresentation of 12p may be related to invasive growth (Rosenberg et al. 2000). In addition, overrepresentation of chromosome arm 12p is seen in testicular CIS adjacent to overt testicular tumors but not in CIS cells without a tumor (Ottesen et al. 2003). In addition to testicular tumors, abnormalities in chromosome arm 12p are detected in 80% of ovarian dysgerminomas (Cossu-Rocca et al. 2006).

5.1 Germ cell tumor development

Gonadal germ cell tumors are suggested to develop from primordial germ cells during embryogenesis in utero. Most testicular tumors develop from a common tumor precursor called testicular carcinoma in situ (CIS), also referred to as intratubular germ cell neoplasia (ITGCN) or testicular intraepithelial neoplasia (TIN). Developmental arrest in the differentiation of the early germ cell lineage is hypothesized to be the key pathogenic event leading to neoplastic transformation of a primordial germ cell or gonocyte into a CIS cell. CIS cells most likely develop from fetal gonocytes in utero. They resemble primordial germ cells and fetal gonocytes morphologically, and they share overlapping expression of several proteins, e.g., Oct-3/4, AP-2γ and c-KIT (Rajpert-De Meyts et al. 2003, Hoei-Hansen et al. 2004, Pauls et al. 2005). Recent genome-wide gene expression studies have revealed more evidence of the origin of CIS from fetal germ cells (Skotheim et al. 2002, Almstrup et al. 2005). As exceptions, infantile germ cell tumors and spermatocytic seminomas do not originate from CIS cells.

Table 3. Characteristics of malignant ovarian germ cell tumors (MOGCTs), and testicular cancer and CIS

<table>
<thead>
<tr>
<th></th>
<th>MOGCTs</th>
<th>Testicular cancer</th>
<th>Testicular CIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence /100 000</td>
<td>0.41</td>
<td>4.8</td>
<td></td>
</tr>
<tr>
<td>Surgical Treatment</td>
<td>USO</td>
<td>Orchidectomy</td>
<td>Orchidectomy</td>
</tr>
<tr>
<td>+ staging</td>
<td></td>
<td>+ RPLND (Stage ≥II)</td>
<td>(unilateral CIS)</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>Stage I: -</td>
<td>Stage I: -</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Stage II-IV:</td>
<td>Stage II-IV:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BEPx3 (no residua)</td>
<td>BEPx3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>BEPx4 (residua)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Radiation therapy</td>
<td>Dysgerminoma</td>
<td>Seminoma</td>
<td>Bilateral CIS</td>
</tr>
<tr>
<td>Prognosis</td>
<td>Stage I: &gt;99 %</td>
<td>Stage I: &gt;99 %</td>
<td>Cancer in 70 % in</td>
</tr>
<tr>
<td></td>
<td>Stage ≥II: &gt;75 %</td>
<td>Stage ≥II: 50-90 %</td>
<td>7 years</td>
</tr>
</tbody>
</table>

USO, unilateral salpingo-oophorectomy; BEP, bleomysin, etoposide, cisplatin; RPLND, Retroperitoneal lymph node dissection
The gonads share common development early in embryogenesis. Thus, gonadal germ cell tumors display similarities in morphology, histology, treatment and prognosis (Table 3, Table 4). In addition, the presence of a few families with both ovarian and testicular germ cell tumors suggests a possible genetic etiology (Galani et al. 2005). Germ cell tumors are derived from cells of the germ cell lineage in which maturation is blocked (Figure 5). Dysgenetic gonads are associated with improper maturation of germ cells, thus these patients have an increased risk of germ cell tumors. In dysgenetic gonads gonadoblastoma is considered as a counterpart for testicular CIS. The presence of Y-chromosome material in cases of Turner’s syndrome is associated with an increased risk of gonadoblastoma (Mancilla et al. 2003). Other risk factors of testicular germ cell tumors (TGCTs) are a previous contralateral testicular tumor, undescended testis, and testicular tumors among first-degree relatives (Dong et al. 2001, Bromen et al. 2004). In addition, increased fetal exposure to maternal hormones, and low birth weight have been linked to TGCTs (Cook et al. 2008). Moreover, some postnatal characteristics have been associated with an increased risk of testicular cancer: early puberty, tallness, and subfertility (Doria-Rose et al. 2005, McGlynn et al. 2007).

Figure 5. Origin of various histological subtypes of gonadal germ cell tumors. PGCs: primordial germ cells, YST: yolk sac tumor, IT: immature teratoma, EC: embryonal carcinoma

5.2 Testicular dysgenesis syndrome

Testicular cancer is associated with testicular dysgenesis syndrome (TDS). In TDS all three testicular cell lineages (Sertoli, Leydig, germ cell) show improper function or maturation (Skakkebaek et al. 2001). As a consequence, clinical manifestations of TDS include hypospadias, cryptorchidism, poor semen quality and increased risk of testicular cancer. The risk of a contralateral TGCT is elevated in cases of undescended testis, thus supporting the theory of TDS being associated with testicular cancer (Moller et al. 1996). Neonatal or perinatal exposure to estrogens have been suggested as risk factors of TDS. The synthetic estrogenic drug diethylstilbestrol (DES) was formerly used during pregnancy to prevent abortions and other pregnancy-related complications (Palmlund et al. 1993). Later, DES was found to be associated with an
increased risk of vaginal cancer in female offspring and testicular malformations, impaired sperm quality and testicular cancer in male offspring (Storgaard et al. 2006, Rubin 2007).

5.3 Clinicopathological characteristics of gonadal germ cell tumors

Germ cell tumors are histologically a heterogeneous group of tumors (Figure 5, Table 4). The most common group in both the testis and the ovary is the germinoma group (ovarian dysgerminomas/testicular seminomas). Tumors may also harbor a mixture of different subtypes. Bilateral ovarian germ cell tumors are uncommon, with the exception of 10–15 % of cases of dygerminoma. In 5–10 % of cases of ovarian germ cell tumor, patients are diagnosed with a benign cystic teratoma adjacent to the malignant tumor or in the other ovary. The majority of ovarian germ cell tumor patients present with abdominal pain, which is associated with an abdominal mass in most cases. In testicular cancer patients the most common symptom is a hard swelling within a testis, mostly without any pain. Gonadal germ cell tumor diagnosis is histological after the primary surgery. Most ovarian and testicular germ cell tumors are diagnosed as Stage I tumors. More advanced ovarian tumors metastasize by way of the peritoneal surface or by lymphatic spread, whereas hematogenous spread is more common in testicular cancer. The testicular precursor CIS is detected clinically by ultrasonography, often in connection with microlithiasis, and confirmed by surgical biopsy. The risk of testicular cancer among CIS patients is 70 % within 7 years (von der Maase et al. 1986).

Table 4. Classification and relative incidence (%) of malignant gonadal germ cell tumors.

<table>
<thead>
<tr>
<th></th>
<th>Ovarian</th>
<th>Testicular</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysgerminoma/semionoma</td>
<td>40</td>
<td>50</td>
</tr>
<tr>
<td>Yolk sac tumor</td>
<td>20</td>
<td>1</td>
</tr>
<tr>
<td>Immature teratoma</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Embryonal carcinoma</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>Choriocarcinoma</td>
<td>&lt;2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Polyembryoma</td>
<td>&lt;2</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Mixed</td>
<td>20</td>
<td>33</td>
</tr>
</tbody>
</table>

5.4 Serum and tissue tumor markers

Elevated concentrations of serum tumor markers are of value in the diagnosis of germ cell tumors. The serum tumor markers α-fetoprotein (AFP) and the β subunit of human gonadotropin (hCGβ) are virtually diagnostic of germ cell tumors. Levels of AFP are elevated in yolk sac tumors whereas hCGβ concentrations are elevated in cases of choriocarcinoma and embryonal carcinoma (Table 5). In addition, serum levels of lactate dehydrogenase (LDH) are elevated in connection with some of the tumors. The level of LDH is of limited sensitivity, specificity and positive predictive value (Venkitaraman et al. 2007). Concentrations of LDH are also elevated
in cases of hemolytic anemia, chronic liver disease, chronic pancreatitis, congestive heart failure, collagen-vascular disease and muscular dystrophy. Serum LDH is of value as a prognostic factor in the diagnosis of TGCTs, but there can be false-positive findings when used in follow-up. A diagnostic tissue marker in CIS cells is placental-like alkaline phosphatase (PLAP) (Giewercman et al. 1991). PLAP is normally synthesized by trophoblasts after the first trimester of pregnancy (Manivel et al. 1987). It is a marker of PGCs and it is also seen in human oogonia, but not in oocytes after birth, while its activity is lost just after germ cells enter meiosis (Stoop et al. 2005). However, the biological function of PLAP in CIS cells is unknown.

Pathohistological diagnosis of germ cell tumors is based on histological evaluation and distinctive immunohistological markers (Table 6). Germinomas have a characteristic histological appearance with primitive germ cells resembling primordial germ cells separated by fibrous septa and infiltrated by lymphocytes (Ulbright 2005, Roth et al. 2006). Germinomas may contain syncytiotrophoblastic cells producing hCG. In some cases germinomas may be confused with YSTs or Sertoli cell tumors (Ulbright 2005, Ulbright 2008, Looijenga 2009).

Yolk sac tumors appear in a variety of histological patterns, most of them staining positively for AFP and containing hyaline globules, thick bands of basement membrane material and Schiller-Duvel bodies resembling fetal glomeruli (Roth et al. 2006). Solid forms of YSTs may be confused with DGs and clear cell adenocarcinomas and endometrioid carcinomas (Ulbright 2008).

Embryonal carcinomas are composed of groups of large primitive cells with overlapping nuclei and distinct cell borders resembling those of the embryonic disc (Roth et al 2006). Solid forms of EC may be confused with DGs or some variants of YSTs. Embryonal carcinomas stain positively for PLAP.

Choriocarcinomas consist of cytotrophoblasts surrounded by syncytiotrophoblasts, thus staining positively for hCG, human placental lactogen, inhibin, and low molecular weight cytokeratin, and sometimes PLAP (Roth et al. 2006).

Immature teratomas are composed of embryonic tissue, but some mature tissue may be present (Roth et al. 2006). Immaturity manifests predominantly as immature neuroepithelial tissue. Immature teratomas are graded from 1 to 3 based on the amount of immature neuroepithelium.
Table 5. Serum tumor markers in malignant gonadal germ cell tumors.

<table>
<thead>
<tr>
<th>Histological type</th>
<th>AFP</th>
<th>hCGβ</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysgerminoma/seminoma</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>Yolk sac tumor</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Immature teratoma</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>Choriocarcinoma</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Embryonal carcinoma</td>
<td>+/-</td>
<td>+</td>
</tr>
<tr>
<td>Polyembryoma</td>
<td>+/-</td>
<td>+</td>
</tr>
</tbody>
</table>

Table 6. Immunohistochemical markers used in differential diagnosis of gonadal germ cell tumors.

<table>
<thead>
<tr>
<th>OCT3/4</th>
<th>c-KIT</th>
<th>SOX2</th>
<th>SOX17</th>
<th>CD30</th>
<th>AFP</th>
<th>hCG</th>
<th>PLAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysgerminoma/seminoma</td>
<td>+</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Embryonal carcinoma</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Yolk sac tumor</td>
<td>-</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+/-</td>
</tr>
<tr>
<td>Choriocarcinoma</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+/–</td>
</tr>
</tbody>
</table>

Modified from Looijenga 2009.

5.5 Treatment and survival of gonadal germ cell tumor patients

Surgical treatment of gonadal germ cell tumors is fertility-sparing unilateral oophorectomy or orchiectomy, even in advanced stages (Table 3). Earlier, before the introduction of combination chemotherapy, virtually all patients died of the disease. Currently, platinum-based combinations are the standard treatment for patients with other than Stage I disease. BEP (bleomycin, etoposide, cisplatin) combination chemotherapy is the gold-standard treatment, both in ovarian and testicular germ cell tumors. Compared with the previously used PVB (cisplatin, vinblastine, bleomycin) combination chemotherapy is the gold-standard treatment, both in ovarian and testicular germ cell tumors. Compared with the previously used PVB (cisplatin, vinblastine, bleomycin) combination chemotherapy is the gold-standard treatment, both in ovarian and testicular germ cell tumors. The POMB-ACE (cisplatin, Oncovin-vincristine, methotrexate, bleomycin, actinomycin-D, cyclophosphamide, etoposide) combination has been used in advanced and aggressive testicular cancer and in advanced ovarian germ cell tumors (Murugaesu et al. 2006).

Malignant gonadal germ cell tumors are curable diseases with survival rates exceeding 95 %. Prognostic factors of malignant ovarian germ cell tumors have been stage, amount of residual tumor, histological subtype and raised concentrations of serum tumor markers. In addition, the size of the tumor is prognostic in testicular cancer.

Given that the majority of germ cell tumor patients survive the disease, the long-term effects of the treatments are important as regards their effects on fertility and health. Potential complica-
tions after chemotherapy include an increased risk of cardiovascular diseases, neurotoxicity, hearing dysfunction, renal failure and secondary malignancy. Acute nonlymphoblastic leukemia is the most common secondary malignancy, being associated with a 0.9 % risk in testicular cancer patients (Boshoff et al. 1995). In a recent study, following conservative surgery, fertility was preserved in most patients with malignant ovarian germ cell tumors, given that 76 % achieved at least one pregnancy (Tangir et al. 2003). In testicular cancer patients, chemotherapy is associated with temporary azoospermia (Lampe et al. 1997). However, fertility rates are 30 % lower in testicular cancer patients than in the normal population (Fossa et al. 2000), thus, indicating presence of related testicular dysgenesis syndrome.

**Aims of the Study**

The aim of this study was to identify novel diagnostic and prognostic tools for evaluation of malignant gonadal germ cell tumors. In addition, the aim was to further reveal the biology behind the formation and differentiation of germ cell tumors.

**The specific aims of this study were:**

1. To characterize the role of GATA factors, their cofactors, and their target genes in testicular and ovarian germ cell tumors.


3. To reveal the role of the estrogen signalling pathway in ovarian germ cell tumors.

4. To provide further evidence of the fetal origin of testicular CIS and germ cell tumors.

5. To assess the prognostic value of serum concentrations of AFP, hCGβ and CA 125 in malignant ovarian germ cell tumors.
Patients, Samples and Methods

1. Malignant ovarian germ cell tumor patients (I, II, III)

Table 7. Summary concerning patients with malignant ovarian germ cell tumors (n = 30).

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean age, years (± SD)</td>
<td>30.0 (± 4.9)</td>
<td></td>
</tr>
<tr>
<td>Mean follow-up time, months (range)</td>
<td>92 (2–205)</td>
<td></td>
</tr>
<tr>
<td>Histological distribution</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysgerminoma (DG)</td>
<td>11</td>
<td>37</td>
</tr>
<tr>
<td>Immature teratoma (IT)</td>
<td>10</td>
<td>33</td>
</tr>
<tr>
<td>Yolk sac tumor (YST)</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>Others*</td>
<td>3</td>
<td>10</td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>21</td>
<td>70</td>
</tr>
<tr>
<td>II-III</td>
<td>9</td>
<td>30</td>
</tr>
<tr>
<td>Treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USO</td>
<td>17</td>
<td>81</td>
</tr>
<tr>
<td>TAH and/or BSO</td>
<td>4</td>
<td>19</td>
</tr>
<tr>
<td>Chemotherapy (platinum/non-platinum)</td>
<td>18 (16/2)</td>
<td>86</td>
</tr>
<tr>
<td>Stage II-III</td>
<td></td>
<td></td>
</tr>
<tr>
<td>USO</td>
<td>2</td>
<td>22</td>
</tr>
<tr>
<td>TAH and/or BSO</td>
<td>7</td>
<td>78</td>
</tr>
<tr>
<td>Chemotherapy (platinum/non-platinum)</td>
<td>9 (9/0)</td>
<td>100</td>
</tr>
</tbody>
</table>

* ‘Others’ included two embryonal carcinomas and one mixed type with DG and YST components.

USO = unilateral salpingo-oophorectomy, TAH = total abdominal hysterectomy, BSO = bilateral salpingo-oophorectomy

2. Tissue samples (I–V)

2.1. Human malignant ovarian germ cell tumor samples

Approval for the study was obtained from the Ethics Committee of the Department of Obstetrics and Gynecology, University of Helsinki, and from the National Authority for Medicolegal Affairs. Tumor samples (n = 14), originally collected for diagnostic purposes at the Department of Obstetrics and Gynecology, Helsinki University Central Hospital, between 1982 and 2002 were used for the present study.

2.2. Human fetal testicular, testicular CIS and tumor samples

The Regional Committee for Medical Research Ethics in Denmark approved the use of anonymised human tissue samples for gene expression studies.

Ten normal fetal testicular samples (gestational weeks 15–41) were obtained from tissue
archives of the Department of Pathology, Rigshospitalet, Copenhagen, Denmark. The tissues had been stored after autopsy of material from induced or spontaneous abortions and stillbirths, mainly in connection with placental or maternal problems. Fetal age was estimated from the date of the last menstrual bleed, and the developmental stage was based on the foot size of the fetus. Specimens of testicular tumors and the adjacent tissue, which usually contains CIS, were obtained after orchidectomy performed for therapeutic purposes. The tissue sections were fixed in either buffered formalin or paraformaldehyde and subsequently embedded in paraffin. In total, 10 CIS and 11 tumor samples (6 seminomas and 5 non-seminomas) were analyzed by immunohistochemistry.

3. Human germinoma-derived cell line (II, III, V)

The NCC-IT human germinoma cell line (Teshima et al. 1988) was obtained from The American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (Gibco, Invitrogen Corporation, Carlsbad, CA, USA) supplemented with fetal bovine serum (10 %) and penicillin-streptomycin (1 %).

4. Immunohistochemistry (I–IV)

The primary antibodies used for immunohistochemistry are presented in Table 8. The anti-SNURF antibody has been described previously (Hakli et al. 2005). The monoclonal antibody to AMH (MIS) was a gift from Dr. R. Cate (Biogen, Cambridge, MA, USA). The tissue sections from paraffin-embedded tissue blocks were deparaffinized and rehydrated using descending concentrations of ethanol. Antigen retrieval was performed by incubating the slides in 0.1 M citric acid (pH 8.0) at approximately 100 °C for 20 min. Endogenous peroxidase activity was blocked with 3% H₂O₂ in water for 5 min. Incubation with the primary antibody was carried out overnight at 4 °C. An avidin-biotin immunoperoxidase system was used to visualize bound antibody (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA), with 3,3’-diaminobenzidine (Sigma, St. Louis, MO, USA) or aminoethyl carbazole (Zymed, San Francisco, CA, USA) as chromogens. The sections were counterstained with hematoxylin. In negative control experiments PBS replaced the primary antibody.

After immunohistochemical staining, sections were analyzed under a light microscope. Scoring of the antigens was based on the staining intensity of at least 10 % of the tumor cells.
Table 8. Antibodies used in immunohistochemistry.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Company</th>
<th>Catalog#</th>
<th>Host</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-2γ</td>
<td>Santa Cruz</td>
<td>sc-12762</td>
<td>Monoclonal Mouse</td>
<td>1:200</td>
</tr>
<tr>
<td>BMP-2</td>
<td>Santa Cruz</td>
<td>sc-6895</td>
<td>Polyclonal Goat</td>
<td>1:100</td>
</tr>
<tr>
<td>ERα</td>
<td>DakoCytomation</td>
<td>M7047</td>
<td>Monoclonal Mouse</td>
<td>1:50</td>
</tr>
<tr>
<td>ERβ</td>
<td>AbD Serotec</td>
<td>MCA1974S</td>
<td>Monoclonal Mouse</td>
<td>1:50</td>
</tr>
<tr>
<td>FOG-2</td>
<td>Santa Cruz</td>
<td>sc-10755</td>
<td>Polyclonal Rabbit</td>
<td>1:100</td>
</tr>
<tr>
<td>GATA-4</td>
<td>Santa Cruz</td>
<td>sc-1237</td>
<td>Polyclonal Goat</td>
<td>1:200</td>
</tr>
<tr>
<td>GATA-6</td>
<td>Santa Cruz</td>
<td>sc-9055</td>
<td>Polyclonal Rabbit</td>
<td>1:500</td>
</tr>
<tr>
<td>HNF-4</td>
<td>Santa Cruz</td>
<td>sc-6566</td>
<td>Polyclonal Goat</td>
<td>1:200</td>
</tr>
<tr>
<td>Ihh</td>
<td>Santa Cruz</td>
<td>sc-1782</td>
<td>Polyclonal Goat</td>
<td>1:100</td>
</tr>
<tr>
<td>INHα</td>
<td>AbD Serotec</td>
<td>MCA951S</td>
<td>Monoclonal Mouse</td>
<td>1:500</td>
</tr>
<tr>
<td>Oct-3/4</td>
<td>Santa Cruz</td>
<td>sc-8629</td>
<td>Polyclonal Goat</td>
<td>1:1000</td>
</tr>
<tr>
<td>SF-1</td>
<td>ABR Affinity</td>
<td>PA1-24565</td>
<td>Polyclonal Rabbit</td>
<td>1:2000</td>
</tr>
</tbody>
</table>

5. Western blotting (V)

Protein from cultured NCC-IT cells was extracted using a commercial NucleoSpin® RNA/protein kit (Machery-Nagel, Düren, Germany). Total protein (5 µg) was separated by 7.5 % SDS-PAGE and transferred to PVDF membranes. Non-specific binding was blocked with 5 % nonfat milk in 0.1 % Tween-TBS buffer for 1 h. The membrane was incubated overnight with primary (AP-2γ) antibody, followed by secondary antibody and subsequent visualization by using Enhanced Chemiluminescence Plus Kits (Amersham Biosciences Inc., Arlington Heights, IL). Beta-actin was used as a loading control.

6. Semi-quantitative RT-PCR (III, V)

Cells were collected at different time points (24–120 h) after stimulation, and total RNA was isolated using RNeasy kits (Qiagen, Hilden, Germany). Purified RNA (1 µg) was reverse-transcribed and 2 µL of the reaction mixture was used for each PCR. The primers and thermal cycler conditions are presented in Table 9. The numbers of cycles used for PCRs were 32 for ERα, 32 for ERβ, 30 for SNURF, and 30 for β-actin. Agarose gel electrophoresis (2 %), run in the presence of SybrSafe™ DNA gel stain (Invitrogen, Carlsbad, CA, USA) demonstrated PCR products of expected size (Figure 2A, B) for each of the PCR primer pairs. Threshold cycles were normalized to those for β-actin. The intensities of the bands were analyzed by using the Bio-Rad Gel Doc program (Bio-Rad Laboratories, Hercules, CA, USA) and quantified by using Quantity One software (Bio-Rad Laboratories).
Table 9. Primers used in semiquantitative PCRs.

<table>
<thead>
<tr>
<th>Forward</th>
<th>Reverse</th>
<th>Expected size (bp)</th>
<th>Annealing T (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AP-2γ 5’-gacgccatgttgtgaaaataacc-3’</td>
<td>5’-gaaagtgagggtgcgcttgtat-3’</td>
<td>269</td>
<td>52</td>
</tr>
<tr>
<td>ERα 5’-gagacatgagagctgccaac-3’</td>
<td>5’-ccacgcagctgtaaagct-3’</td>
<td>439</td>
<td>70</td>
</tr>
<tr>
<td>ERβ 5’-ggccgacaaggaggttggt-3’</td>
<td>5’-ttcatgccttggtaactc-3’</td>
<td>519</td>
<td>70</td>
</tr>
<tr>
<td>SNURF 5’-tgactacccatactccgacaagcc-3’</td>
<td>5’-gcgtgtctgctgctcatctctctc-3’</td>
<td>298</td>
<td>60</td>
</tr>
<tr>
<td>β-actin 5’-cgggaaatcgcgtgacattaaag-3’</td>
<td>5’-ttcgtgatgcgcacagactcc-3’</td>
<td>213</td>
<td>72</td>
</tr>
</tbody>
</table>

7. Stimulation of NCC-IT cells with estradiol (III)

The cells were plated on 12-well dishes at a density of 25 × 10⁵ per well and incubated in RPMI-1640 (phenol red-free) supplemented (10 %) with dextran-charcoal-treated fetal bovine serum (HyClone, Logan, UT, USA) for 48 h before stimulation. NCC-IT cells were stimulated with 17β-estradiol (E2) (Sigma, St. Louis, MO, USA) at concentrations of 1 to 1000 nmol/L for 24–120 h to determine time- and dose-responses for the expression of ERs and their co-activator SNURF. For control experiments, the anti-estrogen ICI 182,780 (Tocris Cookson Ltd., Bristol, UK) was added at a concentration of 1000 nmol/L simultaneously with 1 nM E2. The concentrations of E2 and anti-estrogen were based on those used in corresponding experiments by others (Martin et al. 2005). All incubations were performed in duplicate and repeated at least three times. Untreated and vehicle-only-treated controls were included in each experiment.

8. Cell transfection and siRNA (V)

NCC-IT cells were plated on 6-well plates 24 hours prior to transfection. They were subsequently transfected with Lipofectamine 2000 as instructed by the supplier (Invitrogen). Control cells were untreated or treated with Lipofectamine 2000. AP-2γ siRNA (small interfering RNA) was purchased from Thermo Fisher Scientific Inc. (Dharmacon Inc., Chicago, IL, USA). Transfection was performed for each experiment according to the supplier’s instructions, in antibiotic-free media. The media were changed 4 hours after transfection. The cells were collected for RNA and protein analyses 24 hours after transfection.

9. Flow cytometric analysis (III, V)

To analyze cell proliferation, an APC BrdU Flow Kit (BD Biosciences, San Jose, CA, USA) was used, following the staining protocol described by the manufacturer. After E2 or anti-estrogen (ICI 182,780) stimulation, the cells were labelled with BrdU (an analog of the DNA precursor thymidine) at a concentration of 10μmol/L. Cells were harvested at different time points (6 to 48 h), fixed, and stained with an anti-BrdU-specific APC fluorochrome according to the manufacturer’s protocol. Cells with incorporated BrdU, indicating proliferating cells, were analyzed by flow cytometry (FACSAria, BD Biosciences).
10. Statistical analysis (II, III)

The probability of survival was estimated by the Kaplan–Meier method. When analyzing overall and disease-specific survival, the event was death. Data collected after cell stimulation is expressed as mean ± SEM derived from three independent experiments, all with two replicates, and adjusted to a value of 1.0 for the mean of the first control culture. The E2 and ICI 182,780 stimulation data were analyzed by using Student’s t test. A p value < 0.05 was considered statistically significant. The statistical software used was SPSS, versions 13.0–16.0 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

1. Biology of gonadal germ cell tumors

1.1 Pluripotency and self-renewal in ovarian germ cell tumors (II, IV)

The origin of germ cell tumors is suggested to be the early primordial germ cell. Primordial germ cells resemble embryonic stem cells, which are characterized by pluripotency and self-renewal capacity. AP-2γ and Oct-3/4 play key roles in embryonic stem cell survival and pluripotency (Hansis et al. 2000, Pauls et al. 2005). Thus, it may be speculated that these factors also play roles in germ cell tumorigenesis. In the present work AP-2γ and Oct-3/4 were analyzed in the MOGCT samples and both of these factors were present in DGs but mostly absent in YSTs and ITs (Figure 6, Table 10) (II). Expression of these pluripotent markers suggests that DGs represent primitive germ cell tumors. In contrast, pluripotency is lost in more differentiated MOGCT subtypes such as yolk sac tumors. The expression of these pluripotent markers in early fetal gonadal germ cells and in dysgerminomas may indicate that this tumor type originates from early fetal germ cells.

<table>
<thead>
<tr>
<th>Markers of pluripotency</th>
<th>Markers of endodermal differentiation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AP-2γ</td>
</tr>
<tr>
<td>Dysgerminoma</td>
<td>+</td>
</tr>
<tr>
<td>Yolk sac tumor</td>
<td>-</td>
</tr>
<tr>
<td>Immature teratoma</td>
<td>+/-</td>
</tr>
</tbody>
</table>

In addition to the pluripotent factors Oct-3/4 and AP-2γ, self-renewal factors may also play a role in germ cell tumorigenesis. The protein BMI1 is involved in self-renewal of stem cells (Park et al. 2003). In mice BMI1 is expressed in undifferentiated spermatogonia and its overexpression increases their proliferation (Zhang et al. 2008b). BMI1 is also expressed in human testicular germ and Sertoli cells (Sanchez-Beato et al. 2006). However, in testicular cancer BMI1 is expressed in only 30% of the tumors (Sanchez-Beato et al. 2006). In contrast, in the
The present study BMI1 was expressed in all histological subtypes of MOGCTs and in all samples (Figure 6) (unpublished results). Thus, the proto-oncogene BMI1 might play a role as a germ cell self-renewal factor, allowing malignant germ cell tumor development.

Figure 6. Expression of AP-2γ, Oct-3/4 and BMI1 in ovarian dysgerminomas (left column A, C, E), and yolk sac tumors (right column B, D, F). Scale bar 200 µm, original magnification x10. Lines one and two are reprinted from a publication in Tumor Biology (2008), Salonen et al., with permission from S. Karger AG, Basel (II). Line three presents unpublished data based on the present study.

1.2 CIS and fetal origin of testicular germ cell tumors (IV)

Carcinoma in situ cells represent the origin of most testicular germ cell tumors. These cells resemble fetal gonocytes and embryonic stem cells. GATA-4 is an essential transcription factor in testicular development. In the present study the role of GATA-4 was elucidated in fetal testicular cells and in testicular cancer precursor cells (IV). The present study confirmed GATA-4 expression both in fetal and adult Sertoli and Leydig cells (IV). Similarly to its expression in somatic cells of the fetal testis, it is also expressed in fetal ovarian granulosa cells (Vaskivuo et al. 2001). A subset of early fetal testicular germ cells at the 15th gestational week expressed GATA-4 (Table 11, Figure 7) (IV). Of the early fetal gonocytes, 72–81 % expressed GATA-4. GATA-4 was not, however, expressed later in fetal or in adult germ cells (IV). In contrast to early fetal testicular gonocytes, fetal oocytes do not express GATA-4 (Vaskivuo et al. 2001). These data indicate that GATA-4 may have a function in early fetal gonocyte development,
possibly in connection with their differentiation. In contrast to germ cells, in testicular Sertoli and Leydig cells GATA-4 has functions throughout fetal and adult life (IV). Similarly to GATA-4, GATA-6 was expressed in fetal Sertoli cells and in adult somatic cells (IV). However, GATA-6 was not found in testicular fetal and adult germ cells (IV). Thus, GATA-6 has a role only in testicular somatic cells but not in germ cell function. Taken together, although germ cells develop from similar primordial germ cells in both sexes, different transcription pathways are expressed in female and male germ lines during development.

**Figure 7.** Expression of GATA-4 in fetal gonocytes (A) at the 15th gestational week (arrow heads) and (B) at the 20th gestational week. Expression of GATA-4 (C) and FOG-2 (D) in testicular CIS cells (arrow heads). Modified from Salonen et al., submitted (IV).

Carcinoma *in situ* cells expressed GATA-4 in heterogeneous manner, with 24 to 93 % (mean 68 %) of the cells showing positive expression of immunohistological staining. When present, the type of adjacent tumor (seminoma or non-seminoma) was not associated with the amount of GATA-4-positive CIS cells, although the lowest degree of positivity (24 %) was seen in a CIS specimen not adjacent to any tumor type. Thus, GATA-4 expression in early fetal gonocytes and its absence later in fetal and adult germ cells provide additional evidence that CIS cells originate from early fetal gonocytes. The developmental down-regulation of GATA-4 in germ cells is similar to AP-2γ down-regulation during fetal development. However, GATA-4 was already absent by the 17th gestational week (IV), in contrast to AP-2γ, which is down-regulated later (after the 22nd gestational week) (Hoei-Hansen et al. 2004). Thus, the present data provide further evidence of the very early development of CIS cells during the first 15 gestational weeks.
Table 11. GATA-4 expression in fetal and adult testicular tissue. Based on the present study (IV).

<table>
<thead>
<tr>
<th></th>
<th>FETAL</th>
<th></th>
<th>ADULT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>15th gest. week</td>
<td>17th gest. week</td>
<td></td>
</tr>
<tr>
<td>Germ cell</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sertoli cell</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Leydig cell</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

1.3 Function of GATA-4 in fetal testicular and CIS cells (IV)

GATA-4 plays a role in testicular development. Thus, GATA-4 target genes as well as GATA-4’s co-factor FOG-2 are essential in gonadal differentiation and hormonal regulation. In the present study, FOG-2 was expressed in fetal somatic Sertoli and Leydig cells (IV). In addition, the GATA-4 downstream target genes SF-1 and INHα were expressed in fetal as well as in adult somatic cells (IV), as expected (Rabinovici et al. 1991, Hanley et al. 1999). Another GATA-4 target gene, AMH, was expressed in fetal and immature Sertoli cells (IV), as also reported previously (Tilmann et al. 2002). Thus, GATA-4 may have different functions in various testicular cells during different stages of development, as shown by differential expression of its target genes. In addition to GATA-4, AMH is regulated by SRY and Sox9 (Figure 2) (de Santa Barbara et al. 2000). Thus, besides GATA-4, additional upstream regulators of GATA-4 target genes may play important roles during different developmental stages of gonadal development.

Of the GATA-4 target genes, SF-1 was heterogeneously expressed in CIS cells. However, other GATA-4 target genes, AMH and INHα, were mostly negative in CIS specimens. Thus, in testicular CIS cells at least one of the GATA-4-regulated genes was expressed, supporting the concept of an active role for GATA-4 in driving target gene transcription in CIS cells.

Given that interaction of GATA-4 and FOG-2 is essential in normal testicular development and function (Tevosian et al. 2002), their expression in CIS samples (90 %) provides evidence of the normal function of GATA-4 in these cells (Figure 7) (IV). In contrast, FOG-2 was expressed only in some of the seminomas (33 %), whereas GATA-4 protein was present in all studied seminomas (IV). There may well exist some unknown cofactors in addition to FOG-2 functioning in these germ cell tumors in concert with GATA-4. It is plausible that GATA-4 may not regulate its normal target genes during testicular tumor development, in the absence of FOG-2.

Steroidogenic factor-1 is expressed in mouse primordial germ cells (Hinshelwood et al. 2005). In contrast, in the present study SF-1 was not expressed in human fetal gonocytes (IV). Moreover, SF-1 expression was absent in all non-seminomas and most seminomas, but it was expressed in CIS cells (IV). In normal fetal and adult Sertoli and Leydig cells SF-1 was expressed (IV). Thus, the expression of SF-1 in CIS cells and its absence in germ cells provide evidence of abnormal differentiation. CIS cells develop from primordial germ cells, but along with their development the normal gene expression pattern may be disturbed, as shown by the Sertoli
cell-like expression of SF-1.

1.4 Estrogen signalling pathway in MOGCTs (III, V)

It has been suggested that estrogens have a role in testicular germ cell tumorigenesis during embryogenesis (Toppari et al. 1996, Skakkebaek et al. 2001). We speculated that estrogens could also have a role in the formation of ovarian germ cell tumors. The peak incidence of MOGCTs occurs soon after puberty, thus activation of the pituitary-ovarian axis causing a steroid hormone burst may be hypothesized to stimulate the formation of these tumors. As ERα and ERβ are expressed in fetal oocytes (Vaskivuo et al. 2005), they may also be present in germ cell tumor precursor cells. In line with our hypothesis, both estrogen receptors were expressed in oocytes of normal ovaries and all MOGCT subtypes (III), providing the possibility of estrogen action in these tumors.

Estrogen actions are modified by various co-regulators. SNURF is an ER co-activator and it is expressed in murine fetal and postnatal germ cells (Hirvonen-Santti et al. 2004). In the present study, expression of SNURF was localized to human oocytes and all MOGCT subtypes (III). Thus, by regulating steroid hormone action, SNURF may contribute to the transformation of an oocyte into a germ cell tumor. Ovarian dysgerminomas expressed SNURF (III), which is in contrast to the situation in a previously reported study of testicular seminomas, with nearly absent expression of SNURF (Hirvonen-Santti et al. 2003). Thus, the function of ERs may differentially be altered by different co-regulators in various gonadal germ cell tumors.

Estrogen receptor beta has been suggested to have a suppressive role in various tumors. In epithelial ovarian cancer expression of ERβ is down-regulated, whereas that of the ERα is similar or increased when compared with normal ovary (Bardin et al. 2004). In the present study, the expression of ERβ was stronger than that of ERα in most of the MOGCTs (III). Thus, in contrast to epithelial ovarian cancer, ERβ may not play a suppressive role in germ cell tumorigenesis (Lazennec et al. 2001). The putative effect of estrogens on the development of ovarian tumorigenesis may also depend on the age of the subject. The use of oral contraceptives in fertile women has a protective effect against ovarian epithelial cancer, whereas in postmenopausal women the use of estrogen products may increase the risk of ovarian cancer (Rossing et al. 2007, Lurie et al. 2008). In the case of germ cell tumors the possible effect of estrogen on primordial germ cells occurs early in life, maybe even during fetal life. Thus, estrogens may have a different role and effect on the young female gonad and germ cell tumorigenesis compared with epithelial ovarian cancer in elderly women.

To study the effects of estrogens on germ cell tumors in vitro, stimulation studies were performed using the human germinoma-derived NCC-IT cell line. In line with the results on the tumor samples, both ERα and ERβ and their co-activator SNURF were expressed in NCC-IT cells (III). Stimulation of NCC-IT cells with estradiol increased the expression of both ERs (Figure 8) and the increased expression was counteracted by concomitant treatment with anti-estrogen (III). However, E2 did not have an effect on proliferation of NCC-IT cells; nor did the anti-estrogen (III). This could be due to ERs counteracting the effects of each other. Similarly
as in murine ovaries (Hirvonen-Santti et al. 2004), SNURF expression was up-regulated in NCC-IT cells treated with estradiol in the present study (III). Thus, SNURF may play a role in the estrogen signalling pathway in germ cell tumors (Figure 8). In addition to its genomic actions, estradiol has more rapid non-genomic actions through membrane-mediated activation of extracellularly regulated kinase and protein kinase A, resulting in stimulation of proliferation of seminoma cells (Bouskine et al. 2008). Thus, also the non-genomic actions of estradiol may differ from one cell type to another.

Figure 8. Effect of estradiol (10 nM) on the expression of ERα, ERβ and SNURF in NCC-IT cells after 24 to 72 hours of treatment. The data are expressed as mean ± SEM derived from three independent experiments (* p < 0.05, ** p < 0.005, C: control). Reprinted from a publication in Molecular and Cellular Endocrinology (2009), Salonen et al., with permission from Elsevier (III).

Estrogen signalling has an effect on the expression of AP-2γ in breast and prostate cancer cells (Orso et al. 2004, Zhang et al. 2007). In breast cancer cells estrogen increases AP-2γ expression (Orso et al. 2004). In prostate cancer cells AP-2γ is an upstream regulator of ERβ (Zhang et al. 2007). Similarly, as with the expression of ERs and SNURF, the expression of AP-2γ in NCC-IT cells was enhanced in response to E2 treatment (unpublished data, Figure 9) (V). The data provide further evidence of estrogen-modulated regulation of AP-2γ in germinoma cells. siRNA-mediated knockdown of AP-2γ reduced its protein expression levels by approximately 70 % (Figure 9) (V). Moreover, proliferation of germinoma cells was reduced by approximately 40 % following silencing of AP-2γ with siRNA (unpublished data) (V). Thus, estrogen signalling in germinoma cells may also act via the AP-2γ pathway. The estradiol peak at puberty can be speculated to up-regulate ERβ, and also AP-2γ, which is essential for primordial germ cell survival (Hoei-Hansen et al. 2004). Survival of primordial germ cells in turn may enable the formation of germ cell tumors.
Figure 9. A. Stimulation with estradiol increased the expression of AP-2γ mRNA in NCC-IT cells. B. Blocking of AP-2γ by siRNA in NCC-IT cells had an effect on its protein expression as analyzed by Western blot. Analysis of the intensity of the bands was based on visual evaluation. C: control, Tr: transfection vehicle control. Based on the present study (unpublished data; V).

2. Diagnosis and prognosis of gonadal germ cell tumors

2.1 Diagnostic tools for gonadal germ cell tumors (I, II, IV)

Preoperative diagnosis of gonadal germ cell tumors is based on clinical findings, imaging studies (ultrasonography) and assay of serum tumor markers. Final histopathological diagnosis is made by morphological examination of the tumor after primary surgery. Gonadal germ cell tumors typically occur among young males and females; thus a germ cell tumor is to be considered in differential diagnosis in the case of a young patient presenting with suspected gonadal tumor. The serum tumor markers AFP and hCGβ are of value in diagnosis of the various germ cell tumor subtypes (Gershenson 1993). Concentrations of AFP are elevated mainly in cases of yolk sac tumors, whereas those of hCGβ are elevated in patients with choriocarcinomas and embryonal carcinomas. In the present study levels of serum AFP were elevated in half and those of hCGβ in one third of the MOGCT patients (II). In concordance with previous data, AFP levels were elevated in yolk sac tumor patients, whereas hCGβ levels were abnormally high in embryonal carcinoma patients (II).

Serum concentrations of the commonly used marker in cases of epithelial ovarian cancer, CA 125, were elevated in more than half of the MOGCT patients (II). In a previous study levels of CA 125 were elevated in 40 % of MOGCT patients (Deligeoroglou et al. 2004). In the present study, CA 125 levels were elevated in the majority of the yolk sac tumor (67 %) and immature teratoma (60 %) patients, but in fewer than half of the dysgerminoma (43 %) patients (II). Assay of CA 125 is probably not of value in the differential diagnosis of malignant ovarian tumors given that its levels are also elevated in cases of the more common epithelial ovarian cancer as well as in some noncancerous gynecological conditions such as pregnancy, endometriosis, pelvic inflammatory disease and some diseases resulting in the presence of ascites (Halila et al. 1986, Halila et al. 1988, Perkins et al. 2003).

Most of the tumors associated with elevated serum CA 125 concentrations also expressed CA 125 antigen in the tumor tissue (II). However, some tumor samples collected from patients with
increased serum CA 125 levels did not reveal any immunohistochemically detectable CA 125 (II). Thus, the lack of CA 125 expression in tumor tissues despite positive serum findings suggests that sources other than the tumor itself, such as the peritoneal mesothelial cells, may be responsible for the secretion of CA 125 into the circulation, as a non-specific reaction to tumor growth.

In addition to serum markers, tissue markers were evaluated. Similarly to what has been reported previously (Cheng et al. 2004), we found that immunohistochemical detection of Oct-3/4 was typical of dysgerminomas (Table 10) (II). In addition, another marker of pluripotency, AP-2γ, was specific for DGs (Figure 6, Table 10) (II). Thus immunohistochemical expression of Oct-3/4 and AP-2γ may be used for differential diagnosis of MOGCTs.

Markers specific for endodermal differentiation, including GATA-4, GATA-6, HNF-4, BMP-2 and Ihh, were all expressed in ovarian yolk sac tumors (I). In addition, GATA-4 was also expressed in DGs (Table 10) (I). GATA-4 and GATA-6 are essential factors in normal yolk sac development, and the present results, therefore, suggest that YST cells have maintained capacity of GATA-4 and GATA-6 production. Given that GATA-6 and HNF-4 were present only in yolk sac tumors (I), they could be of value in histological diagnosis of this germ cell tumor subtype.

Testicular seminomas and ovarian dysgerminomas are considered to be counterparts. In line with the expression of GATA-4 in ovarian DGs, testicular seminomas expressed GATA-4 (Table 12) (IV). In contrast, ovarian DGs were GATA-6-negative, whereas most of the testicular seminomas (67%) were GATA-6-positive (I, IV). In addition, GATA-6 was absent in all non-seminomas (IV). This data on the differential expression of the transcription factor GATA-6 in ovarian DGs and testicular seminomas provides evidence of variation in the transcriptional pathways in these tumors, although they have been considered to be close female-male counterparts.

In contrast to testicular seminomas and immature teratomas, embryonal carcinomas were totally devoid of detectable expression of GATA-4 (IV). Moreover, none of the target genes (AMH, SF-1, INH-α) were expressed in embryonal carcinomas, nor were GATA-6 or FOG-2 (IV).

Table 12. Comparison of GATA-4, GATA-6 and FOG-2 expression in ovarian and testicular germ cell tumors as detected by immunohistochemistry. Based on the present study (I, IV).

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>GATA-4</th>
<th>FOG-2</th>
<th>GATA-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysgerminoma</td>
<td>+/-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Seminoma</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
<tr>
<td>Yolk sac tumor (ov)</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Embryonal ca (te)</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Immature teratoma (ov)</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Immature teratoma (te)</td>
<td>+/-</td>
<td>+/-</td>
<td>+/-</td>
</tr>
</tbody>
</table>

ov, ovarian; te, testicular
2.2 Prognostic tools for malignant ovarian germ cell tumors (II)

Since the introduction of platinum-based combination chemotherapy, MOGCTs have changed dramatically from deadly to curable disease. Currently, the 5-year survival rate approaches 100% (Pectasides et al. 2008). Among the present subjects, survival was somewhat lower (73 %) than in other recent studies (Table 13) (II). This could be a result of the low number of patients studied. In addition, in the present study the mean age of the patients was somewhat higher than in previous studies (II), thus possibly affecting the overall survival rate. However, different histological subclasses show some variation as regards survival rate, YSTs being associated with modest survival. In the present study, patients with DGs had the highest survival rate (II), in line with the results of previous studies (Table 13).

The main prognostic factors as regards ovarian germ cell tumors have been histological type, surgical stage and the presence of residual tumor tissue (Mitchell et al. 1999, Nawa et al. 2001, Lai et al. 2005). Levels of the serum tumor markers AFP and hCGβ are often elevated in patients with MOGCTs. AFP and hCGβ cannot be used as prognostic markers for all subtypes of MOGCTs, given their specificity to yolk sac tumors and choriocarcinomas, respectively. However, elevated levels of serum AFP and hCGβ have prognostic value in some cases of MOGCTs as assessed by univariate and multivariate analyses in a recent study (Murugaesu et al. 2006). Given that levels of AFP are elevated mostly in yolk sac tumors, a remarkably elevated AFP concentration (> 1000 kU/L) has had prognostic value in some studies (Mitchell et al. 1999, Nawa et al. 2001). In the present study the AFP level did not have a prognostic value (II), and others have reported similar findings (Mayordomo et al. 1994). The serum tumor marker CA 125 is associated with epithelial ovarian cancer and the preoperative serum CA 125 concentration is an independent prognostic factor (Cooper et al. 2002). In the present study 56 % of the MOGCT patients had elevated serum CA 125 levels (II). Moreover, increased preoperative serum CA 125 values were associated with poor prognosis (Figure 10) (II).

![Figure 10](image-url). Kaplan–Meier analysis of probability of survival of MOGCT patients. A. Normal vs. elevated preoperative serum levels of CA 125 (p < 0.05). B. Age < 30 vs. age ≥ 30 years (p < 0.05). Left figure reprinted from a publication in Tumor Biology (2008), Salonen et al., with permission from S. Karger AG, Basel (II).
In addition to an elevated serum CA 125 concentration, presence of residual tumor was associated with poor prognosis (II). The same observation has also been reported earlier (Lai et al. 2005). Also patients over 30 years of age had a more adverse outcome in the present study (Figure 9) (II). In contrast, age has not been a significant factor in some previous studies (Nawa et al. 2001). Most MOGCT patients are adolescents or young adults. In the present study the mean age (30.0 ± 4.9 [SD] years) of the patients was higher than in previous studies (Murugae-su et al. 2006) (II); thus the older age of the patients may explain the somewhat poorer prognosis seen among the present subjects.

Even though most MOGCT patients survive the disease, some patients still succumb to the malignancy. Novel prognostic markers may be of value in the identification of patients with more adverse outcome, so as to provide them with extended follow-up and/or non-traditional treatment modalities, e.g. high-dose chemotherapy, which has been used previously in cases of ovarian yolk sac tumors, with improved outcome (Kang et al. 2008).

Table 12. Comparison of recently reported series of patients with malignant ovarian germ cell tumors.

<table>
<thead>
<tr>
<th>Authors</th>
<th>Study years</th>
<th>n</th>
<th>Median age, years (range)</th>
<th>Mean or median FU, months (range)</th>
<th>SU (%)</th>
<th>DG (%)</th>
<th>IT SU</th>
<th>YST SU</th>
<th>MIX/OTH SU</th>
<th>Stage I/II/IV %</th>
<th>USO (%)</th>
<th>No CT (%)</th>
<th>Platinum-based CT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ayhan et al. 1995</td>
<td>1964-93</td>
<td>67</td>
<td>(Mean)26 (2-245)</td>
<td>58.5 (2-245)</td>
<td>60.6</td>
<td>48</td>
<td>19</td>
<td>15</td>
<td>18</td>
<td>66/4/30</td>
<td>42</td>
<td>49</td>
<td>28</td>
</tr>
<tr>
<td>Zanetta et al. 2001</td>
<td>1982-96</td>
<td>169</td>
<td>21 (8-41)</td>
<td>67 (8-180)</td>
<td>95</td>
<td>41</td>
<td>28</td>
<td>17</td>
<td>14</td>
<td>67/3/20</td>
<td>81</td>
<td>38</td>
<td>61</td>
</tr>
<tr>
<td>Tangir et al. 2003</td>
<td>1975-95</td>
<td>106</td>
<td>17 (6-49)</td>
<td>122 (24-384)</td>
<td>86.6</td>
<td>34</td>
<td>33</td>
<td>12</td>
<td>13</td>
<td>64/8/28</td>
<td>77</td>
<td>17</td>
<td>29</td>
</tr>
<tr>
<td>Lai et al. 2004</td>
<td>1984-03</td>
<td>93</td>
<td>23 (7-64)</td>
<td>66 (12-236)</td>
<td>97.4</td>
<td>34</td>
<td>31</td>
<td>25</td>
<td>10</td>
<td>63/3/16</td>
<td>94</td>
<td>31</td>
<td>NDA</td>
</tr>
<tr>
<td>Salonen et al. 2008</td>
<td>1980-04</td>
<td>31</td>
<td>26.5 (11-72)</td>
<td>92 (2-205)</td>
<td>73</td>
<td>37</td>
<td>33</td>
<td>20</td>
<td>10</td>
<td>71/6/23</td>
<td>63</td>
<td>13</td>
<td>93</td>
</tr>
<tr>
<td>Total</td>
<td>1964-04</td>
<td>538</td>
<td>22.1 (6-72)</td>
<td>88.6 (2-384)</td>
<td>84.3</td>
<td>37</td>
<td>30.7</td>
<td>16.7</td>
<td>14.3</td>
<td>68/4/18</td>
<td>70</td>
<td>28</td>
<td>58</td>
</tr>
</tbody>
</table>

FU = follow-up, SU = overall survival, DG = dysgerminoma, IT = immature teratoma, YST = yolk sac tumor, MIX/OTH = mixed or other, USO = unilateral salpingo-oophorectomy, RT = residual tumor, NDA = no data available, CT = chemotherapy
3. Clinical significance of the studies

Patients with malignant gonadal germ cell tumors are commonly young women and men. Treatment and prognosis of these tumors are strikingly different in comparison with other gonadal malignancies. This study was carried out to shed further light on the molecular basis of these tumors, and to improve the diagnosis and follow-up of these patients. One of the specific aims was to define novel diagnostic and prognostic markers of MOGCTs. In addition, new molecular pathways of potential importance in germ cell tumorigenesis were assessed. The data are ultimately needed also in the development of future therapies.

Conclusions and Future Perspectives

1.1 Conclusions

1. The expression of endodermal markers, the transcription factors GATA-4 and GATA-6, their cofactors and target genes was characterized in gonadal germ cell tumors. In ovarian germ cell tumors GATA-4 was expressed in dysgerminomas and yolk sac tumors, whereas GATA-6 was expressed only in yolk sac tumors. In addition, downstream endodermal target genes of GATA-4 HNF-4, BMP-2 and Ihh were expressed in yolk sac tumors, but were mostly absent in dysgerminomas. Seminomas, the testicular counterparts of dysgerminomas, also expressed GATA-4. In contrast to dysgerminomas, most of the seminomas also expressed GATA-6. Although gonadal germ cell tumors share some characteristics, these tumors are different as regards expression of some of the transcription factors possibly playing important roles in the formation and maintenance of different germ cell tumors.

2. Markers of pluripotency, AP-2γ and Oct-3/4, were specifically expressed in dysgerminomas, but were mainly absent from other types of ovarian germ cell tumor. Thus, they may be used as additional tools in the differential diagnosis of germ cell tumors. The expression of these markers provides also evidence of the pluripotent nature of dysgerminomas compared with other more differentiated germ cell tumor subtypes.

3. Both estrogen receptors (ERα and ERβ) and their co-activator SNURF were expressed in all ovarian germ cell tumor subtypes. Moreover, the human germinoma-derived cell line NCC-IT expressed both ERs and SNURF. Also estradiol treatment up-regulated mRNA expression of ERs and SNURF in these cells, effect which was counteracted by the anti-estrogen ICI 182,780. Collectively, the data suggest that estrogen signalling pathways may have roles in ovarian germ cell tumorigenesis.

4. In the testis, GATA-4 was expressed in early fetal germ cells at the 15th gestational week, being absent later in gestation and in adult germ cells. This transcription factor was also present in most of the premalignant CIS cells. GATA-4 was also expressed in testicular seminomas, but it was absent in embryonal carcinomas – tumors that have lost the germ cell phenotype. These findings suggest a role of GATA-4 in fetal germ cell differentiation, and they support the
concept of a fetal origin of testicular CIS cells.

5. Elevated preoperative levels of the serum tumor marker CA 125 were associated with adverse outcome in patients with malignant ovarian germ cell tumors. Assay of serum CA 125 provides a new tool for the evaluation and follow-up of patients with malignant ovarian germ cell tumors.

1.2 Future perspectives

Risk factors connected to ovarian germ cell tumors are still poorly understood. Epidemiological studies of ovarian germ cell tumors could be used to reveal novel risk factors. It would also be interesting to compare the female and male counterparts of gonadal germ cell tumors, i.e. dysgerminomas and seminomas, as regards epidemiological data as well as various underlying and associated conditions.

Estrogen pathways may play a role in the development of germ cell tumors. Given that various ER co-regulators modify ER actions, while the affinity for estradiol is equal, ERα- and ERβ-specific co-activators may be of interest, revealing possibly differential outcomes in the proliferation of germ cell tumors. Given that ERα and ERβ have different and opposite roles in estrogen signalling, it would be of great interest to study the possibly different roles of these receptors in germ cell tumors by using siRNA silencing of the gene products.

Various genes are involved in gonadal differentiation. GATA-4 is involved in testicular differentiation and function and it is expressed in germ cell tumors. Genes involved in ovarian differentiation such as WNT4, DAX1, FOXL2, RSPO1 could also play roles in gonadal germ cell tumor development, and their expression in these neoplasms should be studied.

To understand the causes behind germ cell tumors we need to learn more about factors involved in germ cell development, differentiation and migration. In future, iPS cells may provide tools for experimental models of germ cell tumors in vitro, revealing novel molecular pathways in normal embryogenesis and in addition, in tumorigenesis. However, germ cell tumor development is a combination of genetic, molecular and environmental factors. Thus, we should connect basic molecular studies and epidemiological studies in future to provide further evidence of germ cell tumor development, and the data may later lead to possible novel therapeutics and prevention of these tumors.
Acknowledgments

This study was carried out at the Research Laboratory of the Hospital for Children and Adolescents, Biomedicum Helsinki, and in the Departments of Gynecology and Obstetrics, and Children’s Hospital, University of Helsinki, Finland. I wish to acknowledge the heads of the departments: Dr. Jari Petäjä, Dr. Veli Ylitalo, Professor Maija Haukkamaa, Professor Mikael Knip, Professor Olli Jänne, Professor Olavi Ylikorkala, and Professor Jorma Paavonen for providing excellent research facilities.

I had the privilege to join the National Clinical Graduate School (CLIGS), which provided me with financial support and valuable scientific education. The Clinical Graduate School in Pediatrics/Gynecology and the Pediatric Graduate School are acknowledged for funding and education. The Finnish Medical Society Duodecim, the Finnish Gynecological Association, and Helsinki University Central Hospital Research Funds are acknowledged for financial support.

Professor Olli Carpén and Professor Eero Lehtonen are acknowledged for careful review of this thesis and their valuable comments. Members of my excellent follow-up group, Professor Olli Carpén and Professor Juha Tapanainen, are acknowledged for their constructive comments and support throughout my PhD project.

I owe my deepest gratitude to my supervisors, Professor Markku Heikinheimo and Adjunct Professor Oskari Heikinheimo. It has been a great privilege to work with two most encouraging and dedicated supervisors. Oskari encouraged me onto the path of this PhD project. His enormous positivity and great enthusiasm has guided me throughout this work. Markku led me into the world of laboratory science and introduced me to his laboratory group of FOGs. He has provided excellent research facilities for this project. Markku’s great attitude towards science, medicine, and life outside the lab, and his understanding of the balance of these three has inspired me throughout my journey.

I thank my outstanding collaborators, Docent Ralf Bützow for his most valuable knowledge of gynecologic pathology, Docent Arto Leminen for his excellent help with the tumor data, Professor Ulf-Håkan Stenman for his knowledge of serum tumor markers, Professor Jorma Palvimo for his help with the estrogen receptor study, Professor Jorma Toppari for his knowledge of the testis, Professor Ewa Rajpert-De Meyts for her excellent knowledge of testicular germ cell tumors, and Dr. Niels Garem, Dr. Susanna Mannisto, Docent Nina Nupponen and Dr. Kirmo Wartiovaara.

Very valuable and skillful technical assistance in the laboratory was provided by Taru Jokinen, Sari Linden, Tiuke Helmiö and Saija Kotola. Dr. Nick Bolton is acknowledged for English language editing of this thesis.

I have enjoyed my time in the lab with the great group of FOGs. Hanna Haveri has been most valuable and inspiring company, sharing great discussions. Helka Parvianinen has shared her valuable experience and advice. In addition to Helka and Hanna, Antti Kyrölähti, Mar-
jut Kauppinen, Mikko Anttonen, Anniina Färkkilä, Jenni Elo, ‘adopted’ Reeta Pökkylä, and
the ‘old FOGs’ Ilkka Ketola and Sanne Kiiveri, have shared the ups and downs of scientific
research and have made my days in the lab a pleasure. In addition, Noora, Saija and Sauli have
added to the great working atmosphere around the lab.

My dear friend Birgitta is thanked for all her tremendous support in work, science and other
aspects of life. Many of my friends have been of very great support and company over these
years. Sami, Mikko, Päivi and Hanna are especially thanked for excellent lunch breaks.

My dear friend Johanna is acknowledged for her help with editing this thesis. Johanna, Tomi
& Laura, Piku, Mervi and my mother-in-law Ellen have been most valuable, sharing their time
with my dear son Oliver.

I warmly thank my family for their support and strength throughout the years. I especially
thank my dear brother Anssi for his tremendous encouragement and endless conversations
about science on my PhD road.

Finally, I thank my husband Pasi for his love, support and positive attitude during this journey,
and my son Oliver for showing me a whole new way of approaching this world that we live in.

Jonna Salonen
Helsinki, September 2009
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