Molecular Studies on Pathogenesis, Prognostic Factors, and New Treatment Options for Ovarian Granulosa Cell Tumors

Anniina Färkkilä

Children’s Hospital
and
Department of Obstetrics and Gynecology
University of Helsinki
Finland

The National Graduate School of Clinical Investigation
and
Pediatric Graduate School

ACADEMIC DISSERTATION

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Supervisors

Professor Markku Heikinheimo, MD, PhD
Children’s Hospital, University of Helsinki
Helsinki, Finland

Docent Leila Unkila-Kallio, MD, PhD
Department of Obstetrics and Gynecology, Helsinki University Central Hospital
Helsinki, Finland

Docent Mikko Anttonen, MD, PhD
Department of Obstetrics and Gynecology, Helsinki University Central Hospital
Helsinki, Finland

Reviewers

Professor Seija Grénman, MD, PhD
Department of Obstetrics and Gynecology, Turku University Central Hospital
Turku, Finland

Professor Seppo Parkkila, MD, PhD
School of Medicine, University of Tampere
Tampere, Finland

Official opponent

Professor Olli Carpén, MD, PhD
Department of Pathology, University of Turku
Turku, Finland

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To my family
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Granulosa Cell Tumor (GCT) is a hormonally active and highly vascularized subtype of ovarian cancer, constituting 5% of all ovarian malignancies. GCTs are characterized by an indolent, albeit often unpredictable, course of disease, with a 5-year survival rate of over 90%. Recurrences occur in 20-30% of GCT patients, even in early-stage disease, and sometimes unexpectedly late after the primary tumor. Initial tumor stage is the only prognostic factor in GCTs, and molecular prognostic factors are lacking. Further, the treatment of advanced or recurrent GCT is difficult, leading to increased mortality and underscoring the need for biologically targeted treatments for aggressive GCTs.

GCTs are thought to arise from the proliferating granulosa cells of preovulatory follicles. The molecular mechanisms leading to GCT formation are likely to include regulators of granulosa cell proliferation and apoptosis; however, the pathogenesis of GCTs remains unknown. We studied regulators of granulosa cell function and tumor angiogenesis in GCT pathogenesis by utilizing tumor tissue and patient serum samples and cell culture assays. The objectives of this study were to find new molecular prognostic factors and to identify targets for new biological treatments for GCT.

Anti-Müllerian Hormone (AMH) is a crucial regulator of granulosa cell function that belongs to the large transforming growth factor-β (TGF-β) family of growth factors. GCTs express AMH and knockout mouse models targeting TGF-β/AMH signaling suggest that AMH acts as a growth inhibitor in GCT pathogenesis. We found that GCTs expressed the AMH receptors, with the AMH type II receptor (AMHRII) being characteristic of GCTs. AMH expression was decreased in large GCTs, and recombinant AMH inhibited growth of GCT cells in vitro. The results support the premise that AMH acts as a growth inhibitor in GCTs, and AMH and AMHRII emerge as targets for treatment of GCT.

Vascular Endothelial Growth Factor-A (VEGF) is a key factor in tumor angiogenesis and also regulation of granulosa cell proliferation and function in the ovary. VEGF has been successfully targeted in the treatment of several forms of cancer. We found that VEGF and its functional receptor VEGFR-2 are highly expressed in GCTs; VEGFR-2 was also expressed in the active, phosphorylated form. GCTs produced significant amounts of VEGF that could also be detected in the serum of GCT patients. In cell culture assays, the inhibition of VEGF by soluble anti-VEGF antibody (bevacizumab) inhibited growth and induced apoptosis of GCT cells. These results indicate an auto- or paracrine pro-tumorigenic role of VEGF in GCTs and encourage targeting VEGF and VEGFR-2 in the treatment of aggressive GCTs.

In search of new molecular prognostic factors, we utilized a tumor tissue microarray of 80 primary GCT patients. Transcription factor GATA4 was previously found to be associated with GCT pathogenesis and to delineate an aggressive subset of GCTs. Epidermal Growth Factor Receptors (EGFR/HERs) are regulators of normal granulosa cell function and overexpressed in many cancer types. HER2 is a known oncogene and a target for
treatment in breast and gastric cancer. The roles of HERs in GCT prognosis have thus far been unknown. We found, in contrast to previous studies, that initial tumor stage was not prognostic of tumor recurrence, and up to 20% of the stage Ia GCTs recurred. High expression of both GATA4 and HER2, and high nuclear atypia were prognostic of tumor recurrence, also in early-stage tumors. In multivariate analyses of molecular prognostic factors, GATA4 was superior to HER2, and high GATA4 expression led to a 4-fold increase in recurrence risk. GATA4 expression was also prognostic of shorter disease-specific survival along with higher tumor stage (II-III) and nuclear atypia. These results suggest that GATA4 could be used in prognostic assessment of GCTs, especially in early-stage GCTs.

Taken together, these studies have provided novel insight into the pathogenesis of GCTs and will potentially improve the prognostic evaluation and the development of biological treatment options for GCT patients.
List of original publications

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals. The original publications are reproduced with the permission of the original copyright holders. In addition, some unpublished data are presented.


* The authors contributed equally to the study.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AHR</td>
<td>adjusted hazard ratio</td>
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<tr>
<td>ALK</td>
<td>activin receptor-like kinase</td>
</tr>
<tr>
<td>AMH</td>
<td>anti-Müllerian hormone</td>
</tr>
<tr>
<td>AMHRII</td>
<td>anti-Müllerian hormone receptor II</td>
</tr>
<tr>
<td>Bcl2</td>
<td>B-cell lymphoma-2</td>
</tr>
<tr>
<td>BMP</td>
<td>bone morphogenic protein</td>
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<tr>
<td>BrdU</td>
<td>bromodeoxyuridine</td>
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<tr>
<td>BVZ</td>
<td>bevacizumab</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>DAPI</td>
<td>4′,6-diamino-2-phenylindole hydrochloride</td>
</tr>
<tr>
<td>DFS</td>
<td>disease-free survival</td>
</tr>
<tr>
<td>DSS</td>
<td>disease-specific survival</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>FCS</td>
<td>fetal calf serum</td>
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<tr>
<td>FFCS</td>
<td>female fetal calf serum</td>
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<tr>
<td>FOG-2</td>
<td>friend of GATA-2</td>
</tr>
<tr>
<td>FOXL2</td>
<td>forkhead box protein L2</td>
</tr>
<tr>
<td>FSH</td>
<td>follicle-stimulating hormone</td>
</tr>
<tr>
<td>GATA4</td>
<td>GATA binding protein 4</td>
</tr>
<tr>
<td>GCT</td>
<td>granulosa cell tumor</td>
</tr>
<tr>
<td>GDF</td>
<td>growth and differentiation factor</td>
</tr>
<tr>
<td>Her</td>
<td>heregulin</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>hypoxia-inducible factor 1α</td>
</tr>
<tr>
<td>HR</td>
<td>hazard ratio</td>
</tr>
<tr>
<td>IHC</td>
<td>immunohistochemistry</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>LH</td>
<td>luteiniing hormone</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>MVD</td>
<td>microvessel density</td>
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<tr>
<td>ng</td>
<td>nanograms</td>
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<tr>
<td>OR</td>
<td>odds ratio</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>pVEGFR-2</td>
<td>phosphorylated VEGFR-2</td>
</tr>
<tr>
<td>rhAMH</td>
<td>recombinant human AMH</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SF-1</td>
<td>steroidogenic factor-1</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-β</td>
</tr>
<tr>
<td>TRAIL</td>
<td>tumor necrosis factor-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>TTMA</td>
<td>tumor tissue microarray</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor-A</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>VEGF receptor-1</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>VEGF receptor-2</td>
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</table>
Introduction

Ovarian cancer is the fifth most common cancer in women, and, despite recent developments in diagnostics and treatments, the leading cause of death from gynecological cancer worldwide. Granulosa cell tumor (GCT) is the second most common subtype of ovarian cancer, representing 5% of all ovarian malignancies. GCTs are hormonally active and highly vascularized tumors that are characterized by an indolent course of disease, with a 5-year survival rate of over 90%. However, recurrences occur in 20-30% of patients, also those with early-stage disease, leading to higher mortality. The pathogenesis and factors affecting prognosis of GCTs are largely unknown.

GCTs are thought to arise from the rapidly proliferating granulosa cells of preovulatory follicles. During folliculogenesis granulosa cells proliferate and interact through endocrine and paracrine mechanisms to prepare the oocyte for fertilization. Initially, several primordial follicles are recruited to enter the rapid growth phase in which the granulosa cells proliferate. This phase is dependent on autocrine and paracrine actions of intraovarian factors and proceeds independently of pituitary gonadotropins. After one of the follicles is cyclically selected, the growth of the dominant follicle becomes dependent on gonadotropins and proceeds to ovulation and subsequent corpus luteum formation. The remaining secondary follicles undergo atresia, and the granulosa cells die of programmed cell death, i.e. apoptosis. The balance between granulosa cell proliferation and apoptosis is strictly controlled by several autocrine and paracrine growth factors such as follicle-stimulating hormone (FSH), anti-Müllerian hormone (AMH), and transforming growth factor-β (TGF-β).

Growth factors are naturally occurring proteins that act via a paracrine mechanism to promote cellular growth, proliferation, and differentiation. Growth factors bind to their specific receptors commonly located on the surface of the target cells. The binding of the growth factor to its receptor initiates a strictly controlled cascade of intracellular signals that are mediated through phosphorylation of specific amino acid residues on the receptors, signaling molecules and protein kinases. The target of these events is the activation of transcription factors, proteins that bind specific sites of DNA to initiate transcription of DNA to mRNA, leading to altered protein synthesis. This strictly controlled activation or inactivation of transcription factors determines cell-specific gene expression and protein synthesis, leading to altered cellular proliferation and differentiation.

It is commonly known that tumor formation occurs due to genetic changes that lead to growth advantage and the transformation of normal cells into malignant cells. This involves misregulation of cell proliferation and apoptosis, which are likely to be fundamental processes in GCT pathogenesis. This study focuses on investigating the expression and function of granulosa cell growth and transcription factors in GCT pathogenesis. The purpose of this study was to find new target molecules for prognostic evaluation and for the development of new treatment options for GCT patients.


Anti-Müllerian hormoni (AMH) on tärkeä granuloosasolujen säätelijähormoni. Tutkimustulosten mukaan AMH ilmentyi voimakkaasti pienissä GSK:ssa ja esti lisäksi kasvainsolujen kasvua osoittaen AMH:n toimivan kasvunrajoitettavaksiekä GSK:ssa. GSK:t ilmensivät vahvasti myös AMH:n reseptori II:ta, joka on mahdollinen kohde uusille syöpähoidoille.


GATA4 on tärkeä granuloosasolujen geeninsäätelijä, joka on tämän tutkimuksen tulosten mukaan uusi itsenäinen ennustetekijä GSK:ssa. Vahva GATA4:n ilmentymiseen liittyi nelinkertainen riski taudin uusiutumiselle sekä kohonnut riski myös tautisepsiselle kuolemalle. HER2 on tunnettu syöpägeeni ja munasarjan toiminnan säätelijä, jota vastaan on käytössä kohdennettuja syöpähoidoja. Tutkimustulosten mukaan HER2:n vahva ilmentyminen ennusti GSK:n aggressiivistä käyttäytymistä. HER2 on siten mahdollinen kohde biologisille syöpähoidoille myös GSK:ssa.

Review of the literature

Ovarian cancer is classified into three groups based on histopathological patterns that reflect the various cell types present in the ovary: epithelial ovarian tumors, sex cord stromal tumors, and germ cell tumors (Ries 2007). The majority (80-90%) of ovarian cancers are derived from the surface epithelium of the ovary: the epithelial ovarian tumors. Sex cord stromal tumors arise from the sex cord and stromal components and represent 8% of all ovarian tumors. Germ cell tumors are derived from the primordial germ cells of the embryonic gonad. Granulosa cell tumors (GCTs) are the predominant form of sex cord stromal tumors, representing 90% of this subgroup. Other subtypes of sex cord stromal tumors include thecoma-fibromas, Sertoli-Leydig cell tumors, gynandroblastomas, and sex cord tumor with annular tubules.

1. Granulosa cell tumors

GCTs are hormonally active ovarian neoplasms characterized by a long natural history and overall a favorable prognosis (reviewed in (Schumer 2003) and (Jamieson 2012)). GCTs are divided into two distinct subtypes based on histology and clinical characteristics: juvenile and adult GCT. Juvenile GCT (JGCT) comprises only 5% of all GCTs (Young 1984a). JGCT is generally diagnosed in children and adolescents, with a median age at diagnosis of 7-8 years (Calaminus 1997). The predominant presenting symptoms in JGCT are abdominal pain and endocrine manifestations, commonly precocious pseudopuberty. Juvenile GCT typically presents at an early stage and the prognosis is favorable, although at advanced stages the clinical course may be more aggressive (Young 1984a; Powell 1993; Calaminus 1997; Merras-Salmio 2002). Although extremely uncommon, the juvenile type can be found in adults and the adult type in children.

Adult GCT (hereafter referred to as GCT) comprises 95% of all GCTs and is the subject of this study. The incidence of GCT has generally been reported to be between 0.58 and 1.6/100 000 (Stenwig 1979; Bjorkholm 1981; Ohel 1983); in Finland, the incidence was reported to be 0.47/100 000 (Unkila-Kallio 1998). No specific risk factors are known for the development of GCT; menopausal status and parity (Evans 1980; Young 1984b; Malmstrom 1994) or the use of fertility drugs (Unkila-Kallio 2000) or oral contraceptives ("The reduction in risk of ovarian cancer associated with oral-contraceptive use." 1987) are not associated with the risk of GCT. Further, the risk of GCT is not associated with germline mutations of BRCA1 or BRCA2, unlike epithelial ovarian cancer (Koul 2000).

1.1 Clinical presentation and diagnosis

GCTs most commonly present during the perimenopausal or early postmenopausal period, with a median age at diagnosis between 50 and 54 years (Schumer 2003). At diagnosis,
the tumor is usually confined to one ovary, but may be large with a mean tumor diameter of 9-12 cm (Malmstrom 1994; Nosov 2009; Sun 2012). The vast majority (70-90%) of GCTs are diagnosed at Stage I (Malmstrom 1994; Auranen 2007; Ayhan 2009; Sun 2012) (Table 1).

Table 1. Staging of ovarian cancer according to the International Federation of Gynecology and Obstetrics (FIGO 2009)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
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<tr>
<td>Ia</td>
<td>The tumor is confined to the ovary/ovaries. Tumor is in one ovary and the ovary capsule is intact. No malignant cells in the abdominal cavity.</td>
</tr>
<tr>
<td>Ib</td>
<td>Tumor is in both ovaries but the ovary capsule is intact. No malignant cells in the abdominal cavity.</td>
</tr>
<tr>
<td>Ic</td>
<td>The tumor is limited to one or both ovaries. The ovary capsule is ruptured, tumor reaches the ovary surface, or malignant cells are detected in the abdominal cavity.</td>
</tr>
<tr>
<td>II</td>
<td>The tumor involves one or both ovaries and has extended to the pelvis.</td>
</tr>
<tr>
<td>III</td>
<td>The tumor involves one or both ovaries with microscopically confirmed peritoneal metastases outside the pelvis and/or regional lymph node metastasis. Includes liver capsule metastases.</td>
</tr>
<tr>
<td>IV</td>
<td>Distant metastasis beyond the peritoneal cavity, and liver parenchymal metastasis.</td>
</tr>
</tbody>
</table>

The most commonly presenting symptoms are abnormal uterine bleeding caused by the excess estrogen production by the tumor, and abdominal symptoms caused by a large pelvic mass. In premenopausal patients, GCTs may cause menstrual irregularities, menorrhagia, amenorrhea, and infertility (Unkila-Kallio 2000; Ayhan 2009; Sun 2012). In older women, postmenopausal bleeding is the most common symptom (Evans 1980; Ohel 1983; Cronje 1998). The tumor-produced estradiol may result in endometrial hyperplasia or even endometrial adenocarcinoma in 5-10% of patients (Fox 1975; Stenwig 1979; Evans 1980; Unkila-Kallio 2000; Auranen 2007). In addition, patients with GCT are at increased risk of breast cancer, with a reported incidence of 3.7-20% (Ohel 1983). GCTs are also highly vascularized and may present with acute abdominal pain and hemoperitoneum caused by tumor rupture (Fox 1975; Stenwig 1979; "Case records of the Massachusetts General Hospital." 1995; Poma 1998).

The gross appearance of a GCT is commonly a solid and cystic tumor mass in which the cyst may contain hemorrhagic fluid (Young 1992). At histological examination, GCT reveals a distinctive appearance (Figure 1 A), containing round to oval, pale cells with characteristic coffee bean grooved nuclei (Figure 1 B, arrowheads) and scarce cytoplasm. GCT presents with a variety of histological patterns, including both well-, and poorly differentiated histologies. The well-differentiated forms are further subdivided into microfollicular, macrofollicular, trabecular, insular, and tubular patterns (Young 1992) based on their appearance. The poorly differentiated forms are characterized by a diffuse (sarcomatoid) pattern and monotonous cellular growth (Figure 1 A). The histological diagnosis of GCT can be challenging, and especially the sarcomatoid subtype can be
mistaken for a poorly differentiated carcinoma on intraoperative frozen section. Detailed analysis and immunohistochemistry (IHC) for inhibin-α and cytokeratin are recommended in clinical pathology for confirmation of GCT diagnosis (Hildebrandt 1997; Cathro 2005; Nofech-Mozes 2012). In retrospective studies utilizing GCT tissue samples, histological re-evaluation of adult GCTs is evermore critical since the mis-diagnosis rates can be as high as 53% (Cronje 1999). Unlike carcinomas, GCTs usually present mild nuclear atypia and few mitotic figures (Young 1992).

Figure 1. *Histological appearance of GCT with hematoxylin staining. Appearance of a typical diffuse (sarcomatoid) subtype of GCT, arrows indicate blood vessels with lumen (A). Coffee bean-like grooved nuclei (arrowheads) in B. Scale bars: 100 µm in A and 50 µm in B.*

1.2 Treatment and follow-up

Surgery is the primary treatment option for GCT; the extent of surgery can be modified according to age and need to preserve fertility. The recommended form of surgery is total abdominal hysterectomy and bilateral salpingo-oophorectomy (SO), including staging procedures (Stuart 2003; Colombo 2007; Pectasides 2008). However, in stage Ia patients, unilateral SO appears to be an accepted course of action, especially in younger patients wishing to preserve fertility (Bjorkholm 1981; Zhang 2007; Lee 2008). The role of surgical staging is not as evident as in epithelial ovarian cancer, but at least peritoneal cytology is recommended (Stuart 2003). Occasionally peritoneal/para-aortic lymph node dissection, omentectomy, and peritoneal biopsies are performed (Schumer 2003), although the prognostic significance of these staging factors is not well defined (Lee 2008; Fotopoulou 2010; Thrall 2011). Adjuvant therapy is recommended only in advanced stages or recurrent disease (Stuart 2003).

The surgical treatment of recurrent GCTs is often challenging (Fotopoulou 2010), and patients commonly experience several relapses and are thus subjected to multiple operations and treatment modalities (Auranen 2007; Lee 2008). Most recurrences are
intra-abdominal, and hepatic metastasis is rather common (Auranen 2007; Lee 2008; Fotopoulou 2010), whereas lymphatic or distant metastasis is rarely seen. No standard guidelines exist for the treatment of recurrent GCT; however, it is commonly agreed that the removal of recurrent mass should be performed, and recurrent GCT patients show good survival after optimal cytoreduction with or without adjuvant chemotherapy (Al-Badawi 2002). Data on the efficacy of adjuvant therapies are limited due to lack of prospective randomized studies, and the current literature consists of case reports and small retrospective reviews of patient files (Auranen 2007). Chemotherapy treatment consists of platinum-based combination treatments, showing response rates of 60-90% (Homesley 1999; Pautier 2008). Radiotherapy and hormonal treatment (GnRH antagonists, tamoxifen, and aromatase inhibitors) are sometimes used (Kauppila 1992; Freeman 2006), although the long-term effects of these modalities remain unknown. The limited effects of current non-surgical treatments underscore the need for new biologically targeted treatments for advanced or recurred GCT patients.

In Finland, the follow-up of GCT patients has traditionally been similar to that of epithelial ovarian cancer patients, consisting of clinical controls at 3- to 12-month intervals over 5 years. After 3-5 years, hospital controls cease and patients are referred to a general physician or a private gynecologist. The clinical control comprises a physical examination combined with a pelvic ultrasound and blood tests/serum markers. X-ray or CT scans are used only with suspicion of recurrence. Inhibin B is the serum marker currently used in GCT patient follow-up, but it has certain limitations. Serum inhibin B levels can be elevated also in other ovarian tumors, especially in mucinous ovarian cancers (Robertson 2007), and inhibin B levels fluctuate during the menstrual cycle leading to false-positive results in premenopausal women. Further, normal inhibin B levels do not rule out ovarian malignancy (Mom 2007).

1.3 Survival and prognostic factors

In contrast to epithelial ovarian cancer, GCT is considered to be of low malignant potential and is characterized by a slow and indolent growth with a tendency towards late recurrence. The 5-year survival rates of stage I patients ranges from 75% to 95%, being over 90% in most series (Schumer 2003; Colombo 2007), and the 10-year survival is 84-95% (Schwartz 1976; Pankratz 1978). In stage II-IV patients, the survival rates are lower; 5-year survival ranges from 22% to 75% (Stenwig 1979; Bjorkholm 1981; Fujimoto 2001), and 10-year survival is 17-65% (Schwartz 1976; Pankratz 1978). The recurrence rates have varied from 10% to 30% in previous studies (Malmstrom 1994; Cronje 1998; Ayhan 2009; Nosov 2009), being around 20-25% in larger series with longer follow-up periods (Lee 2008; Sun 2012). The mean time to first recurrence has been reported to be 4-8 years (Evans 1980; Malmstrom 1994; Lee 2008; Sun 2012). However, Cronje et al. reported 17% of the recurrences to take place after 10 years (Cronje 1999), and recurrences 30-40 years after the diagnosis have been described (Hines 1996; East 2005). With recurrence, the mortality rises to 60-80% (Fox 1975; Cronje 1999).
Tumor stage at the time of diagnosis is the only factor explicitly related to survival (Miller 1997; Fujimoto 2001; Zhang 2007; Lee 2008; Miller 2008; Ayhan 2009; Sun 2012). Other prognostic factors have been difficult to establish, most likely due to the relative rarity of the disease and the long follow-up period required to include all potentially recurrent tumors. Rupture of the tumor capsule has been implicated as an adverse prognostic indicator, also in stage I GCT (Bjorkholm 1981; Costa 1996; Auranen 2007). Some studies have reported postoperative residual tumor to be a negative prognostic factor, as expected (Bjorkholm 1981; Costa 1996; Sehouli 2004; Auranen 2007; Lee 2008).

Large tumor size has been shown to be an adverse prognostic factor in several studies (Stenwig 1979; Bjorkholm 1981; Chan 2005; Ranganath 2008; Sun 2012), with critical sizes varying from 5 to 25 cm. However, the adjustment of tumor stage is not clearly defined in these studies. Further, many other studies have not found tumor size to be of prognostic significance (Malmstrom 1994; Cronje 1999; Anttonen 2005; Auranen 2007; Lee 2008; Nosov 2009).

Data on age at diagnosis as a prognostic factor are conflicting. Some authors describe an association between older age and poor prognosis (Stenwig 1979; Ohel 1983; Costa 1996), while others have noted that younger age is associated with a poor prognosis (Pankratz 1978; Nosov 2009). Further, no association of patient age with prognosis was found in other studies (Evans 1980; Miller 1997). Parity and reproductive status do not seem to influence outcome in GCTs (Fox 1975; Evans 1980).

Many studies have assessed the value of mitotic activity and nuclear atypia in GCTs, but again the data are conflicting. Some studies have found high mitotic activity to predict worse prognosis (Bjorkholm 1981; Malmstrom 1994; Fujimoto 2001; Miller 2001; Sehouli 2004), while others have contradicted this finding (Costa 1996; Anttonen 2005; Villella 2007; Leuverink 2008). Nuclear atypia has been shown to be associated with aggressive behavior in GCTs by some (Stenwig 1979; Bjorkholm 1981; Ohel 1983; Miller 1997), but not all authors (Kim 2006; Villella 2007). Disparities in these data are probably attributable to the highly subjective assessment of the degrees of mitotic activity and nuclear atypia, and the varying methodologies used. Many other factors related to cell proliferation or DNA integrity, such as Ki67, p53, and DNA aneuploidy, show conflicting data regarding prognosis (King 1996; Auranen 2007; Miller 2008; Pectasides 2008). The different histological subgroups are not associated with prognosis (Auranen 2007). Furthermore, a few other molecular markers have been investigated for prognostic significance, including members of the epidermal growth factor receptor (EGFR) family, tumor suppressor protein p53, and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) receptors (King 1996; Juric 2001; Nosov 2009; Kyronlahti 2010). Molecular prognostic factors are, however, still lacking, and the search is ongoing. Transcription factor GATA4 was reported to be associated with aggressive GCTs also after adjusting for tumor stage (Anttonen 2005), and GATA4 is thus far the most promising prognostic marker for GCT.
All in all, initial tumor stage remains the only solid prognostic factor in GCTs. However, the majority of GCT patients being diagnosed at stage I emphasizes the need for new molecular prognostic factors that are able to predict tumor recurrence. Moreover, molecular prognostic markers are required to identify the high-risk patients who would benefit from adjuvant treatment and an extended follow-up.

2. Pathogenesis

2.1 GCT pathogenesis

GCT cells exhibit many characteristics of proliferating granulosa cells of preovulatory follicles, including the production of estrogen and inhibin (Lappohn 1989; Amsterdam 1997; Lague 2008), expression of the FSH receptor (Fuller 1998), and GATA4 expression (Laitinen 2000; Anttonen 2005). The pathogenesis of GCTs remains largely unknown, but the molecular changes are likely to involve disruption of the signaling pathways that regulate granulosa cell proliferation and apoptosis.

2.1.1 FOXL2 mutation

Cytogenetic studies have shown that GCTs exhibit a relatively stable karyotype compared with other ovarian cancers (Lin 2005; Mayr 2008). Recent developments in highly efficient genomic analyses have allowed more detailed analysis of GCTs. Using whole-transcriptome RNA sequencing technology, Shah et al. identified a single somatic missense mutation in transcription factor FOXL2 (402C-G) in four GCTs (Shah 2009). The predicted consequence at the protein level was the substitution of a tryptophan residue for a highly conserved cysteine residue (C134W). Direct DNA sequencing revealed that the mutation was present in 97% of adult GCTs, as later confirmed by our group (Jamieson 2010) and others (Kim 2010a; Kim 2010b; Al-Agha 2011; Gershon 2011; Hes 2011). The lack of this mutation in juvenile GCTs, other sex cord stromal tumors, or other tumor types suggests that it is pathognomonic to adult GCT (Shah 2009; Jamieson 2010; Al-Agha 2011; Gershon 2011).

FOXL2 is a forkhead transcription factor that plays a crucial role in regulating follicular development in the normal ovary (Schmidt 2004; Uda 2004), and it is abundantly expressed in the granulosa cells of preovulatory follicles (Moumne 2008). During embryonic development FOXL2 regulates ovarian and granulosa cell differentiation (Schmidt 2004). In GCTs, the functional role of FOXL2 mutation is unraveled, but functional analyses suggest that FOXL2 acts as a tumor suppressor in normal granulosa
cells and that the C134W mutation impairs FOXL2’s ability to mediate apoptosis (Lee 2005; Kim 2011).

2.2 AMH and TGF-β signaling pathway

Anti-Müllerian Hormone (AMH), also known as Müllerian Inhibiting Substance (MIS), is a 140 kilo Dalton (kDa) dimeric glycoprotein that belongs to the large transforming growth factor-β (TGF-β) growth factor family. During embryonic development AMH is expressed solely in the Sertoli cells of the male, causing the embryonic regression of the Müllerian ducts, the precursors of the uterus, fallopian tubes and upper vagina (Lee 1993). In the male, Sertoli cells produce AMH during fetal development, and the testes continue the production throughout life, regulating Leydig cell steroidogenesis (Josso 2006). In the female, the sex-dimorphic pattern is lost postnatally and AMH is also expressed in granulosa cells of the ovary (Visser 2005). The biological effects of AMH are mediated through a transmembrane serine/threonine kinase type II receptor (AMHRII) that is specifically expressed in the gonads and in mesenchymal cells adjacent to the Müllerian ducts. In the adult female, AMHRII is expressed in granulosa cells and at low levels in ovarian stroma and endometrium (Bakkum-Gamez 2008; Song 2009). Low levels of AMHRII are also present in non-gynecological tissues, including liver parenchyma, kidney tubules, breast ducts, exocrine pancreas, and bronchiolar epithelium (Bakkum-Gamez 2008). In gynecological cancers, AMHRII is highly expressed in cancers of the ovary, endometrium, cervix, and breast (Masiakos 1999; Ha 2000; Renaud 2005; Bakkum-Gamez 2008; Song 2009).

2.2.1 AMH expression and function in the ovary

AMH expression is undetectable in the fetal ovary and weak in the postnatal ovary, but after puberty its expression starts in the granulosa cells of small preantral follicles (Figure 2) (Visser 2005; Broekmans 2008). The expression is highest in granulosa cells of large preantral and small antral follicles, and diminishes in the next stages of follicle development. AMH is no longer expressed during the final stages of follicle growth, and AMH expression also disappears in atretic follicles. In granulosa cells, AMH expression is regulated by steroidogenic factor-1 (SF-1), GATA4 (Tremblay 1999; Anttonen 2003), and FSH (Taieb 2011). The granulosa cell-produced AMH regulates follicular development by inhibiting the initial recruitment of primordial follicles (Visser 2006). FSH is the gonadotrophin that stimulates antral follicle growth, and AMH regulates the cyclic recruitment of follicles by decreasing the responsiveness of small antral follicles to FSH (Visser 2006).
Figure 2. Role of AMH in folliculogenesis. The oocytes are shown in black, granulosa cell layers in gray and follicular fluid in white. AMH is produced predominantly in the small antral follicles; the thickness of the gray arrow represents the relative amount of AMH production. AMH inhibits the initial recruitment and FSH-dependent cyclic recruitment. Modified from Broekmans 2008.

The actions of AMH in the adult ovary are autocrine and paracrine in nature, but the release of AMH from granulosa cells leads to measurable serum levels, which are proportional to the number of developing follicles in the ovaries. The recent development of highly sensitive, standardized ELISA to evaluate serum AMH has raised interest in clinical applications (Streuli 2009). Serum AMH is a unique marker in the evaluation of ovarian function, especially when considering the reproductive capacity in women (reviewed in (Broekmans 2008; Visser 2012)).

2.2.2 AMH signaling: the TGF-β/BMP- pathway

AMH belongs to the large TGF-β superfamily of highly conserved but functionally diverse groups of growth factors involved in numerous physiological processes during pre- and postnatal life (Massague 2000a). In vertebrates, there are at least 35 ligands in the TGF-β superfamily that signal through seven transmembrane receptors. The different TGF-β superfamily ligands can form active signaling complexes by binding to one or more combinations of the receptors in the TGF-β/bone morphogenic protein (BMP) pathway, allowing cell-specific responses (Massague 2000b).

The complex regulation of the TGF-β/BMP pathway relies on the phosphorylation of different downstream effectors (Smads) by specific ligand-receptor binding (reviewed in (Schmierer 2007)). The ligands comprise TGF-β-type ligands (TGF-β, activin, and nodal) as well as BMP-type ligands (AMH, BMP, and growth and differentiation factors (GDFs)). The receptors of this pathway are classified into type I (activin receptor-like
kinases, ALKs) and type II receptors that form heterodimers upon ligand binding (Figure 3). Downstream of the receptors the signal is conveyed by the Smads, which are transcription factors that transmit the signaling to the nucleus and regulate gene expression. Different Smads are activated by different ligands; TGF-β, activin, and nodal activate Smad2/3, and AMH, BMPs, and GDFs Smad1/5/8. Also the type I receptors are divided based on different Smad activation; ALK4, ALK5, and ALK7 activate Smad2/3, and ALK1, ALK2, ALK3, and ALK6 activate Smad1/5/8.

**Figure 3.** TGF-β/BMP signaling pathway. Upon ligand binding, type I and type II receptors form heterodimers, autophosphorylate and subsequently phosphorylate specific downstream Smads. The activated Smads form a transcription complex with a co-Smad (Smad4), which then enters the nucleus and binds specific sites of DNA to control gene transcription. Modified from Schmierer 2007.

### 2.2.3 TGF-β signaling and AMH in GCT tumorigenesis

Imbalances in TGF-β/BMP signaling are likely to contribute to GCT pathogenesis, and several mouse models suggest that overactivity of TGF-β-type signaling and Smad2/3 contributes to GCT formation. Matzuk et al. first reported this phenomenon in α-inhibin-deficient mice that developed sex cord stromal tumors of granulosa cell origin through overactive activin/TGF-β signaling (Matzuk 1992). The mechanism was further elaborated when the downstream Smad3 was additionally deleted and the tumor formation was delayed in these mice (Li 2007a; Li 2007b). Moreover, in mice lacking Smad1/5 in
granulosa cells the overactivity of Smad 2/3 resulted in the formation of aggressive GCTs (Pangas 2008; Middlebrook 2009); however, these tumors resembled more the juvenile GCTs. In addition, GCTs developed in mice when the BMP type I receptors ALK3 and ALK6 were deleted from granulosa cells (Edson 2010). These findings suggest that the BMP/AMH-type pathway acts as a tumor suppressor in normal granulosa cells.

Previous studies have shown that AMH is expressed in GCTs (Ragin 1992; Rey 2000; Anttonen 2005). However, in large human and mouse GCTs, AMH tissue expression levels are reduced (Dutertre 2001; Anttonen 2005). AMHRII is expressed in human GCTs at higher levels than in other ovarian cancer types (Salhi 2004; Song 2009), and AMHRII has been shown to be functional and to activate Smad1 upon AMH stimulation in murine GCTs (Dutertre 2001). Moreover, AMH has been suggested as a therapeutic agent in AMHRII-expressing human cancers; AMH inhibits the growth of AMHRII-positive cancer cells in vitro (Masiakos 1999; Ha 2000) and in vivo (Stephen 2002). This growth inhibition is mediated through AMHRII and results in a block in cell cycle progression, with subsequent apoptosis (Masiakos 1999; Ha 2000). The functional role of AMHRII in GCTs remains obscure.

GCTs express high levels of AMH in the tissues, and high levels of AMH can also be detected in the serum of GCT patients (Rey 2000). Previous studies suggest that AMH is a serum marker for GCT (Long 2000; Rey 2000). Serum AMH has also been reported to positively correlate with tumor size (Chang 2009). The clinical use of AMH has thus far been discouraged because of the lack of studies describing the value of AMH in a larger pool of GCT patients. A comparative study with the current marker inhibin B in a single patient cohort is needed to validate the clinical use of AMH in GCT patients (Streuli 2009).

2.3 Tumor angiogenesis: VEGF and endostatin

Tumor growth relies on mechanisms of angiogenesis - the growth of new blood vessels from pre-existing vasculature - to supply oxygen and nutrients. The angiogenic process is driven by growth factors of which Vascular Endothelial Growth Factor-A (VEGF) seems to be the most important in tumors (reviewed in (Ferrara 2004)). VEGF is a 21 kDa protein originally found to induce proliferation, sprouting, migration, and tube formation of vascular endothelial cells (reviewed in (Ferrara 2003)). During embryogenesis VEGF is required for hematopoiesis, vasculogenesis, and angiogenesis and Vegf knockout is embryonic lethal (Ferrara 2003). During embryonic development VEGF plays a crucial role in organ growth and development. In the adult, VEGF is expressed during neo-angiogenesis; in the ovary and endometrium during the menstrual cycle, in wound healing, and in tumors (reviewed in (Tammela 2005)). At least six VEGF isoforms of variable amino acid number are produced through alternative splicing; VEGF_{121}, VEGF_{165}, and VEGF_{189} are the major forms produced by most cell types (Robinson 2001) (Figure 4). The different isoforms differ in the heparin-binding domain, resulting in various
extracellular matrix (ECM) binding properties (Ferrara 2003); VEGF$_{121}$ is a freely diffusible protein, VEGF$_{189}$ is almost completely bound to the ECM, and VEGF$_{165}$ has intermediate properties. The ECM binding forms a biological regulatory mechanism that allows the ECM bound isoforms to be released in a diffusible form by plasmin cleavage. In addition, VEGF$_{165}$, but not VEGF$_{121}$, interacts with coreceptors, such as the neuropilins, to enhance VEGFR-2 signaling (Soker 2002).

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**Figure 4.** The three major VEGF isoforms are formed as a result of alternative mRNA splicing and differ by their ECM binding properties. The proteins are depicted from N to C terminus and the exons are numbered below the bar. Binding sites for VEGFR-1 and VEGFR-2 are marked with black stripes and the plasmin cleavage site with an arrow. Modified from Robinson 2001.

![Figure 4](image-url)

VEGF binds to two tyrosine kinase receptors, VEGFR-1 (Fms-like kinase-1, Flt-1) and VEGFR-2 (Fetal liver kinase-1 (Flk-1) or kinase-insert domain receptor (KDR)), expressed primarily in endothelial cells (Ferrara 2003) (Figure 5). VEGFR-1 and VEGFR-2 are also expressed in osteoblasts, inflammatory cells, and hematopoietic stem cells. VEGFR-1 expression is upregulated during angiogenesis and in hypoxia, unlike VEGFR-2 (Gerber 1997). The expression of VEGFR-2 is autoregulated; VEGF upregulates its expression (Shima 1995). Both VEGF receptors are essential for embryonic vasculature formation and hematopoiesis, and knockout mice are embryonic lethal (Shalaby 1997), although mice lacking only the intracellular tyrosine kinase part of VEGFR-1 are viable and have only slightly impaired angiogenesis in adults (Hiratsuka 1998).

The tyrosine kinase receptors consist of an extracellular ligand-binding domain, a single transmembrane region, and an intracellular domain with intrinsic protein kinase activity. The tyrosine kinase receptors typically form hetero- or homodimers upon activation. The binding of VEGF to its receptor causes the autophosphorylation of specific intracellular tyrosine residues, which in turn initiates multiple signaling cascades that utilize, for instance, the mapkinase, protein-kinase C, and protein-kinase B pathways to induce endothelial cell survival, proliferation, angiogenesis, and vasopermeability. Although VEGF binds to VEGF-R1 and -2 with similar affinity, the phosphorylation of VEGF-R1 is weak, and VEGF-R2 is thus considered the main mediator of VEGF function (Tammela...
Furthermore, VEGF-R1 also exists in a soluble extracellular form, which may act as regulator of VEGF function, and is associated with pathological conditions such as preeclampsia and ovarian hyperstimulation syndrome (Tammela 2005; Pau 2006).

![Summary of VEGF signaling in tumorigenesis](image)

**Figure 5.** Summary of VEGF signaling in tumorigenesis. VEGF is produced as a 21 kDa protein that is subsequently glycosylated and forms a 45 kDa homodimer. VEGF is secreted by the tumor in response to hypoxia, growth factors, and cytokines. The VEGF receptors, usually expressed on blood endothelial cells, are composed of seven immunoglobulin-like extracellular domains (spheres), and an intracellular tyrosine kinase domain (ovals). After VEGF binding to VEGFR-2, the intracellular tyrosine residues of VEGFR-20 intrinsically phosphorylate, leading to an angiogenic response in endothelial cells. VEGFR-1 activation leads to recruitment of endothelial progenitor cells (EPCs). VEGFR-1 is also found as a soluble form (sVEGFR-1) that acts as a decoy receptor and binds VEGF. Bevacizumab (BVZ), a humanized monoclonal antibody used in cancer therapy, binds soluble VEGF and inhibits its binding to the VEGFRs. Modified from Tammela 2004 and Ferrara 2003.
2.3.1 VEGF expression and signaling in the ovary

VEGF plays a crucial role in regulating female reproductive function (Lam 2005). In the human ovary, VEGF is crucial for follicular angiogenesis and the development and maintenance of the corpus luteum (Geva 2000), and its expression is regulated by gonadotrophins (Mattioli 2001). In preovulatory follicles, VEGF is mainly expressed in the interstitial tissue and theca layers, whereas in ovulatory and post-ovulatory follicles VEGF is abundantly expressed in granulosa and granulosa-lutein cells (Geva 2000; Balasch 2004).

Granulosa cells express VEGF (Balasch 2004; Rolaki 2007), and its expression parallels follicular angiogenesis; VEGF expression increases with increasing follicle size, is highest during ovulation, and gradually diminishes in the developed corpus luteum (Geva 2000; Greenaway 2004). VEGF expression also disappears in atretic follicles (Greenaway 2004). In granulosa cells, VEGF expression is upregulated by estradiol, progesterone, FSH, and luteinizing hormone (LH) (Shimizu 2007). During follicular development VEGF is crucial for granulosa cell survival by inhibiting apoptosis (Greenaway 2004; Shin 2006; Kosaka 2007). Further, VEGF inhibition with a soluble decoy receptor suppresses granulosa cell proliferation, follicular development (Wulff 2002), and granulosa cell function (i.e. AMH expression) (Thomas 2007). These survival and function-promoting signals seem to be mediated by VEGFR-2 (Greenaway 2004).

VEGFR-1 and VEGFR-2 are expressed in granulosa cells (Otani 1999; Greenaway 2004; Shimizu 2007) and more intensively in the granulosa-lutein cells of the corpus luteum (Otani 1999; Sugino 2000). The expression of VEGFR-2 colocalizes with VEGF expression, suggesting an auto-regulatory loop in granulosa cells (Greenaway 2004).

2.3.2 VEGF in tumors and anti-VEGF cancer treatments

In growing tumors, hypoxia is the main stimulator of VEGF expression through hypoxia-inducible factor 1α (HIF-1α), but also many cytokines and growth factors, such as TGF-α and TGF-β, insulin-like growth factor-1, and interleukins 1 and 6, stimulate VEGF expression (Ferrara 2003). The VEGF secreted by the tumor binds to VEGFR-2 expressed in the endothelial cells of adjacent blood vessels, initiating neo-angiogenesis to supply oxygen and nutrients to the tumor (Figure 5). VEGF is expressed in the majority of solid tumors, and also in some hematological malignancies, and its expression correlates with disease progression and survival (Ferrara 2002; Ferrara 2003). Serum VEGF is also frequently elevated in the serum of cancer patients, and the levels correlate negatively with prognosis in, for example, breast (Salven 1999), prostate (Jones 2000), lung (Salven 1998), and colorectal cancers (Takeda 2000).

In ovarian cancer, both tumor tissue and serum VEGF expressions correlate negatively with prognosis (Yamamoto 1997; Cooper 2002; Li 2004). Small series report VEGF tissue
expression also in GCTs (Juric 2001; Li 2004; Schmidt 2008). However, the association of VEGF expression with prognosis in GCTs remains to be elucidated. Moreover, serum VEGF levels or the expression of VEGF receptors in GCTs have not been characterized prior to this study.

Bevacizumab (BVZ) is a humanized monoclonal antibody that binds soluble VEGF and inhibits its function (Figure 5). BVZ was approved as an anti-cancer drug by the U.S. Food and Drug Administration in 2004 and has been successfully used in the treatment of breast, lung, colorectal, and renal cancers (Jubb 2010), and in epithelial ovarian cancer (Penson 2010; Perren 2011). Clinical responses of BVZ have been reported also in some GCT patients (Kesterson 2008; Tao 2009), and a phase II clinical trial on BVZ in sex cord stromal tumors is ongoing (www.clinicaltrials.gov). In ovarian cancer, the clinical responses have not been as long-standing as anticipated, and the treatment has caused severe adverse effects such as bowel perforation (Burger 2011; Tanyi 2011). New cancer drugs targeting the VEGF receptors are being developed and extensively studied in clinical trials, with promising anti-tumor activity and hopefully less toxicity (Teoh 2012).

2.3.3 Endostatin

Endostatin, a 20 kDa C-terminal fragment of collagen XVIII, is an endogenous inhibitor of endothelial cell proliferation and angiogenesis (O'Reilly 1997; Folkman 2006). Endostatin inhibits tumor angiogenesis by both downregulating the pro-angiogenic factors, including VEGF and VEGFR-2, and upregulating anti-angiogenic factors, such as thrombospondin (Abdollahi 2004). Endostatin also induces endothelial cell apoptosis by downregulating the anti-apoptotic B-cell lymphoma-2 (Bcl-2) (Dhanabal 1999).

Elevated serum endostatin levels have been reported in various cancers, including those of the breast (Zhao 2004), kidney (Feldman 2000), and lung (Suzuki 2002). The exact source of circulating endostatin is still unknown, but it is thought to be released from the ECM of blood vessel walls and basement membranes by certain proteases, particularly in the liver (Schuppan 1998). Endostatin has been a candidate for antiangiogenic cancer therapy (Herbst 2002). However, the mechanism of endostatin action as a cancer treatment is incompletely known and the clinical responses have remained modest (Karamouzis 2009).

2.4 GATA4 in ovarian function and GCTs

The GATA factors are an evolutionarily conserved group of transcription factors that bind a specific A/T-GATA-A/G motif found in the regulatory regions of numerous genes (reviewed in (Viger 2008)). In vertebrates, the GATA family consists of six members (GATA1 to GATA6) that are crucial regulators of development and differentiation (Weiss 1995). GATA1/2/3 regulate the development of the brain, spinal cord, and inner ear and
the differentiation of hematopoietic cell lineages. GATA4/5/6 are primarily expressed in tissues of mesodermal and endodermal origin such as the heart, gut, and gonads.

GATA4 is a zinc finger transcription factor that plays a crucial role during fetal development; GATA4 null mutation is embryonic lethal due to defects in heart development (Molkentin 1997). GATA4 is required also for normal ovarian and testicular development (Bielinska 2007; Manuylov 2008). In the adult, GATA4 plays a role in follicular development, with granulosa cells being the major site of GATA4 expression (Laitinen 2000). During follicular development GATA4 expression is spatiotemporally regulated; the expression initiates in the proliferating granulosa cells of small preantral follicles, peaks in the antral follicles, and diminishes rapidly during ovulation, being virtually non-existent in luteal glands (Heikinheimo 1997; Laitinen 2000; Vaskivuo 2001; Anttonen 2003). In the ovary, FSH and TGF-β regulate the expression of GATA4 (Heikinheimo 1997; Anttonen 2006). GATA4 has been shown to regulate granulosa cell proliferation and function (Anttonen 2006; Kyronlahti 2008), moderating many factors crucial for normal follicular development, including AMH (Tremblay 1999; Anttonen 2006), aromatase (Tremblay 2001b), and α-inhibin (Anttonen 2006). A recent study on heterozygous and granulosa cell-specific conditional GATA4 knockout mice showed that GATA4 is crucial for normal follicular development and function (Kyronlahti 2011).

In GCTs, GATA4 is expressed at levels comparable to preovulatory granulosa cells (Laitinen 2000; Anttonen 2005). Moreover, high expression of GATA4 has been associated with higher stage GCT and increased recurrence risk in GCT patients (Anttonen 2005). Further, GATA4 putatively plays a role in GCT pathogenesis by inhibiting apoptosis; GATA4 activates the expression of the anti-apoptotic Bcl-2 and protects GCT cells from apoptosis (Kyronlahti 2008; Kyronlahti 2010).

2.5 EGF receptors in ovarian function and GCTs

Epidermal growth factor (EGF) is crucial during gonadal development and regulates granulosa cell function (Schomberg 1983). EGF is a member of a large group of growth factors that includes TGF-α, heparin-binding EGF-like growth factor, amphiregulin, epiregulin, betacellulin, epigen, and neuregulins (reviewed in (Conti 2006)). These ligands bind four related receptors, the EGF receptors. The complex network of signals is regulated by different combinations of ligands binding to homo- and heterodimeric receptors, leading to different cell-specific responses.

EGF receptors are a group of transmembrane tyrosine kinase receptors that includes the EGF receptor EGFR/hererugin-1 (HER1), HER2/neu, HER3, and HER4. In the ovary, EGF receptor family members promote granulosa cell proliferation and survival (Conti 2006; Zandi 2007), and normal EGFR signaling seems to be required for FSH response and steroidogenesis in granulosa cells (Jamnongjitt 2005; Wayne 2007). No specific ligand has been found for HER2, but it can be transactivated via heterodimerization with other
HER2 receptors (Hynes 2005). After ligand binding, HER3 and HER4 dimerize and activate intracellular pathways to promote survival and proliferation; HER3 lacks the intracellular kinase domain, but contributes to intracellular signaling through heterodimerization with other HER receptors (Lemmon 2009).

Overexpression and/or mutation of EGFR and HER2 have been shown to contribute to the progression of several human cancers, including brain, lung, breast, and ovarian cancers (Hynes 2005; Zandi 2007). The EGF receptors have been extensively studied in cancer therapy and are targeted with monoclonal antibodies or tyrosine kinase inhibitors in clinical trials (reviewed in (Tsujioka 2010)). GCTs also express EGFR (Leibl 2006) (N. Andersson, unpublished data), and positive expression has been found to be associated with worse prognosis (Nosov 2009). Further, EGFR inhibition was shown to induce apoptosis in GCT cells (N. Andersson, unpublished data). HER2, HER3, and HER4 are also expressed in GCTs (Furger 1998; Leibl 2006) (see also Section 2.5.1), and HER3-4 potentially mediate GCT cell survival in a GCT cell line (Furger 1998). The functional role of HER2 in GCT cells has not been studied.

### 2.5.1 HER2 oncogene

HER2 is overexpressed in 30% of breast cancers as a result of amplification of the gene encoding for HER2 (*HER2*) (Hicks 2008). This amplification is highly associated with worse overall survival in breast cancer (Slamon 1987). In epithelial ovarian cancer, HER2 is overexpressed in 17-44% and amplified in 7-14% of tumors. Some studies have reported an association between HER2 expression and worse clinical outcome (Fajac 1995; Felip 1995; Lassus 2004). HER2 is a therapeutic target in cancer therapy; currently, there are two drugs in clinical use targeting HER2 in the treatment of breast and metastatic gastric cancer: a monoclonal antibody trastuzumab (Herceptin®) and a small molecule HER2 tyrosine kinase inhibitor lapatanib (Tykerb®) (Stern 2012).

Data on HER2 expression in GCTs are somewhat conflicting; two studies reported positive expression in GCTs (King 1996; Furger 1998), while others found GCTs to be negative for HER2 (Kusamura 2003; Leibl 2006; Mayr 2006; Menczer 2007). HER2 is also expressed in GCT cell lines KGN (N. Andersson, unpublished data), and COV343 (Furger 1998). Copy number alterations of the *HER2* gene were not described in a study including GCTs (Mayr 2006).
Aims of the study

This study was undertaken to investigate regulators of tumor angiogenesis and granulosa cell function in GCT pathogenesis and to find new molecular prognostic factors and treatment options for GCT patients.

Specific aims of the study were as follows:

1) to investigate the functional role of AMH signaling and the role of VEGF and its receptors in GCT pathogenesis

2) to evaluate the possibility of targeting AMH and VEGF pathways in the treatment of GCTs

3) to identify new molecular prognostic markers for GCT
Materials and Methods

1. Patients (I-IV)

The clinical data of 118 GCT patients diagnosed at Helsinki University Central Hospital from 1956 to 2010 were retrospectively collected. Fifty-four GCT patients were recruited with their informed consent to give blood samples and fresh tumor tissue upon surgery for expression analyses and cell culture experiments. All living GCT patients no longer in follow-up were invited for a clinical control, and 41 patients were clinically examined to gain an extended follow-up. The rest were followed from hospital files and the causes of death were collected from death certificates retrieved from the Finnish Causes of Death Registry. The Ethics Committee of Helsinki University Central Hospital (197/E9/06) and the National Supervisory Authority of Welfare and Health in Finland (decision number 244/05.01.00.06/2009) approved the study protocol.

2. Serum samples (II)

Seventy-four serum samples of 54 GCT patients were collected from August 2007 to November 2011. The samples were prepared and stored at -80°C until analysis. Data on patient hemoglobin, leukocyte, hematocrite, and platelet counts were collected from samples drawn on the same days.

3. Tissue samples (I-IV)

A tumor tissue microarray (TTMA) of 80 primary and 13 recurrent GCTs from 90 patients was previously constructed (Anttonen 2005). Paraffin-embedded sections of the TTMA consisted of quadruple core samples of 93 GCTs on a single slide, and the tumor subtype, degree of nuclear atypia, and mitotic index were defined as described elsewhere (Anttonen 2005) in accordance with WHO 2003 guidelines (F.A. Tavassoli 2003). The tumor size and histological characteristics in the TTMA are summarized in Table 2. Thirty-four fresh tumor tissue samples were collected from May 1994 to August 2009, and the samples were snap-frozen in liquid nitrogen and stored in -80°C until analyses. The ovaries from three premenopausal women operated on for cervical cancer were used as controls. From December 2007 to September 2010, we obtained eight primary and six recurrent GCTs for primary cell cultures (see Section 4).
MATERIALS AND METHODS

Table 2. Summary of tumor size and histological parameters of 80 primary and 13 recurrent GCTs in the TTMA.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Level</th>
<th>Primary GCT (n=80) n (%)</th>
<th>Recurrent GCT (n=13) n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor size</td>
<td>&lt;10 cm in diameter</td>
<td>48 (60.0)</td>
<td>10 (23.1)</td>
</tr>
<tr>
<td></td>
<td>≥10 cm in diameter</td>
<td>32 (40.0)</td>
<td>3 (76.9)</td>
</tr>
<tr>
<td>Subtype</td>
<td>Differentiated</td>
<td>56 (70.0)</td>
<td>3 (23.1)</td>
</tr>
<tr>
<td></td>
<td>Sarcomatoid</td>
<td>24 (30.0)</td>
<td>10 (76.1)</td>
</tr>
<tr>
<td>Nuclear atypia</td>
<td>Low</td>
<td>62 (77.5)</td>
<td>9 (69.2)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>18 (22.5)</td>
<td>4 (30.8)</td>
</tr>
<tr>
<td>Mitotic index</td>
<td>Low</td>
<td>60 (75.0)</td>
<td>8 (61.5)</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>20 (25.0)</td>
<td>5 (38.5)</td>
</tr>
</tbody>
</table>

4. Cell lines (I,III)

The KGN cell line (Dr. T. Yanase, Kyushu University, Fukuoka, Japan) was cultured as previously described in DMEM/F12 containing 10% FFCS (Nishi 2001). The GCT cell cultures were established as described elsewhere (Kyronlahti 2010); the fresh tumor sample was retrieved straight from the operation theater in cold PBS, mechanically minced, and incubated in 0.5% collagenase (Sigma-Aldrich Corporation, St. Louis, MO, USA) for 2 hours, filtered through a 140 µm filter mesh, washed two times with cell culture supernatant, and the single cells were then plated for experiments in the DMEM/F12 containing 10% FFCS without passaging. KGN cells were used All of the primary GCT cells as well as the KGN cells harbored the c.402C→G (p.C134W) mutation in FOXL2.

5. Expression analyses (I-IV)

5.1 Immunohistochemistry and scoring of the results (I-IV)

Paraffin-embedded sections (6 µm in thickness) of the TTMA and normal ovaries were subjected to IHC with the antibodies presented in Table 3. The sections were deparaffinized with xylene and rehydrated with ethanol incubations. Antigen retrieval was performed with 10 mM citric acid in a microwave oven for 10–20 min, and endogenous peroxidase was blocked with 3% hydrogen peroxide. Immunoperoxidase stain was performed as described elsewhere (Anttonen 2005) using an avidin-biotin immunoperoxidase system (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA, USA) and DAB (Sigma, St. Louis, MO, USA) to visualize the bound antibody. The
sections were counterstained with hematoxylin. Images were acquired with an LS Leica DMRXA microscope using 20x and 100x magnifications, connected to an Olympus DP70 camera and a DCP controller image acquisition program. The intensity of staining and the percentage of positive cells were scored for each antigen from four cores of each tumor by two independent researchers, and the tumors were divided into subgroups of high, medium, low, and negative, depending on the antigen. The threshold levels were defined individually for each antigen depending on the immunoreactivity and the localization of the antigen. Disagreements were resolved by a joint review. Tumor blood vessels were visualized with α-CD34 antibody, and the number of tumor blood vessels was counted per visual field. The microvessel density (MVD) was graded as high with $\geq 60$ microvessels per visual field and as low with <60 vessels per visual field.

Table 3. Antibodies utilized in IHC and Western blotting. * The antibody was kindly provided by Dr. Isabelle Navarro-Teulon (INSERM U896, Montpellier, France).

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Company</th>
<th>Catalog no</th>
<th>Dilution in IHC/WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMHRRII*</td>
<td>* (see heading)</td>
<td></td>
<td>1:50/1:5000</td>
</tr>
<tr>
<td>Smad1</td>
<td>Abcam</td>
<td>ab55476</td>
<td>1:50/1:1000</td>
</tr>
<tr>
<td>Smad2</td>
<td>Invitrogen</td>
<td>51-1300</td>
<td>1:200/-</td>
</tr>
<tr>
<td>Smad3</td>
<td>Invitrogen</td>
<td>51-1500</td>
<td>1:400/-</td>
</tr>
<tr>
<td>Smad4</td>
<td>Santa Cruz Biotechnology</td>
<td>Sc-7966</td>
<td>1:200/-</td>
</tr>
<tr>
<td>Smad5</td>
<td>Abcam</td>
<td>ab40771</td>
<td>1:50/1:2500</td>
</tr>
<tr>
<td>P-Smad1/5</td>
<td>Cell Signaling Technology</td>
<td>#9511S</td>
<td>1:100/1:2500</td>
</tr>
<tr>
<td>P-Smad2/3</td>
<td>Cell Signaling Technology</td>
<td>#3101S</td>
<td>1:500/-</td>
</tr>
<tr>
<td>VEGF</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-152</td>
<td>1:50/-</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-316</td>
<td>1:100/-</td>
</tr>
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<td>VEGFR-2</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-6251</td>
<td>1:200/1:500</td>
</tr>
<tr>
<td>P-VEGFR-2</td>
<td>Abcam</td>
<td>Y1214</td>
<td>1:70/1:500</td>
</tr>
<tr>
<td>CD34</td>
<td>DAKO</td>
<td>M7165</td>
<td>1:50/-</td>
</tr>
<tr>
<td>HER2</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-33684</td>
<td>1:100/-</td>
</tr>
<tr>
<td>HER3</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-415</td>
<td>1:50/-</td>
</tr>
<tr>
<td>HER4</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-283</td>
<td>1:50/-</td>
</tr>
<tr>
<td>Cleaved caspase-3</td>
<td>Cell Signaling Technology</td>
<td>#9661L</td>
<td>-/1:500</td>
</tr>
<tr>
<td>Bcl2</td>
<td>DAKO</td>
<td>MO887</td>
<td>-/1:500</td>
</tr>
<tr>
<td>Cyclin D2</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-598</td>
<td>-/1:1000</td>
</tr>
<tr>
<td>Beeta actin</td>
<td>Santa Cruz Biotechnology</td>
<td>sc-1616</td>
<td>-/1:5000</td>
</tr>
</tbody>
</table>

5.2 PCR and quantitative PCR (I-III)

The primers listed in Table 4 were either designed with IDT SciTools software or retrieved from the literature, and tested with in-silico PCR using the UCSC Genome Bioinformatics website for recognizing the correct mRNA transcript. The RNA was
isolated with a Nucleospin RNA/Protein kit (catalog no. 740 933.250, Macherey-Nagel, Düren, Germany) and purified with an RNA purification kit (Nucleospin RNA Clean up kit, catalog no. 740 948.50) according to instructions. Semi-quantitative PCR was performed as described previously (Salonen 2009). For qPCR, first-strand cDNA synthesis was performed from 0.8 µg of total RNA using SYBR GREEN RT-PCR reagents and random hexamers (Applied Biosystems, Foster City, CA, USA) according to instructions. Standard curve method was applied; purified mRNA from the KGN cell line or from pooled GCT samples was used as a standard. Analysis was performed with SYBR Green RT-PCR reagents and an ABI PRISM 7700 sequence detection system (Applied Biosystems) according to manufacturer’s instructions. All analyses were performed in triplicate.

Table 4. Primers utilized in PCR and quantitative PCR (qPCR).

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Primers 5’-3’</th>
<th>Amplicon size</th>
<th>PCR type</th>
</tr>
</thead>
</table>
| AMH        | F: CGCCTGGTGGTCTCTACAC  
R: GAACCTCAGCGAGGGTGTT | 60 bp | qPCR |
| AMHRII     | F: TGTGTCTCCCGGTATCCG  
R: AATGTGGCTGTGCTGAGGC  
F: TTTGGGGCTTTGGGGCATTTAC  
R: GATGCCGAGACAGTGAT | 164 bp | qPCR  
238 bp | PCR |
| ALK2       | F: TTAAAAGGCACAAAAACAAAGA  
R: CGTACAACGATCCCATTTCA | 423 bp | PCR |
| ALK3       | F: TTTTAGGCAACCAAGGAAAG  
R: TGGTAGATCAAGGACATCA | 156 bp | PCR |
| ALK6       | F: CTCAAGGAGGCGATCTGAGCA  
R: GCGGCCCTCAATGCAGGGAT | 437 bp | PCR |
| Smad1      | F: GCGGCATATTGGAAAGGAG  
R: CCTGGGGCCATTTAAAGAT  
R: ACCTGGTTTCCAGCCCA | 429 bp | PCR |
| Smad5      | F: GCGAAAAGGAAGCTGTGGT  
R: ACCTGGTTTCCAGCCCA | 320 bp | PCR |
| Beta-actin | F: CTGACGGGGCACTCGTAGAT  
R: CAGACAGCACTGTGGTGGC | 174 bp | qPCR |
| VEGF       | F: TGCAGATTATGGAAAGGAG  
R: AATGTGGCTGTGCTGAGGC  
F: TTTGGGGCTTTGGGGCATTTAC  
R: GATGCCGAGACAGTGAT | 82 bp | qPCR |
| VEGFR-2    | F: GGAAGCTCCTGAGAATGCTGT  
R: GAGGATATTTCTGGGAGCC | 139 bp | qPCR |
| GAPDH      | F: TCAATTCCTGGTATGCAAGC  
R: TTACTCCTTTGAGGAGGCATGT | 80 bp | qPCR |
5.3 Western blotting (I, III)

The extracted protein was separated by 7.5% SDS-PAGE and transferred onto an Immobilon-P membrane (Millipore, Bedford, MA, USA). Beta-actin was used as a loading control. Nonspecific binding was blocked with 5% nonfat milk in 0.1% Tris-buffered Tween-saline buffer. Primary and secondary antibodies were incubated for 1 h at room temperature. The proteins were visualized with the Enhanced Chemiluminescence Plus Kit (Amersham Biosciences Inc., Piscataway, NJ, USA).

5.4 ELISA assays (I-III)

The serum and cell culture supernatant samples were analyzed in duplicate according to manufacturers’ instructions with enzyme-linked immunosorbent assays (ELISAs): VEGF #DVE00 (R&D Systems, Minneapolis, MN, USA), endostatin #DNST0 (R&D Systems), and AMH #DSL-10-14400 (Beckman Coulter Inc., Webster, TX, USA).

5.5 Silver in situ hybridization (IV)

Paraffin-embedded sections of the TTMA were utilized for Silver in situ hybridization (SISH) for the HER2 DNA and chromosome 17 probes with Inform® (Ventana Medical Systems, Tucson, AZ, USA). The automated SISH was performed according to the manufacturer’s instructions (Dietel 2007), with slight modifications; the hybridization time for the HER2 probe was 10 and for the chromosome 17 probe 8 h.

6. Cell cultures and treatments (I, III)

The primary GCT and KGN cells were subjected to AMH, BVZ, or control treatments for 1-5 days. BVZ 25 mg/ml was purchased from Genentech/Roche (San Francisco, CA, USA). Recombinant human AMH (rhAMH) was purified and tested for bioactivity as described earlier (Donahoe 2003). The cells were treated at different concentrations and followed by apoptosis, proliferation, cell viability assays (see Section 6.1), or protein extraction (Nucleospin RNA/Protein kit, catalog no. 740 933.250, Macherey-Nagel, Düren, Germany). For cell culture supernatant collection, 200 000 primary GCT cells and KGN cells were plated to 6-well chambers containing 2 ml of DMEM/F12 with 10% FFCS, the culture supernatant was collected after 2-4 days (depending on the confluence), and the supernatants were stored at -20°C until analysis.
6.1 Apoptosis, proliferation, and cell viability assays

For apoptosis analyses, 20000 KGN and primary GCT cells were seeded on 96-well plates (Caspase assay, see below) or 8-well glass chambers (DAPI staining, see below). Caspase-Glo® 3/7 assay (Promega Corporation, Madison, WI, USA) was utilized according to the manufacturer’s instructions. DAPI staining was performed as described (Kyronlahti 2010), and the percentage of apoptotic cells in 1200-3000 total cells was determined with ImageJ™ 1.42q software (National Institutes of Health, Bethesda, MD, USA).

For cell proliferation analyses, a bromodeoxyuridine (BrdU) staining kit (Invitrogen, Carlsbad, CA, USA) was utilized according to the manufacturer’s instructions, and the proportion of BrdU-positive cells was determined from 2000–3000 cells on 8-well glass chamber slides. For cell viability assays, a tetrazolium dye (MTT)-based cell growth determination kit (#CGD1, Sigma-Aldrich Corporation) was utilized for cell viability assay; 10000 KGN cells were plated on 96-well plates, and the kit was utilized according to the manufacturer’s instructions.

7. Database and statistical analyses (I-IV)

File Maker Pro 10.0v3 software was used to construct a database of the patients, including full clinical data, serum samples, tissue samples, and follow-up and survival data. The patients were encoded with study ID numbers. The data consisted of up to 500 data fields per patient, and the sample data were linked to patient details based on study ID number. The data were exported as a Microsoft Excel file and transported to statistics software for analyses. The clinical data of the patients regarding recurrences and survival were updated twice during this study: in September 2009 and in May 2011. The results on IHC, quantitative PCR analyses, and serum analyses were stored in the database.

The statistical analyses were carried out with JMP® 9.0.0 software (SAS Institute Inc., Cary, NC, USA). The immunohistochemical data and the categorical variables were analyzed with contingency tabling (2x2) and chi-square or Fisher’s exact tests when appropriate. The continuous variables were analyzed with linear regression using Pearson’s correlation coefficient. The serum data were tested for normal distribution with Shapiro-Wilks test and analyzed with one-way ANOVA and Student’s t-test upon normal distribution, or with Wilcoxon/Kruskall-Wallis and Wilcoxon matched-pairs sign-rank tests when differing from normal distribution (serum VEGF data). Upon comparison with normally distributed variables, a logarithmic transformation was performed on the serum VEGF data. The cell culture data and mRNA expression data were analyzed with one-way ANOVA, followed by Dunnett’s or each pair Student’s t-test. Kaplan-Meier was performed according to the methodology using a log-rank test. Multivariate analyses were conducted using a nominal logistic regression model and a Cox proportional hazards model. P<0.05 was considered statistically significant.
Results and Discussion

1. Expression profile of GCTs (I-IV)

Tumor growth is dependent on the expression of growth and transcription factors that regulate cell proliferation and survival as well as angiogenesis. We set out to analyze protein and mRNA expressions of several factors closely related to granulosa cell function and angiogenesis in GCTs: AMH and its receptors, VEGF and its receptors, and EGF receptors.

1.1 Protein expression profile in TTMA

The TTMA of 80 primary and 13 recurrent GCTs was analyzed with immunohistochemistry for the different antigens. Based on the intensity of staining and the percentage of positive cells, the tumors were further divided into “high” and “low” expressing groups, the latter also including the negative tumors. A summary of the TTMA scoring results of all of the factors studied here, as well as the results on tumor MVD and HER2 gene amplification is presented in Table 5.

AMHRII and its signaling cascade components were expressed in GCTs, also in phosphorylated, i.e. activated, state. VEGF and its receptors were expressed, and VEGFR-2 also in the phosphorylated state in the majority of GCTs. HER2-4 expression was also detected; however, amplification of the HER2 gene was rare. The correlations and biological roles of the factors are further discussed below (Sections 2-4).

The TTMA proved to be a good tool for screening and evaluating expression levels of the different factors. Although subjective, two independent researchers performed the analysis, and a consensus of the classification of the tumors to two or three expression levels was achieved for all available samples. For some of the samples, the core samples represented only connective tissue or were lacking from the section, and therefore, the numbers do not total 93 in all analyses. Compared with evaluating 93 serial slides for expression levels, the TTMA putatively provides a more reliable and reproducible tool to screen for prognostic factors and biological associations between different tumors (Kononen 1998). Based on the complete clinical details and follow-up data of the patients, we were able to screen for prognostic factors for GCT in the TTMA (see Section 4).
Table 5. Summary of expression levels of the studied factors and the degree of microvessel density (MVD) and HER2 gene amplification (SISH) in the TTMA of 80 primary and 13 recurrent GCTs. The tumors were divided to "High" and "Low" expression groups; the “Low” group also includes negative tumors. “Ampl” in HER2 SISH indicates low-level (3-6) amplification of HER2 gene. GATA4 expression was previously described in Anttonen 2005.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Level</th>
<th>Primary GCT n(%)</th>
<th>Recurrent GCT n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMHRII</td>
<td>High</td>
<td>45 (57.0)</td>
<td>6 (54.5)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>34 (43.0)</td>
<td>5 (45.5)</td>
</tr>
<tr>
<td>Smad1</td>
<td>High</td>
<td>37 (48.6)</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>46 (51.3)</td>
<td>8 (66.7)</td>
</tr>
<tr>
<td>Smad2</td>
<td>High</td>
<td>53 (67.1)</td>
<td>12 (92.3)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>26 (32.9)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>Smad3</td>
<td>High</td>
<td>32 (43.2)</td>
<td>4 (30.7)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>42 (56.8)</td>
<td>9 (69.2)</td>
</tr>
<tr>
<td>Smad4</td>
<td>High</td>
<td>58 (76.3)</td>
<td>12 (92.3)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>18 (23.6)</td>
<td>1 (7.7)</td>
</tr>
<tr>
<td>Smad5</td>
<td>High</td>
<td>57 (76.0)</td>
<td>5 (41.7)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>18 (24.0)</td>
<td>5 (38.5)</td>
</tr>
<tr>
<td>P-Smad1/5</td>
<td>High</td>
<td>49 (62.8)</td>
<td>7 (58.3)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>29 (37.1)</td>
<td>5 (41.7)</td>
</tr>
<tr>
<td>P-Smad2/3</td>
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<td>52 (66.7)</td>
<td>10 (83.3)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>26 (33.3)</td>
<td>2 (16.7)</td>
</tr>
<tr>
<td>VEGF</td>
<td>High</td>
<td>56 (72.7)</td>
<td>9 (81.8)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>21 (27.3)</td>
<td>2 (18.2)</td>
</tr>
<tr>
<td>VEGFR-1</td>
<td>High</td>
<td>11 (14.3)</td>
<td>5 (45.5)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>66 (85.7)</td>
<td>6 (54.5)</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>High</td>
<td>71 (92.2)</td>
<td>11 (100.0)</td>
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<td></td>
<td>Low</td>
<td>6 (7.8)</td>
<td>0</td>
</tr>
<tr>
<td>P-VEGFR-2</td>
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<td>4 (33.3)</td>
</tr>
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<td>Low</td>
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<td>8 (66.7)</td>
</tr>
<tr>
<td>MVD</td>
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</tr>
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<td></td>
<td>Low</td>
<td>60 (75.9)</td>
<td>6 (50.0)</td>
</tr>
<tr>
<td>HER2</td>
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<td>17 (22.7)</td>
<td>4 (33.3)</td>
</tr>
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<td>58 (77.3)</td>
<td>8 (66.7)</td>
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<td>HER2 SISH</td>
<td>Ampl</td>
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<tr>
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<td>No</td>
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<td>12 (100.0)</td>
</tr>
<tr>
<td>HER3</td>
<td>High</td>
<td>18 (23.1)</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>60 (76.9)</td>
<td>8 (66.7)</td>
</tr>
<tr>
<td>HER4</td>
<td>High</td>
<td>54 (72.9)</td>
<td>10 (83.3)</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>20 (27.0)</td>
<td>2 (16.7)</td>
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<td>GATA4</td>
<td>High</td>
<td>34 (42.5)</td>
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</tr>
<tr>
<td></td>
<td>Low</td>
<td>46 (57.5)</td>
<td>8 (61.5)</td>
</tr>
</tbody>
</table>
1.2 mRNA expression profiles

The mRNA of 34 freshly frozen GCT samples was analyzed with quantitative PCR for the expression of the factors listed in Table 6. Because of variation in the conditions between analyses and standard curves, all correlations between factors should be made from a single PCR run, and using the same reference gene. This was achieved in the study; however, the results are only comparable with the genes analyzed in the same setting. Therefore, the results can be summarized through rough estimates when compared with the reference gene (Table 6). In addition, there were only 11 samples for which both mRNA and IHC were available in the TTMA, and for most of the factors the numbers were too small to detect correlations between mRNA and protein expression levels. The roles of the different factors are further discussed below (Sections 2-4).

Table 6. Summary of quantitative PCR analysis of mRNA expression levels in 28 primary and 6 recurrent GCT samples. + indicates low expression, ++ indicates medium expression, and +++ high expression compared with the reference gene (GAPDH or Beta-actin).

<table>
<thead>
<tr>
<th>Factor</th>
<th>mRNA expression level</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMH</td>
<td>+</td>
</tr>
<tr>
<td>AMHRII</td>
<td>+</td>
</tr>
<tr>
<td>VEGF</td>
<td>++</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>+++</td>
</tr>
</tbody>
</table>
2. AMH is a growth inhibitor of GCTs (I)

AMH is a crucial regulator of granulosa cell function. AMH is expressed in GCTs (Anttonen 2005), and mouse models suggest a role for AMH in GCT pathogenesis (Pangas 2008; Edson 2010). We elaborated the functional role of AMH in GCTs by characterizing the expression of the AMH receptors and the downstream signaling molecules (Smads) (Section 2.1) and by describing the effect of exogenous AMH on GCT cell proliferation and apoptosis in vitro (Section 2.2).

2.1 Expression of AMH and its receptors in GCTs

The TTMA was utilized for IHC, and 34 tumor tissue samples were used for Western blotting and PCR for the expressions of the AMH and its receptors; the results are summarized in Figure 6. We found that AMHRII expression was characteristic of GCTs; AMHRII expression was detected in 96% of GCTs, while the expression was high in 57% of the tumors. Only three tumors (4%) were negative for AMHRII. Western blotting of AMHRII was positive in all 34 tumors analyzed. Further, all of these tumors expressed AMHRII at the mRNA level. Moreover, the type I AMH receptors ALK2, ALK3, and ALK6 were expressed in GCTs. The AMH receptors were equally expressed in primary and recurrent GCTs. Furthermore, the levels of AMHRII expression were similar to those observed in a previous study of GCTs (Song 2009). However, AMHRII expression was higher in GCTs than reported in epithelial ovarian cancers (Bakkum-Gamez 2008; Song 2009).

AMHRII expression correlated positively with AMH expression at the protein as well as at the mRNA level (I: Figure 1 b, Table 2). At the protein level, AMHRII expression also correlated with P-Smad1/5 expression, indicating active AMH-AMHRII-P-Smad1/5 signaling in GCTs. P-Smad1/5 expression strongly and positively correlated with Smad5 expression, indicating that Smad5 could be the prominent form of phosphorylated Smads in GCTs. AMH protein expression also correlated positively with expression of Smad1, Smad5, and P-Smad1/5. Together, these data indicate that AMH signaling cascade components are expressed by and colocalize in GCTs.

Our group previously described AMH protein expression to be decreased in large GCTs (Anttonen 2005). We could now confirm this result at the mRNA level (Figure 9 B), supporting the role of AMH as a growth inhibitor in GCTs. Moreover, mouse models suggest that disruption in the balance between AMH/BMP-type signaling (Smad1/5) and pro-tumorigenic TFG-β-activin signaling (Smad 2/3) contributes to GCT pathogenesis (Pangas 2008; Middlebrook 2009). Interestingly, we observed that P-Smad2/3 and P-Smad1/5 were equally expressed in GCTs less than 10 cm in diameter (n=55), and a positive correlation existed between the P-Smad1/5 and P-Smad2/3 (p=0.0058). By contrast, in larger tumors (≥10 cm in diameter, n=35), the balance was disrupted and no correlation was found (A. Färkkilä, unpublished data).
To further analyze the role of AMH in GCTs, we measured AMH levels from the cell culture supernatant of the KGN cell line and primary GCT cells. We found that cultured GCT cells produced detectable levels of AMH in the supernatant; average levels were 0.68 ng/ml (range 0.41-1.06 ng/ml) for KGN and 0.77 ng/ml (range 0.43-1.71 ng/ml) for primary GCT cells after 1- to 5-day incubations.

Based on the expression of both AMH and its receptors in GCTs, AMH putatively has an auto/paracrine regulatory function in GCTs. However, Chang et al. reported that at the serum level AMH positively correlates with tumor size (Chang 2009). This discrepancy between tissue and serum AMH levels may be explained by a growth inhibitory role of AMH in the tumor microenvironment; in a subset of tumors, the reduced AMH levels may lead to increased TGF-β-type signaling. These cancer cells then escape the AMH-controlled growth inhibition, leading to increased growth potential of the tumor. A similar phenomenon is seen in large antral follicles of the ovary; the reduced AMH levels in highly proliferating granulosa cells may be one of the factors promoting the rapid growth of the follicle (Weenen 2004). In larger GCTs, how and why AMH levels decrease are unknown.
Figure 6. Summary of the expression analyses of AMH, AMH receptors, and Smad1, Smad5, and phosphorylated Smad1/5 in GCTs (I). AMH type I receptors (Alk2, -3, and -6) and AMHRII, as well as the downstream effectors Smad1 and -5 were readily expressed in GCTs as analyzed with PCR in 26 primary and 4 recurrent (no. 21, 24-26) GCTs (A). Real-time PCR of 28 GCTs revealed decreased AMH levels in large GCTs (p=0.0391) (B). Representative images of the immunostainings in normal granulosa cells (row 1) and GCTs (rows 2-4). High magnification (100x) images of the high-staining group are presented in row 3. An asterisk marks the normal granulosa cells in the lining of an antral follicle (row 1). Scale bars 200 µm in C (A-L).
2.2 AMH treatment of GCT cells in vitro

Based on the expression analyses, AMH is putatively a growth inhibitor in the tumor microenvironment, and we next wanted to explore the functional effects of recombinant human AMH (rhAMH) on GCT cells in vitro. In KGN cells, we saw that rhAMH significantly decreased cell number relative to the control (AMH 0 µg/ml) during 3- and 10-day incubations (Figure 7 A). AMH had, however, no significant effect on cell proliferation (Figure 7 B). Similar results were obtained from two cases of primary GCT cells (Figure 7 C).

**Figure 7.** Summary of the effects of rhAMH on cell number and proliferation of GCT cells (I). The KGN and two primary GCT cell cultures were treated with increasing rhAMH concentrations for 1-10 days and analyzed with MTT assays (A, B) or BrdU incorporation assay (C). The MTT data are presented as the relative number of viable cells compared with the control (AMH 0 µg/ml) (A, B); 10% FFCS was used as a control and 1% FFSC as a control for growth factor deprivation. The proliferation of KGN cells with AMH treatment is shown as a proportion of BrdU-positive cells (C). Data are presented as mean ± SD from two (B) or three independent experiments (A, C).
In view of rhAMH treatment decreasing the cell number without an effect on cell proliferation, we studied whether AMH signaling is active and potentially induces apoptosis in GCT cells. We found that rhAMH was able to induce Smad1/5 phosphorylation in KGN cells, indicating that the signaling cascade is functional in GCTs (Figure 8 A). rhAMH treatment also increased the expression of caspase 3, the enzyme for the committed step in apoptosis (Figure 8 A). Similar results were obtained in a primary GCT cell culture after 2- and 5- day incubations (Figure 8 B). Morphological apoptosis was seen in DAPI staining of the tumor cells after rhAMH treatment (Figure 8 C). In KGN cells, rhAMH treatment induced a significant increase in caspase 3/7 activity (I: Figure 4 B) and in the percentage of apoptotic cells (I: Figure 4 C), altogether resulting in an approximately 2-fold increase in apoptotic cells.

**Figure 8.** AMH induces apoptosis in KGN (A) and primary GCT cells (B, C) (I). Western blotting revealed an increase in Phospho-Smad 1/5 and cleaved caspase-3 with AMH treatment. The levels of Smad1, Smad5, Bcl2, and CyclinD2 remained unchanged, and beta-actin served as a loading control. Similar activation of cleaved caspase-3 was seen in a primary cell culture of a recurrent GCT (B), and DAPI staining confirmed increased morphological apoptosis (C) with AMH treatment.
These results on the apoptosis-inducing effect of AMH on AMHRII-positive GCTs are in line with a previous study from epithelial ovarian cancer cells (Ha 2000). In epithelial ovarian cancer, AMH was able to inhibit growth of AMHRII-positive cancers in vitro (Chin 1991; Masiakos 1999; Ha 2000; Wei 2010) and in vivo (Stephen 2001; Stephen 2002; Pieretti-Vanmarcke 2006a; Pieretti-Vanmarcke 2006b). In these models, AMH effect was characterized by apoptosis and mediated through AMHRII (Masiakos 1999; Ha 2000). However, there are some problems when considering AMH treatment for GCTs. AMH protein must be fully cleaved to gain biological activity and to bind to AMHRII (Pepinsky 1988; MacLaughlin 1992; di Clemente 2010; Papakostas 2010). The production of purified, fully cleaved AMH was achieved only recently (di Clemente 2010), and the therapeutic effects of AMH in GCT remain to be evaluated in a more clinical setting. In GCT patients, AMH levels are also elevated in the serum (Long 2000; Chang 2009), and the mechanism of exogenous AMH as a therapeutic agent is questionable. A promising future alternative is the targeting of AMHRII with activating antibodies (Yuan 2006) or small molecule agonists (Renlund 2008; Wei 2010), especially in cancers expressing high levels of AMHRII such as GCT ((Song 2009) and I). Currently there are no cancer drugs targeting AMH or AMHRII in clinical trials. In view of these findings and the high expression of AMHRII in GCTs, and the low expression in normal tissues, AMHRII is a potential target for cancer therapy in advanced or poor-prognosis GCT patients.
3. VEGF is pro-tumorigenic in GCTs (II, III)

Angiogenesis is a critical step in tumor progression (reviewed in (Carmeliet 2005)). VEGF is a key factor in tumor angiogenesis, while VEGFR-2 is the primary receptor transmitting the VEGF signals (Shalaby 1995). GCTs are highly vascularized tumors, but little is known about the functional role of VEGF in GCTs. We set out to analyze the expression of VEGF and its receptors in GCTs (Section 3.1) and further analyzed the functional role of soluble VEGF in GCTs (Sections 3.2 and 3.3) using tissue samples and cell cultures.

3.1 Expression of VEGF and its receptors in GCTs

The TTMA was stained for VEGF and its receptors and tumor MVD was analyzed concurrently with CD34 staining (Figure 9). We found that VEGF protein was expressed in the vast majority of GCTs; 96% of the tumors were positive, and 75% exhibited high expression (Table 5). The expression of VEGF was higher in GCTs than in granulosa-lutein cells. In GCTs, the expression of VEGFR-1 was weak, whereas VEGFR-2 expression was strong; 99% of the tumors were positive, and high expression was seen in 93% (Table 2). In addition to the tumor blood vessels (arrow in inset of Figure 9 C), VEGFR-2 was also highly expressed in the cytoplasm of the tumor cells (Figure 9), as compared with granulosa-lutein cells. Medium to high levels of the transcripts of VEGF and VEGFR-2 were also present in GCTs (Table 6), whereas low levels of VEGFR-1 were detected only in conventional PCR (data not shown). Moreover, we found that phosphorylated VEGFR-2 (pVEGFR-2) was expressed in 82% of the tumors, and quite surprisingly, the majority of GCTs (95%) expressed P-VEGFR-2 in the nuclei of tumor cells (inset in Figure 9 E). The expressions of VEGF and VEGFR-2 correlated positively at protein and mRNA levels and the expression of pVEGFR-2 correlated positively with that of VEGF, VEGFR-1, and native VEGFR-2. VEGF and the receptors were equally expressed in primary and recurrent tumor samples.

Normally in blood vessel endothelial cells, the expression of VEGFR-2 is autoregulated by VEGF; prolonged exposure of high levels of VEGF lead to decreased expression of the VEGFRs (Wang 2000). In cancer cells, VEGFR-2 is frequently expressed and active (Masood 2001; Fox 2004; Koukourakis 2011) and autocrine and paracrine VEGF-VEGFR-2 signaling has been reported to lead to a survival-promoting autoloop in ovarian (Sher 2009), breast (Weigand 2005), and various other cancers (Masood 2001). As in normal granulosa cells (Greenaway 2004), we found that the expression of VEGF and its receptors colocalize in GCTs. Moreover, GCTs expressed both the ligand and the receptor in an activated state, suggesting auto- or paracrine VEGF-VEGFR-2 signaling in GCTs.

After VEGF binding, VEGFR-2 can be transferred to the nucleus (Feng 1999; Fox 2004; Blazquez 2006). This is in line with the findings in this study; native VEGFR-2 was found to localize on the cell membranes and in the cytoplasm (II), and pVEGFR-2 was predominantly expressed in the nuclei of GCT cells (III). The different VEGF isoforms
also differ in their nuclear localization (Zhang 2000), and the role of nuclear pVEGFR putatively mediates different responses depending on different VEGF isoforms, phosphorylated tyrosine residues, and cell types (Zhang 2000; Fox 2004). In GCTs, nuclear pVEGFR expression correlated with VEGF expression and high tumor MVD, suggesting autocrine VEGF signaling to promote tumor angiogenesis. In addition, the prominent expression of VEGFR-2 also in an activated form provides a molecular basis to target VEGFR-2 also in the treatment of GCTs and support the premise that anti-VEGF treatments have direct effects on GCT cells.
Figure 9. Expressions of VEGF (A), and its receptors VEGFR-1 (B) and VEGFR-2 (C), and phosphorylated VEGFR-2 (E) in GCTs (II, III). CD34 was used to visualize the tumor blood vessels (D). Negative control (F). Scale bars 100 µm in D and 50 µm in the inset in E.
3.2 VEGF and endostatin in serum of GCT patients and tumor cell cultures (III)

Increased levels of circulating levels of VEGF have been reported in ovarian cancer patients and correlate with worse prognosis (Li 2004). We explored the expressions of serum VEGF and endostatin in 54 GCT patients before and after tumor treatment (Figure 10). We found that VEGF levels were elevated in patients with GCT (mean 292.6 pg/ml, range 19.0-1108.4, standard error; SE 43.4) and lower in patients free of disease (mean 150.8 pg/ml, range 7.9-530.4, SE 31.0) (p=0.04, Figure 10 A). Further, in paired analyses of 20 GCT patients, serum VEGF levels decreased significantly after tumor removal (p=0.002). Serum VEGF levels were not significantly higher in patients with larger tumors (≥10 cm in diameter) than in patients with smaller tumors (<10 cm in diameter). The biological variation of serum VEGF is wide even in healthy subjects (Yamamoto 1996), and in this study the serum VEGF levels were not very high in GCT patients; the levels were comparable with those of early stage epithelial ovarian cancer patients (Li 2004). This is in line with the majority of GCTs in our study presenting at an early stage.

Serum endostatin levels have been reported to be elevated in breast and renal cancer patients (Feldman 2000; Zhao 2004). In GCT patients, we found lower serum endostatin levels (mean 135.3 ng/ml, range 67-213, SE 9.1) than in disease free patients (mean 161.3 ng/ml, range 87-264, SE 5.9) (p=0.01, Figure 10 B). We also observed decreased levels of serum endostatin in patients with larger tumors (p=0.02). In paired analyses, serum endostatin levels increased after tumor removal (p=0.01). Serum VEGF and endostatin levels did not correlate with each other, unlike findings in breast (Zhao 2004) and renal cell (Feldman 2000) cancers. Neither serum VEGF nor endostatin levels correlated with patient’s age, tumor treatment, or blood hemoglobin, hematocrite, leukocyte, or platelet counts.

We next analyzed the production of VEGF and endostatin by cultured GCT cells to further evaluate the source of circulating VEGF and endostatin in GCT patients. We found that the KGN cell line and primary GCT cells produced significant amounts of VEGF in the supernatant. This finding suggests that the circulating VEGF is produced by the tumor, which is supported by the decrease in VEGF levels after tumor removal. By contrast, endostatin production by the GCT cells was extremely low. The exact source of circulating endostatin is not known, but it may be proteolytically released from the ECM, particularly in the liver (Schuppan 1998). These data suggest that the high VEGF and low endostatin levels in the serum and in the tumor microenvironment may favor angiogenesis and promote tumor growth in GCTs. Therefore, serum VEGF or endostatin are of limited value as tumor markers for GCT. However, elevated serum VEGF levels may reflect excessive production by the tumor, thus presenting VEGF as a potential target for therapy.
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Figure 10. Serum VEGF is elevated and endostatin is decreased in GCT patients (III). Serum VEGF (A) and endostatin (B) levels were measured from the serum of 54 GCT patients, and the samples were analyzed based on disease presence. WD = with disease, DF = disease-free. An asterisk indicates a significant difference at p<0.05.

3.3 Anti-VEGF treatment of GCTs in vitro (III)

Based on the findings that GCTs produce VEGF and express VEGFR-2 also in activated form, we hypothesized that VEGF acts as an autocrine or paracrine survival factor in GCTs. We next wanted to further elaborate the functional role of endogenous VEGF in GCTs. The GCT cell line (KGN) and primary GCT cells were treated with a humanized monoclonal anti-VEGF antibody bevacizumab (BVZ) to block endogenous VEGF. In KGN cells, we found that BVZ significantly increased caspase 3/7 activity (Figure 11 A) and decreased the number of viable cells (Figure 11 B). The higher dose of BVZ (10 µg/ml) induced roughly 4- to 7-fold increases in apoptotic cells in DAPI analyses. Similar results were seen in six primary GCT cell cultures; a mean 2.5-fold increase in apoptosis and activation of caspase 3/7 occurred (III, Figure 5). In addition, inhibition of endogenous VEGF with BVZ decreased the phosphorylation of pVEGFR-2 (Figure 11 C) in KGN cells.

These results are in line with findings in ovarian and breast cancers, where functionally active VEGFR-2 is expressed on cancer cells, suggesting a survival-promoting VEGF-
RESULTS AND DISCUSSION

VEGFR-2 autoloop in cancer cells (Weigand 2005; Sher 2009; Spannuth 2009). The treatment of ovarian cancer cells with anti-VEGFR-2 treatment suppressed growth in vitro and in vivo (Spannuth 2009). In GCTs, by blocking the endogenous VEGF signalling with BVZ, we could induce apoptosis and a decrease in the number of viable cells. The BVZ doses used in this study were somewhat lower than in other in vitro studies (Sims 2008; Hasan 2011). The relative doses in clinical use are higher as well, corresponding to circulating levels of up to 250 µg/ml (Herbst 2005). BVZ inducing apoptosis in relatively small doses indicates that VEGF is essential for GCT cell survival. These results suggest a survival-promoting VEGF-VEGFR-2 autoloop in GCT cells. Furthermore, our findings implicate that BVZ inhibits growth of GCT cells, and demonstrate in vitro biological activity of BVZ in GCTs.

Targeting VEGF may have severe adverse effects (Tanyi 2011), and tumors may become resistant to anti-VEGF therapy (Abdullah 2011). Targeting of VEGFR-2 shows promise as anti-cancer treatment in advanced non-small cell lung cancer and colorectal cancer patients (Drevs 2007; Schiller 2009). One of the most advanced drugs targeting VEGFR-2 is Ramucirumab®, a fully human VEGFR-2 receptor antagonist that blocks the binding of the ligand to VEGFR-2. Ramucirumab® is currently being investigated in clinical trials for the treatment of several cancers including brain, breast, gastric, lung, colorectal, urinary tract, prostate, and ovarian cancers (www.clinicaltrial.gov). In cancer research, the VEGF-VEGFR-2 pathway may be targeted with other approaches, including small molecule tyrosine kinase inhibitors (reviewed in Saharinen 2011). Based on our results, targeting VEGF/VEGFR-2 may present a therapeutic option also in advanced or recurred GCTs. The effects of VEGF-targeted treatments on GCT patients remain to be further addressed in international, multi-center clinical trials.
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Figure 11. Treatment of KGN cells with bevacizumab induced an increase in apoptosis (A), a decrease in the number of viable cells (B), and a decrease in VEGFR-2 phosphorylation (C) (III).
4. Prognostic factors in GCTs (I-IV)

4.1 Analysis of recurrence and survival of the study cohort

To analyze the GCT recurrences and survival of the study cohort in the TTMA, the clinical data of the patients were collected in the GCT database, and follow-up and survival data were retrieved from hospital files and the Finnish Death Registry. The clinical characteristics of 80 primary GCT patients are summarized in Table 7.

Table 7. Summary of clinical characteristics and their relation to recurrences in a cohort of 80 primary GCT patients. Mp= menopause, C=adjuvant chemotherapy, R= adjuvant radiotherapy

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All patients n=80</th>
<th>No recurrence n=62</th>
<th>Recurrence n=18</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at diagnosis, years</td>
<td>Mean (range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>51.6 (19-87)</td>
<td>52.3 (19-87)</td>
<td>49.2 (28-76)</td>
</tr>
<tr>
<td>Year of diagnosis</td>
<td>Mean (range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mp status:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premenopausal</td>
<td>37 (46.2)</td>
<td>25 (67.6)</td>
<td>12 (32.4)</td>
</tr>
<tr>
<td>Postmenopausal</td>
<td>43 (53.8)</td>
<td>37 (86.0)</td>
<td>6 (14.0)</td>
</tr>
<tr>
<td>Stage I</td>
<td>71 (88.8)</td>
<td>56 (78.9)</td>
<td>15 (21.1)</td>
</tr>
<tr>
<td>Ia</td>
<td>50 (70.4)</td>
<td>40 (80.0)</td>
<td>10 (20.0)</td>
</tr>
<tr>
<td>Ib</td>
<td>1 (1.4)</td>
<td>1 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Ic</td>
<td>20 (28.2)</td>
<td>15 (75.0)</td>
<td>5 (25.0)</td>
</tr>
<tr>
<td>II</td>
<td>6 (7.5)</td>
<td>4 (66.7)</td>
<td>2 (33.3)</td>
</tr>
<tr>
<td>III</td>
<td>3 (3.8)</td>
<td>2 (66.7)</td>
<td>1 (33.3)</td>
</tr>
<tr>
<td>Treatment:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Surgery only</td>
<td>65 (81.3)</td>
<td>50 (76.9)</td>
<td>15 (23.1)</td>
</tr>
<tr>
<td>Surgery + C</td>
<td>12 (15.0)</td>
<td>10 (83.3)</td>
<td>2 (16.7)</td>
</tr>
<tr>
<td>Surgery + R</td>
<td>1 (1.3)</td>
<td>0 (0)</td>
<td>1 (100)</td>
</tr>
<tr>
<td>Surgery + C + R</td>
<td>2 (2.5)</td>
<td>2 (100)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Follow-up time, years</td>
<td>Mean (range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16.8 (0.06 – 36.7)</td>
<td>17.1 (0.06 – 36.7)</td>
<td>15.7 (4.6 – 33.9)</td>
</tr>
<tr>
<td>Survival:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alive</td>
<td>51 (63.8)</td>
<td>44 (86.3)</td>
<td>7 (13.7)</td>
</tr>
<tr>
<td>Dead of GCT</td>
<td>11 (13.8)</td>
<td>1 (9.1)</td>
<td>10 (90.9)</td>
</tr>
<tr>
<td>Dead of other</td>
<td>18 (22.5)</td>
<td>17 (94.4)</td>
<td>1 (5.6)</td>
</tr>
</tbody>
</table>
Eighteen (22.5%) of the patients had a recurrence during the study period. Up to 20% of even stage Ia patients developed a recurrence (Table 7). The median time to recur was 7.0 years (range 2.6-18.4 years, mean 7.7, SD 4.5). Six (33.3%) of the recurrences occurred within 5 years, 14 (77.8%) within 10 years, and 17 (94.4%) within 15 years of the diagnosis. One patient had a recurrence 18.4 years after the primary diagnosis. None of the characteristics in Table 7 was associated with an increased risk of tumor recurrence. Tumor stage was not a prognostic factor for recurrence (Figure 13 A, B). There was no difference in the recurrence probability between stage I and stage II-III patients (Figure 13 A). However, an increased risk of recurrence was seen within 5 years of diagnosis in stage Ic patients relative to stage Ia-b patients (HR 10.64, 95% CI 1.57-208.12, p=0.0142), but the difference was not significant after 10 and 15 years or during the follow-up period (Figure 13 B). The mean time to recur for stage Ia-b patients was 9.3 years (95% CI 5.90-12.62 years), and for stage Ic patients 5.4 years (95% CI 0.42-10.46 years). This may be explained by in stage Ic disease, the tumor cells being disseminated into the abdominal cavity, predisposing to the typical local recurrence of GCT.

Although GCTs are characterized by a tendency towards late recurrence, almost 80% of the recurrences develop within 10 years of the primary diagnosis, and recurrences after 15 years are rather uncommon. Further, only one-third of the recurrences were seen within the first 5 years. In this study, a follow-up of over 10 years was achieved in the majority of the patients alive (n=67, 83.8%), allowing us to reliably evaluate prognostic factors for recurrence. According to these data, a 10-year follow-up would be reasonable to detect most recurrences, at least in high-risk patients.

The disease-specific survival (DSS) rates were similar to those observed in other GCT series (Table 8). The 10-year survival was better for stage I patients than for patients with higher disease stages (II-III) (p=0.0170). If the tumor recurred, the mortality from GCT was 52.6%. The median time to die from GCT was 9.7 years (range 0.06-33.9 years), and the median time to die after the first recurrence was 6.4 years (range 0.3-20.4 years). There was an increased risk of disease-specific death in tumors diagnosed before 1980 (n=23, 28.8%) compared with tumors diagnosed after 1980 (n=57, 71.2%) (odds ratio; OR 5.80, 95% CI 1.50-22.35, p=0.0107) (A. Färkkilä, unpublished data). As there were no differences in recurrence probabilities, this is most probably due to improvements in treatment modalities over the decades, most importantly, the introduction of platinum-based chemotherapy in 1980.
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Table 8. Disease-specific survival rates of 80 GCT patients according to time from diagnosis.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DSS</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 years</td>
<td>97.5%</td>
</tr>
<tr>
<td>Stage I</td>
<td>98.6%</td>
</tr>
<tr>
<td>Stage II-III</td>
<td>88.9%</td>
</tr>
<tr>
<td>10 years</td>
<td>92.5%</td>
</tr>
<tr>
<td>Stage I</td>
<td>95.8%</td>
</tr>
<tr>
<td>Stage II-III</td>
<td>66.7%</td>
</tr>
<tr>
<td>15 years</td>
<td>91.3%</td>
</tr>
<tr>
<td>Stage I</td>
<td>94.4%</td>
</tr>
<tr>
<td>Stage II-III</td>
<td>66.7%</td>
</tr>
<tr>
<td>If recurred*</td>
<td>47.4%</td>
</tr>
</tbody>
</table>

* mean follow-up time 16.8 years

These data confirm that GCT usually presents with an indolent course of disease, with a mean survival of 13.5 years from diagnosis. However, 20-30% of the tumors recur and ultimately over 50% of the recurred patients die of GCT, with a mean survival of 7.8 years from the first recurrence. In view of these results, the major challenges in the treatment of GCT patients are identification of prognostic markers able to predict tumor recurrence and early detection and effective treatment of recurred disease.

4.2 Prognostic factors for recurrence and survival of GCTs (IV)

Molecular prognostic factors are needed for GCT, especially for stage I patients. We evaluated the protein expression profiles of the factors studied in the TTMA (Table 5) in relation to prognosis and survival. In summary, neither VEGF, its receptors, nor tumor MVD was associated with prognosis. AMHRII and its signaling cascade components were not associated with prognosis in GCTs, in contrast to findings in epithelial ovarian cancer (Bakkum-Gamez 2008). Of the EGF receptors, HER3 and HER4 were unrelated to prognosis, as was EGFR expression (N. Andersson, unpublished). Of the molecular prognostic factors, HER2 and GATA4 were associated with tumor recurrence (see Section 4.2.1), and GATA4 was also associated with survival of GCT patients (see Section 4.2.2).

Age at diagnosis, tumor mitotic index, histological subtype, and tumor size did not predict tumor recurrence or survival (II, IV). In univariate analysis, nuclear atypia was not associated with tumor recurrence (IV); however, in Kaplan-Meier analysis, high nuclear atypia was prognostic of shorter DFS (Figure 13 F) and DSS (Figure 14 E) (see Sections 4.2.1 and 4.2.2).
Expression of the factors at mRNA level was not associated with prognosis. This is probably attributable to the relatively small number of primary tumors (n=28). Another factor contributing to the lack of association of mRNA levels with recurrence is the short follow-up time; 50% (n=14) of the tumors were diagnosed after 2004, and 29% (n=8) were diagnosed after 2008.

### 4.2.1 Prognostic factors for recurrence in GCTs

HER2 is a known oncogene and its overexpression is associated with worse prognosis in various cancer types. We stained the TTMA for the expression of EGF receptors HER2, HER3, and HER4. The expressions are summarized in Table 5.

HER3 and HER4 were readily expressed in GCTs (Table 5). We found positive expression of HER2 in 98% of the GCTs, while only 2% were either very low or negative for HER2. In 90% of the tumors, HER2 was also expressed in the phosphorylated form. This finding contradicts the studies reporting GCTs to be negative for HER2 (Kusamura 2003; Leibl 2006; Mayr 2006; Menczer 2007). These mostly immunohistochemical studies have used various methods and antibodies. Some of them have applied the HercepTest®, which was originally developed for screening of breast cancer (Leibl 2006; Mayr 2006). In breast cancer, amplification of the HER2 gene leads to up to 100-fold overexpression of the oncoprotein (Press 1993). However, in GCTs the HER2 gene was rarely amplified ((Mayr 2006) and this study). This may lead to milder overexpression in GCTs, which might be undetectable by the HercepTest®. In support of this notion, we found relatively high levels of HER2 mRNA transcript in all 34 GCTs when analyzed with quantitative PCR (N. Andersson, unpublished data). Based on these results, HER2 expression may be transcriptionally mediated in GCTs. This is in line with the findings in epithelial ovarian carcinomas, where HER2 protein overexpression was not as strongly associated with gene amplification as in breast cancer (Lassus 2004). Representative images of low and high expression groups of HER2 and GATA4 are presented in Figure 12.
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Figure 12. *TTMA of 80 primary and 12 recurrent GCTs was stained for GATA4 (Anttonen 2005) (A, B) and HER2 (C, D), and the tumors were divided into low and high expression groups (IV).*

The protein expressions of HER2 and GATA4 correlated positively (p=0.0006) in all tumors. Further, both high HER2 and high GATA4 expressions were associated with higher tumor stage (stages II-III and Ib-III; p<0.05). No correlations were found with the other clinicopathological parameters of Tables 2 and 7.

In univariate analyses, high expression of HER2 and GATA4 was associated with tumor recurrence, even in stage Ia tumors (contingency tabling, data not shown). In Kaplan-Meier analyses, high expression of HER2 (HR 3.15, 95% CI 1.20-8.00) (Figure 13 C) and high expression of GATA4 (HR 4.04, 95% CI 1.52-12.64) (Figure 13 D) were prognostic of shorter disease-free survival (DFS). The DFS was even shorter when the two factors were both highly expressed in the tumor (HR 6.61, 95% CI 1.98-25.44) (Figure 13 E). Both high GATA4 (stage-adjusted HR; AHR 3.96, 95% CI 1.45-12.57, p=0.006) and high HER2 (AHR 3.02, 95% CI 1.11-7.94, p=0.03) predicted DFS independently of tumor stage. However, in multivariate stage-adjusted analyses, GATA4 was superior to HER2 in predicting recurrence (Table 3 C in IV).

According to these data, HER2 is expressed in the majority of GCTs, with high expression being associated with higher stage and increased recurrence risk. HER2 may thus be considered a target for treatment for the more aggressive GCTs, and as in breast cancer
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(Slamon 2001) HER2 immunostaining could be used to select patients for targeted therapy. In multivariate analyses, however, GATA4 seemed superior to HER2 in delineating the prognosis of CCT patients. Overall, keeping in mind the lack of HER2 gene amplification in GCTs and the lack of HER2 overexpression with the clinically available test (HercepTest ®), the single analysis of HER2 expression is likely to be of limited use in the prognostic evaluation of GCT patients. However, combined with GATA4, HER2 may provide additional information on the biological behavior of GCTs.

Nuclear atypia was prognostic of shorter DFS (HR 3.00, 95% CI 1.10-7.66) (Figure 13 F), and in Cox regression analyses high nuclear atypia was an independent prognostic factor for DFS (Table 9 A). However, after adjusting for stage, the AHR, its 95% CI, and p-value of nuclear atypia were not very different from those of GATA4 (Table 9 A). Moreover, the combined high expression of HER2 and GATA4 was an even stronger independent prognostic factor when analyzed with nuclear atypia (Table 9 B). High expression of both HER2 and GATA4 was an independent prognostic factor also when studied only in stage Ia (HR 11.5, 95% CI 1.76-79.41, p=0.0126) and stage I (HR 5.62, 95% CI 1.45-23.48, p=0.0146) tumors.

Whether a tumor has high- or low-grade nuclear atypia is the subjective opinion of a pathologist, with great inter- and intra-observer variation. In this study, the same pathologist assigned the degree of nuclear atypia for all samples. In clinical pathology, this is rarely achievable and may complicate the use of nuclear atypia as a prognostic factor in GCTs.

Table 9. Cox regression models for DFS with HER2, GATA4, and nuclear atypia (A) and combined expression of HER2 +GATA4 and nuclear atypia (B). HR: hazard ratio, AHR: stage-adjusted HR.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Expression level</th>
<th>A</th>
<th>B</th>
</tr>
</thead>
<tbody>
<tr>
<td>HER2</td>
<td>High</td>
<td>2.19</td>
<td>6.30</td>
</tr>
<tr>
<td>GATA4</td>
<td>High</td>
<td>2.70</td>
<td>2.81</td>
</tr>
<tr>
<td>Nuclear atypia</td>
<td>High</td>
<td>2.81</td>
<td>2.81</td>
</tr>
<tr>
<td>HER2 + GATA4</td>
<td>High + High</td>
<td>6.30</td>
<td>6.30</td>
</tr>
<tr>
<td>Nuclear atypia</td>
<td>High</td>
<td>1.44</td>
<td>1.44</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

Figure 13. Kaplan-Meier curves for disease-free survival to the first recurrence of 80 GCT patients according to tumor stage (A, B), expression of HER2 (C), GATA4 (D), HER2 and GATA4 (E), and nuclear atypia (F) (IV). Log-rank test, *: significant difference at p<0.05, **: significant difference at p<0.01. The p-value in E was derived from comparison between the low+low and high+high groups.
4.2.2 Prognostic factors for survival in GCTs

Next we analyzed the factors affecting survival in GCTs. We found that patients with stage II-III tumors had shorter DSS (Figure 14 A), although one must bear in mind that the number of stage II-III patients was relatively small (n=9). The DSS was not different in stage Ia patients compared with stage Ib-III patients (Figure 14 B). HER2 expression was not associated with survival (Figure 14 C). High GATA4 expression was prognostic of shorter DSS; in Kaplan-Meier estimates, the 10-year DSSs were 84.2% (SE 6.2%) for high GATA4 and 97.8% (SE 2.2%) for low GATA4 expression (p=0.0383) (Figure 14 D). High nuclear atypia was also prognostic of DSS, and the 10-year survival rates were 76.3% (SE 10.4%) for high nuclear atypia and 96.4% (SE 2.5%) for low nuclear atypia (p=0.0155) (Figure 14 E). In multivariate analyses, stage II-III and high nuclear atypia were both independently prognostic of worse DSS (IV: Table 3 D). In stage I and stage Ia tumors, both GATA4 and nuclear atypia were prognostic to shorter DSS (Table 10).

Table 10. GATA4 and nuclear atypia as prognostic factors for DSS in stage I and stage Ia GCTs (IV).

<table>
<thead>
<tr>
<th>Factor</th>
<th>Stage I HR (95% CI) p-value</th>
<th>Stage Ia HR (95% CI), p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GATA4</td>
<td>4.99 (1.14-34.21), 0.0322</td>
<td>7.71 (1.12-151.85), 0.0375</td>
</tr>
<tr>
<td>Nuclear atypia</td>
<td>5.29 (1.16-26.94), 0.0319</td>
<td>18.2 (2.32-369.6), 0.0062</td>
</tr>
</tbody>
</table>

Based on these results, nuclear atypia seemed to be the most potent factor in delineating disease-specific survival of GCT patients. However, one must keep in mind that the number of GCT-related deaths was relatively small in this study (n=11).
Figure 14. Kaplan-Meier curves for disease-specific survival of 80 GCT patients according to tumor stage (A, B), expression of HER2 (C), GATA4 (D), and nuclear atypia (E) (IV). Log-rank test, *: significant difference at $p < 0.05$ **: significant difference at $p < 0.01$. 
4.3 Role of GATA4 in GCTs

According to this study, GATA4 is an important factor in delineating the prognosis of GCT patients; high expression of GATA4 was prognostic of increased risk of recurrences and unfavorable survival. Keeping in mind the essential role of GATA4 in normal granulosa cell function, GATA4 potentially has a crucial role in GCT pathogenesis.

In breast cancer, GATA4 expression positively correlated with HER2 expression (Bertucci 2004), and GATA4 was shown to directly regulate HER2 expression by binding \( \text{HER2} \) promoter (Hua 2009). In GCTs, we found that the expressions of GATA4 and HER2 colocalized in immunohistochemistry, together delineating an aggressive subset of GCTs. However, overexpression of GATA4 was more strongly associated with shorter DFS, and may thus be one of the transcription factors contributing to HER2 protein overexpression in the aggressive GCTs.

GATA4 is also likely to contribute to other factors in this study. AMH is a known target gene of GATA4, and in granulosa cells GATA4 directly upregulates AMH expression by binding to its promoter (Tremblay 2001a; Anttonen 2003). This may well be one of the mechanisms by which AMH expression is regulated in GCT tumorigenesis. However, based on these studies, the most aggressive tumor-promoting conditions involve increased GATA4 and decreased AMH expression. The expressions of GATA4 and AMH did not correlate in the TTMA (Anttonen 2005). The regulatory role of GATA4 in GCT pathogenesis is likely to involve cofactors that interact with GATA4 in AMH regulation such as SF-1 and friend of GATA-2 (FOG-2) (Tremblay 1999; Tremblay 2001b; Anttonen 2003). GATA4 may also have a dual role in regulating the same target gene expression depending on the interplay with different cofactors (Tremblay 2001c; Anttonen 2003).

In granulosa cells, GATA4 was required for TGF-\( \beta \) signaling through interaction with Smad3 (Anttonen 2006), and in GCTs GATA4 has been found to interact with FOXL2 and Smad3 (M. Anttonen, unpublished data). This links GATA4 to the fundamental genetic and signaling cascade changes that may give rise to GCT. Further, GATA4 is directly involved in GCT cell survival, protecting GCT cells from apoptosis (Kyronlahti 2008; Kyronlahti 2010). Moreover, GATA4 may also contribute to tumor angiogenesis in GCTs, as it was shown to bind VEGF promoter and act as a pro-angiogenic factor through VEGF in cardiac cells (Heineke 2007).

Based on this study and the current literature, GATA4 is likely to be a major pathogenetic factor in GCTs. GATA4 is also fundamentally involved in delineating the recurrences and survival of GCT patients. Immunostaining of GATA4 is likely to be useful in prognostic evaluation of GCTs. Currently, there are no known therapies that target GATA4. Transcription factors are difficult to target in cancer therapy due to their ubiquitous expression and important functions in normal tissues. Therefore, the development of GATA4-targeted therapies is likely to involve the GATA4 target genes. In view of our results on GCTs, the most potential GATA4-linked targets for treatment include AMH,
AMHRII, and other members of the TGF-β signaling pathway, VEGF, VEGFR2, and HER2.
Conclusions and future perspectives

1. AMH, its receptor AMHRII, and its signaling cascade components were expressed and active in GCTs. Treatment with rhAMH induced apoptosis in GCT cells. AMH is likely to play an important role in GCT pathogenesis by acting as a growth inhibitor, and AMH and AMHRII are new potential targets for anti-cancer treatment of advanced or recurred GCTs.

2. VEGF and its receptor VEGFR-2 were highly expressed in GCTs. GCTs produced significant amounts of soluble VEGF and expressed VEGFR-2 in an active, phosphorylated form. Further, the inhibition of endogenous VEGF led to a decrease in VEGFR-2 activation and to a significant increase in apoptosis in GCT cells. The results suggest that VEGF acts auto- or paracrinely to promote tumor growth in GCTs. VEGF-targeted treatments are potential treatment options for aggressive GCTs.

3. GATA4 is an important factor in delineating the prognosis and survival of GCT patients and may be useful in the clinicopathological assessment of GCT prognosis. Members of the EGF receptor family are expressed in GCTs and are potential targets for therapy. This study demonstrates that several granulosa cell growth factors play significant roles in GCT pathogenesis and describes potential targets for biological treatment of poor-prognosis GCT patients. Targeting the receptors of the growth factors, such as AMH, VEGF, or EGF, is an attractive option in cancer therapy, especially with high and preferably cancer-specific expression of the receptor. Of these, AMHRII has been shown to be a potential cancer-specific molecule that may be targeted in gynecological cancers with, for instance, immunotherapy.

Modern, extremely efficient, high throughput technologies allow the screening of hundreds of cancer drugs for tumor-specific responses (Iljin 2009), opening up the possibilities to personalize cancer treatments. This is important, especially in rare tumors, such as GCT, where evidence-based data on the efficacy of different regimens from large clinical trials are difficult to obtain. More detailed genetic and proteomic analyses of GCTs would build a basis for the detection of new cancer/GCT-specific molecular and signaling pathways that can be utilized in the search for prognostic factors or treatment options. These studies would also shed further light on the mechanism of how molecular changes, especially FOXL2 mutation, contribute to GCT pathogenesis.

The detailed analysis of our internally large clinical series of over 230 GCT patients allows us to review also the clinical risk factors for recurrences and survival. Analysis of the large clinical cohort will give new information to clinicians and patients on the characteristics of this disease. In addition, with the prospective study started in 2007, we have collected serum samples for the development of better serum markers for GCT, such as AMH. The results of these studies will putatively lead to more reliable detection of GCT recurrence and directly improve the follow-up of GCT patients. These analyses
facilitate the development of clinical guidelines for evidence-based treatment, prognostic evaluation, and follow-up of GCT patients.
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Anna Färkkilä
References


REFERENCES


REFERENCES


Feng, Y., Venema, V. J., Venema, R. C., Tsai, N. and Caldwell, R. B. (1999). "VEGF induces nuclear translocation of Flk-1/KDR, endothelial nitric oxide synthase, and...


Ha, T. U., Segev, D. L., Barbie, D., Masiakos, P. T., Tran, T. T., Dombkowski, D., Glander, M., Clarke, T. R., Lorenzo, H. K., Donahoe, P. K. and Maheswaran, S.
REFERENCES


REFERENCES


REFERENCES


REFERENCES


REFERENCES


Inhibiting Substance enhances subclinical doses of chemotherapeutic agents to inhibit human and mouse ovarian cancer." Proc Natl Acad Sci U S A 103(46): 17426-17431.


Rolaki, A., Coukos, G., Loutradis, D., DeLisser, H. M., Coutifaris, C. and Makrigiannakis, A. (2007). "Luteogenic hormones act through a vascular endothelial growth factor-dependent mechanism to up-regulate alpha 5 beta 1 and alpha v beta 3 integrins,


REFERENCES


REFERENCES


