Université de Montréal

ANTI-VEGFA THERAPY REDUCES TUMOR GROWTH AND EXTENDS SURVIVAL IN A MURINE MODEL OF OVARIAN GRANULOSA CELL TUMOR

par
MAYRA TSOI

Département de biomédecine vétérinaire
Faculté de médecine vétérinaire

Mémoire présenté à la Faculté de médecine vétérinaire en vue de
l'obtention du grade de maître ès sciences (M.Sc.)
en sciences vétérinaires option reproduction

Septembre 2012

Résumé

Les tumeurs des cellules de la granulosa (GCTs) sont des tumeurs avec un potentiel malin ayant tendance à récidiver, provoquant ainsi la mort dans 80% des cas de stade avancé consécutif à une rechute. Bien que les GCTs représentent 5% des tumeurs ovariennes, peu d'études ont évalué les protocoles de traitement adjuvant pour la maladie avancée ou récurrente. Notre but était d'évaluer l'efficacité de la voie de signalisation du facteur de croissance de l'endothélium vasculaire A (VEGFA) comme cible pour le traitement de la GCT utilisant le modèle murin transgénique $Pten^{tm1Hwu/tm1Hwu}$; $Ctnnb1^{tm1Mmt/+}$; $Amhr2^{tm3(cre)Bhr/+}$ (*PCA*) qui reproduit le stade avancé de la maladie humaine. Un anticorps anti-VEGFA a été administré une fois par semaine par voie intrapéritonéale (IP) à partir de 3 semaines d'âge. La thérapie anti-VEGFA a permis une réduction de la taille des tumeurs à 6 semaines d'âge ($p<0.05$) et une prolongation de la survie des animaux traités, lorsque comparé aux animaux contrôles. L'analyse des GCTs a montré une réduction significative de la prolifération cellulaire ($p<0.05$) et de la densité microvasculaire ($p<0.01$) mais aucune différence significative n'a été détectée dans l'apoptose cellulaire. p44/p42 MAPK, un effecteur de la signalisation pour le récepteur 2 de VEGFA (VEGFR2) associé à la prolifération cellulaire, était moins activé dans les tumeurs traitées ($p<0.05$). Par contre, l'activation d'AKT, un effecteur impliqué dans la survie cellulaire, était similaire d'un groupe à l'autre. Ces résultats suggèrent que l'anticorps anti-VEGFA réduit la prolifération cellulaire et la densité microvasculaire chez les souris *PCA* par inhibition de la voie de signalisation VEGFR2-MAPK, inhibant ainsi la croissance tumorale. En conclusion, l'efficacité de la thérapie anti-VEGFA mérite d'être évaluée en essais contrôlés randomisés pour le traitement des GCTs chez l'homme.

Mots clés : tumeur des cellules de la granulosa, thérapie anti-VEGFA, angiogenèse, traitement adjuvant, modèle murin préclinique, signalisation AKT, signalisation MAPK

Abstract

Ovarian granulosa cell tumors (GCTs) are potentially malignant tumors that have a tendency for late recurrence and cause death in 80% of women with advanced GCT due to recurrent disease. Although GCTs represent 5% of ovarian tumors in women, few studies have evaluated adjuvant treatment protocols for advanced or recurrent disease. Our goal was to determine the potential of targeting the vascular endothelial growth factor A (VEGFA) signaling pathway for the treatment of GCT. We used a genetically engineered mouse model, $Pten^{tm1Hwu/tm1Hwu}$; $Ctnnb1^{tm1Mmt/+}$; $Amhr2^{tm3(cre)Bhr/+}$ (*PCA*), which imitates the advanced human disease. A monoclonal anti-VEGFA antibody was administered by intra-peritoneal injection once a week beginning at 3 weeks of age. Anti-VEGFA therapy significantly decreased tumor weights by 6 weeks of age ($p<0.05$) and increased survival in treated animals in comparison to controls. Significant decreases in tumor cell proliferation ($p<0.05$) and microvessel density ($p<0.01$), but no significant difference in apoptosis was found in *PCA* tumors. p44/p42 MAPK, a VEGFA receptor 2 (VEGFR2) signaling effector associated with cell proliferation, was significantly less activated in anti-VEGFA-treated tumors ($p<0.05$). In contrast, AKT activation, a VEGFR2 signaling effector associated with cell survival was similar among all groups. These results suggest that anti-VEGFA therapy effectively reduces cell proliferation and microvessel density in *PCA* mice by inhibition of the VEGFR2-MAPK pathway, resulting in inhibition of GCT growth. We conclude that anti-VEGFA therapy merits further investigation in the form of controlled randomized trials for the treatment of human GCT.

Keywords: granulosa cell tumor, anti-VEGFA therapy, angiogenesis, adjuvant therapy, preclinical mouse model, AKT signaling, MAPK signaling

Table of contents

List of tables

Publication

List of figures

Publication

List of acronyms and abbreviations

AC: adenylyl cyclase

AKT: protein kinase B

AMH: anti-Müllerian hormone

APC: adenomatous polyposis coli

BCL2L2: beta-cell leukemia/lymphoma-2-like protein 2

BEP: bleomycin/etoposide/cisplatin

BMP: bone morphogenetic protein

c-erbB2 : human epidermal growth factor receptor 2

CA: *Ctnnb1*$^{tm1Mmt/+}$; *Amhr2*$^{tm3(cre)Bhr/+}$

CT: computed tomography

CYP19A1: cytochrome P450 aromatase

DSH: disheveled

EGFR: epidermal growth factor

ERK: extracellular regulated kinase

ESR: estrogen receptor

FDA: US Food and Drug Administration

FIGO: International Federation of Gynecology and Obstetrics

Flk-1: fetal liver kinase 1

Flt-1: FMS-like tyrosine kinase 1

FOXL2: forkhead box L2

FSH(R): follicle-stimulating hormone (receptor)

FZD: frizzled receptor

GCT: granulosa cell tumor

GnRH: gonadotropin releasing hormone

GSK3β: glycogen synthase kinase 3 beta

HIF: hypoxia inducible factor

HPF: high power field

HPS: hematoxylin phloxine saffron

IGF1(R): insulin-like growth factor-1 (receptor)

IP: intraperitoneal

IV: intravenous

KDR: kinase insert domain receptor

LH: luteinizing hormone

LRP: low-density lipoprotein receptor-related protein

mAb: monoclonal antibody

MAPK: mitogen-activated protein kinase

MEK: ERK kinase

MRI: magnetic resonance imaging

NSCLC: non-small-cell lung cancer

PA: *Pten*$^{tm1Hwu/tm1Hwu}$; *Amhr2*$^{tm3(cre)Bhr/+}$

PCA: *Pten*$^{tm1Hwu/tm1Hwu}$; *Ctnnb1*$^{tm1Mmt/+}$; *Amhr2*$^{tm3(cre)Bhr/+}$

PDGFR: platelet-derived growth factor receptor

PDK: 3-phosphoinositol-dependent protein kinase

PI3K: phosphoinositide 3-kinase

PKA: protein kinase A

PTEN: phosphatase and tensin homolog

RTK: receptor tyrosine kinase

SCST: sex cord-stromal tumor

SFK: SRC family protein kinase

TCF/LEF: transcription factor, T-cell specific/lymphoid enhancer factor 1

TGF: transforming growth factor

TKI: tyrosine kinase inhibitor

VEGF(R): vascular endothelial growth factor (receptor)

Acknowledgements

I would like to take this opportunity to thank those who made this work possible. To my research director Dr. Derek Boerboom, thank you for answering my endless questions and helping me discover the wonderful world of research. To Dr. Marie-Ève Nadeau, thank you for sparking my interest in oncology. To Dr Marilène Paquet, thank you for your excellent analyses. To my colleagues Marie-Noëlle, Evelyne, Alexandre, Aurore and Charlène, thank you for all your help. Thank you to the members of my advisory committee and to the members of the jury who graciously agreed to assist me in this endeavor. Also, thank you to all those who assisted in the management of the mouse colony. Finally, a special thank you to my boyfriend, family and friends for their endless patience and constant support.

Introduction

Cancer is a comprehensive term that encompasses over one hundred diseases that all share in common the transformation of normal human cells into malignant (cancer) cells (1, 2). Initially, a succession of genetic changes occurs that confer growth advantages to given cells, leading to their conversion to cancer cells and ultimately tumor development (2). In contrast to normal cells, cancer cells are able to sustain proliferation, evade tumor suppression, resist apoptosis, replicate indefinitely, induce angiogenesis, and invade and form metastases (3). These acquired alterations in cell physiology are what dictate tumor growth in combination with signals from the tumor microenvironment and inflammatory mediators (2, 4).

Many different types of cancer affect the female reproductive system, with ovarian cancer being the leading cause of death among gynecological cancers and representing 3% of all cancers in women (1). Three categories of ovarian tumors exist with each one named according to its derived cell type (5). 80-90% of ovarian tumors are epithelial ovarian tumors, followed by sex cord-stromal tumors (SCSTs), which make up 7-8%, and the remaining 1-3% are germ cell tumors (5, 6). Within the category of SCSTs, a given tumor will contain sex cord and stromal components of the developing gonad that may include one or a combination of granulosa cells, theca cells, lutein cells, Sertoli cells, Leydig cells, or fibroblasts (7). The majority are granulosa cell tumors (GCTs), accounting for 70-90% of SCSTs and approximately 5% of all ovarian tumors (6, 8-10). GCTs are considered as potentially malignant tumors characterized by insidious growth and late recurrence (6, 8, 11, 12).

Although some studies have investigated GCT pathogenesis, the mechanisms mediating GCT development remain unclear. One factor that has been identified as a crucial

component of GCT disease is angiogenesis, as these are highly vascularized tumors that express high levels of vascular endothelial growth factor (VEGF), a key mediator of tumor angiogenesis (11, 13, 14). Based on this, angiogenesis was proposed as a therapeutic target for the adjuvant treatment of GCT (11, 13-16).

Animal models can serve as useful tools to evaluate the efficacy of novel therapeutic agents and targets. A genetically engineered mouse model that develops GCT that imitates the advanced disease in women was generated and served as a preclinical model for investigating a novel therapeutic agent that targets VEGF.

This thesis will present the evaluation of an anti-angiogenic therapy for the treatment of GCT in our mouse model. First off, a review of ovarian and GCTs in women will be presented, followed by current knowledge of GCT pathophysiology. Next, descriptions of the existing transgenic mouse models of GCT will be given, followed by a presentation of angiogenic inhibitors. Finally, the findings from this study will be presented and discussed.

Literature Review

Chapter 1: Ovarian Cancer

1.1. Ovarian tumors in women

The American Cancer Society estimates that 22,280 women will be newly diagnosed with ovarian cancer in 2012, of which 15,500 will die from it in the United States alone (1). Ovarian cancer is the leading cause of death among gynecological cancers, accounting for 3% of all cancers in women (1).

Ovarian tumors are classified into three major groups based on histological patterns and cell types; the most common and best described are epithelial ovarian tumors (80-90% of cases), followed by SCSTs (7-8%), and finally germ cell tumors (1-3%) (5, 6). Accounting for 70-90% of SCSTs are GCTs, which represent approximately 5% of all ovarian tumors and are derived from the hormonally active component of the ovarian stroma, the granulosa cell (6, 8-10, 17). The reported incidence of GCT is 0.58 to 1.6/100,000/year (18).

1.2. Granulosa cell tumors in women

1.2.1. Forms

Although GCTs are considered to be rare, they are potentially malignant, tend to recur over long periods, and are therefore of clinical importance (8, 11, 12). They are divided into the adult (95%) and juvenile forms (5%) based on clinical presentation and histological characteristics (8, 18). The adult form mostly occurs in post-menopausal women with the

median age at presentation being 50-54 years old (17) and is characterized by well and poorly differentiated histological patterns with coffee-bean grooved nuclei (6). On the other hand, the juvenile form mostly affects women less than 30 years of age (mean 13-17 years) and is characterized by large luteinized cells with hyperchromatic nuclei lacking nuclear grooves (6, 8, 18). Both the adult and juvenile form have been reported in children and adults, respectively (8, 17, 18).

1.2.2. Clinical presentation

GCT cells share morphological, biochemical, and hormonal features of normal proliferating pre-ovulatory granulosa cells (19, 20). Approximately 70% of GCTs produce hormones that include estradiol, inhibin, and anti-Müllerian hormone (AMH) that are responsible for a variety of nonspecific symptoms such as abdominal pain, distension, bloating, and abnormal uterine bleeding (6, 8,18). Other symptoms may include menstrual irregularities, post-menopausal bleeding, abdominal discomfort, weight loss, presence of a pelvic or abdominal mass, and breast enlargement or tenderness (18). Abnormal uterine bleeding and abdominal pain have been reported as the most common presenting symptoms (18, 21). The tumor mass may compress adjacent organs and cause dysuria and constipation (8). Ascites occurs in 10-28.8% of cases (8, 18, 22). Complications, such as ovarian torsion, haemorrhage, or cystic rupture, often manifest as acute abdominal pain and distension (8, 18). Unfortunately, 12.5-14.3% of patients are asymptomatic and are only diagnosed during routine examinations (21, 22).

Specific to the juvenile form, 80-90% of patients 8 years of age or less present with isosexual precocious pseudopuberty, characterized by precocious breast development, increased pubic hair, vaginal bleeding, and advanced growth (6, 8, 18).

Some pathological conditions have been associated with GCT and include endometrial hyperplasia in 25-50% of women (due to estrogen stimulation of the endometrium), endometrial adenocarcinoma in 8-13% of women over the age of 45, breast cancer in 3% of women, and infertility in 22% of women of childbearing age (8, 18). The juvenile form has been associated with Potter's syndrome, multiple congenital abnormalities, Ollier's disease, and Maffucci's syndrome (18).

1.2.3. Diagnosis

Initial diagnosis is based on clinical features and pelvic examination (8). As the majority of GCTs produce oestrogens that are responsible for various symptoms, this often allows for early detection (17). At diagnosis, many GCTs have already attained a considerable size (average diameter of 12 cm), 95% of cases are unilateral, 85-95% are palpable, and 78-91% are at stage I/IV (i.e. confined to the ovary(ies); according to FIGO classification) (8, 17, 18, 23).

The tumors may appear as a solid mass with hemorrhagic or fibrotic changes, as completely cystic, or with multilocular cystic lesions (18). One classification type categorizes adult-type GCT based on morphologic patterns as either multilocular cystic, thick-walled unilocular cystic, or thin-walled unilocular cystic, homogenously solid or heterogeneously solid (24).

Diagnostic tools include ultrasound, which provides qualitative information about the mass, such as size, echogenicity, and consistency however, CT and MRI are more sensitive exams (8, 17). Uterine biopsy is indicated in cases of postmenopausal bleeding in order to exclude adenocarcinomas (17).

Final diagnosis of GCT is based on histopathology examination (8). Malignant GCTs resemble normal granulosa cells but with round to oval nuclei and coarsely stippled chromatin. Cytoplasm is often faintly eosinophilic and vacuolated. GCTs demonstrate a high degree of cellular polymorphism and a high mitotic rate with focal areas of necrosis and hemorrhage, and evidence of local invasion (25).

1.2.4. Serum tumor markers

Many serum tumor markers have been proposed for the management and surveillance of GCT, although very few are specific (8). The most relevant markers include: inhibin, estradiol, and anti-Müllerian hormone (6, 8, 17, 26).

1.2.4.1. Inhibin

Inhibin is a member of the transforming growth factor (TGF)-β family that is synthesized by normal granulosa cells. It is mostly secreted during the follicular phase of the menstrual cycle and acts by suppressing the synthesis and secretion of follicle-stimulating hormone (FSH) (17, 18). Patients with GCT tend to have elevated inhibin serum concentrations (mostly inhibin B), which has been positively correlated with tumor size (26) and therefore serve to detect GCT and estimate tumor bulk (6). As inhibin levels drop after tumor removal, they also serve to predict and detect recurrence (6, 26, 27). Serum inhibin

levels also drop in postmenopausal women (secondary to the depletion of ovarian follicles), which may serve as a comparative baseline in these women (6, 28). It is important to note, however, that elevated inhibin levels are not specific for GCT, and may be elevated in epithelial ovarian tumors (17).

1.2.4.2. Estradiol

Estradiol is mostly produced by granulosa cells during the reproductive period in women (6). In the majority of GCTs, estradiol is secreted in large amounts and is responsible for many of the tumor-related symptoms (6). Although estradiol might seem like a logical tumor marker for GCT, no correlation between estradiol levels and GCT progression or recurrence has been found (29, 30). This could be due to the fact that estradiol production depends on its precursor, androstenedione, produced by theca cells and in the absence of theca cells in some GCTs, no estradiol is secreted (6, 17, 29). On the whole, estradiol is not considered to be a reliable tumor marker for GCT, especially in comparison to inhibin B and AMH (6, 17, 29).

1.2.4.3. Anti-Müllerian hormone

AMH is another member of the TGFβ superfamily that is expressed by granulosa cells and controls the formation of primary follicles during reproductive life, but is virtually undetectable after menopause (17, 28). Serum AMH levels were found to be elevated in the majority of patients with GCT, undetectable in patients with clinical remission, and elevated several months prior to clinical recurrence (30, 31). In a recent review, inhibin B and AMH

were both found to be useful serum markers for diagnosis and surveillance of patients with GCT (32).

1.2.5. Current treatment

Initial management of GCT involves cytoreductive surgery, which allows for staging, definitive diagnosis, and tumor debulking (8, 17, 18). For women beyond childbearing age, total hysterectomy and bilateral salpingo-oophorectomy are attempted (8, 18). For women of childbearing age who wish to preserve fertility, a unilateral salpingo-oophorectomy is performed, provided that the disease is contained within one ovary (8). In these instances, conservative surgery is often complemented by careful staging and endometrial biopsy to rule out uterine cancer (17, 18). Staging normally includes a partial omentectomy, evaluation of para-aortic and pelvic lymph nodes, peritoneal biopsies, and peritoneal washing cytology (8, 17). If the biopsies are disease free, no further treatment is required.

Adjuvant treatment is recommended for patients with recurrent or advanced disease; essentially for patients with stages II-IV disease or stage I disease associated with large tumor size, high mitotic index, or tumor rupture (8, 17). Standardized adjuvant treatment protocols, however, have yet to be established. Adjuvant therapies include: chemotherapy, radiation therapy, hormonal therapy, and more recently, anti-angiogenic therapy.

1.2.5.1. Chemotherapy

GCT is generally responsive to chemotherapy, which is mostly platinum-based, with bleomycin/etoposide/cisplatin (BEP) being the most frequently used regimen (8, 17, 19). Chemotherapy is indicated for advanced, inoperable, or recurrent disease, when surgery or

radiotherapy is not a viable option (18). In a retrospective study by Park *et al* (19), 13 patients diagnosed with advanced GCT had undergone cytoreductive surgery followed by 6 cycles of adjuvant chemotherapy with BEP. None of the patients who received the full 6 cycles of BEP had recurrent disease with a 5-year disease-free survival rate of 100% (19). Secondary effects to the BEP regimen are non-negligible however, and include myelotoxicity and a risk of secondary acute myelogenous leukemia (18).

1.2.5.2. Radiation therapy

Radiation therapy is indicated as an adjuvant treatment or for recurrent disease (18). Although it has been associated with improved survival in a few studies (33-35), other studies have found no survival advantage (36-38). In a retrospective study by Hauspy *et al* (34), 31 patients diagnosed with GCT had received adjuvant radiotherapy. They found a significantly longer disease free survival in patients who had received radiation therapy (251 months) in comparison to patients who had not (112 months, $p=0.02$) (34). In contrast, Malmström *et al* (36) found no survival benefit in 48 GCT patients treated with radiation therapy. Due to the retrospective nature of these studies, too many variables could have potentially confounded the real efficacy of radiation therapy for the treatment of GCT; large-scale prospective trials would be required in order to elucidate its role in the treatment of recurrent disease or as an adjuvant treatment for GCT.

1.2.5.3. Hormonal therapies

Hormonal therapies include gonadotropin releasing hormone (GnRH) agonists, GnRH antagonists, progestogens, and anti-oestrogens (8). A limited number of case reports have

evaluated their efficacy in the adjuvant treatment of GCT and most often describe last resort attempts in cases of recurrent disease (6). While some studies have shown hormonal therapy to result in stabilized disease, prolonged remission, partial response, and no major side effects (18, 39-42), others show no response whatsoever (43, 44). Although these therapies may indeed be effective in slowing down the progression of GCT disease, it is not possible to draw any reliable conclusions from a handful of case reports. In addition, the fact that the majority of patients were in the final stages of disease and several other treatment options had previously been attempted, it is difficult to attribute the precise contribution of hormonal therapy to the observed effects.

1.2.6. Recurrence and survival

As GCT is often diagnosed at an early stage, survival is considered excellent (17). Still, 80% of cases diagnosed with advanced-stage GCT will die from recurrent disease (45).

GCTs can spread locally by direct extension and intraperitoneal seeding to ovarian serosa, omentum, fallopian tube, and pelvic walls or by hematogenous or lymphatic routes to reach the lungs, liver, brain, skeleton, diaphragm, abdominal wall, and adrenal glands to form metastases (8, 18).

GCT has a reputation for recurrences over long periods, with 40 years being the longest time period of recurrence ever recorded (46). In one study (22), recurrence rate was 5.4% for stage I, 21.1% for stage III, and 40% for stage IV disease; overall recurrence rates vary between 11.2-21% (8, 9, 21). Recurrences are thought to originate from peritoneal seeds, which are points of contact between the primary tumor and abdominal or pelvic structures that remain after removal of the primary tumor and grow very slowly. For this reason, recurrences

most often occur in the abdomen or pelvis and tend to occur between 5 to 10 years after initial diagnosis and treatment (8, 18).

Many predicting factors for recurrence and poor survival have been identified and include: advanced stage at diagnosis, high mitotic index (>10 mitoses/10 HPF), bilaterality, large tumor size (>5cm), tumor rupture, lymphatic system invasion, nuclear atypia, absence of call-exner bodies, presence of residual disease, DNA ploidy, and age (50+) (8, 17-19, 47). Stage is universally accepted as the most important prognostic factor (17, 22, 28) with a 5-year survival of 90-100% for stage I disease, 55-75% for stage II, and 22-50% for stage III and IV, and a 10-year survival of 84-95% for stage I, 50-65% for stage II, and 17-33% for stage III/IV (18). VEGF has also been identified as a prognostic factor. Brown *et al* (48) showed that increased microvessel density and overexpression of VEGF correlated with the presence of distant metastasis and shorter survival in patients with SCSTs. Many other prognostic factors are mentioned in the literature, but have not been consistently reported.

Following diagnosis and treatment, regular examination is recommended every three months for the first two years, and then every four to six months for five years. Afterwards, examinations should be continued for life, as late recurrences tend to occur, and normally include a physical examination, ultrasound, CT scan, and evaluation of tumor markers (8).

Chapter 2: Pathophysiology of GCT

2.1. Etiology

Little is known about the etiology of GCT despite the fact that a number of studies have attempted different approaches to elucidate this. A few studies have examined the GCT

karyotype in order to identify abnormalities (6, 27, 49-51). Another approach has been to focus on pathways involved in normal folliculogenesis, as GCTs display many features of proliferating pre-ovulatory granulosa cells, such as FSHR expression, FSH binding, estrogen synthesis, *GATA-4* expression, and inhibin expression and synthesis (6, 20, 27). Others have studied pathways, oncogenes and tumor-suppressor genes that have frequently been implicated in many cancer types (6, 12, 27, 52-57).

2.1.1. Cytogenetic abnormalities

A distinctive pattern of chromosomal aberrations has been detected in 5-20% of aneuploid GCTs (6, 49). Trisomy 12 has been observed in 14-33% of cases, trisomy 14 in 25-33%, and monosomy 22 in 35-40% (6, 27, 49-51). Although several important genes have been identified on chromosome 12 such as *KRAS 2, KRAG, MDM2* and on chromosome 14 such as *FOS, BCL2L2, TGRβ3*, their precise role in GCT pathogenesis is unclear (49).

2.1.2. Molecular genetics of GCT

2.1.2.1. FSH signaling pathway

Although GCTs express high levels of the FSHR gene, no activating mutations in the FSHR or its associated G proteins have been identified (6, 27). Instead, it is suspected that activating mutations in genes encoding signaling effectors downstream of the FSHR, involving extracellular regulated kinases (ERK) or phosphoinositide 3-kinase (PI3K)/AKT signaling cascades might be involved (Figure 1) (6, 27, 58). Although mutation of the PI3K

subunit genes or inactivation of tumor suppressor phosphatase and tensin homolog (*PTEN*) has been implicated in epithelial ovarian tumors, Bittinger *et al* (59) found no association in GCT.

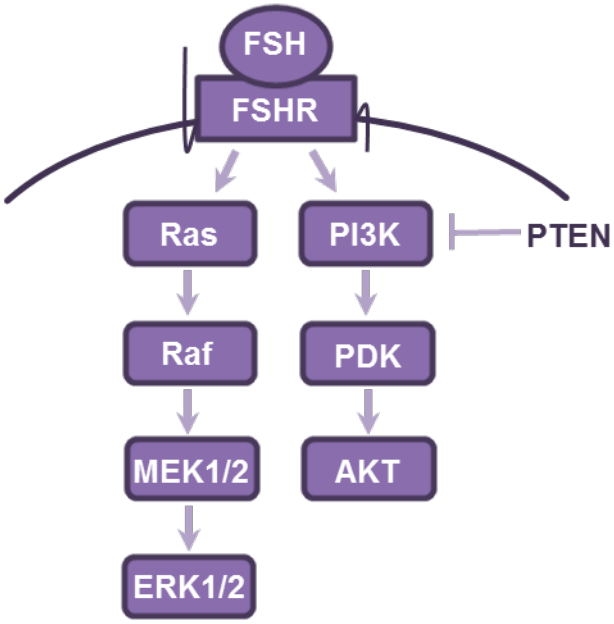


Figure 1

FSH receptor downstream signaling effectors. FSH binding to its receptor FSHR activates both the ERK1/2 and PI3K/AKT intracellular signaling pathways in granulosa cells. AKT = protein kinase B, ERK = extracellular regulated kinase, FSH(R) = follicle stimulating hormone (receptors), MEK = ERK kinase, PDK = phosphatidylinsitol-dependant kinase, PI3K = phosphoinositide 3-kinase, PTEN = phosphatase and tensin homolog.

Adapted from Jamieson & Fuller 2012 and Laguë *et al* 2008.

2.1.2.2. Estrogen receptors

Estrogen synthesis is a crucial part of folliculogenesis with expression of estrogen receptor genes (*ESR1* and *2*) occurring during the proliferative phase in human granulosa cells (27). *ESR2* was found to be predominantly and abundantly expressed in GCTs, in comparison to *ESR1* (27, 60). As approximately 70% of GCTs produce estrogens, it is suspected that these receptors might play a role in stimulating granulosa cell growth and differentiation (20).

2.1.2.3. *GATA-4* expression

Another feature of proliferating granulosa cells is GATA-4 expression. GATA-4 is a member of the GATA family of transcription factors that regulates the expression of many

important genes in the gonads, whose expression was found to be activated in primary follicles in conjunction with the activation of granulosa cell proliferation (61). Anttonnen *et al* (61) reported high levels of GATA-4 in 44% of primary GCTs and a significant positive correlation between GATA-4 expression and clinical stage and risk of recurrence. This group later reported a correlation between GATA-4 expression and *Bcl-2*, an anti-apoptotic gene controlled by GATA-4 (62). The authors suspect that an increase in GATA-4 expression up-regulates *Bcl-2* activation thereby promoting granulosa cell survival and inhibiting apoptosis in GCT tumorigenesis (62).

2.1.2.4. Inhibin expression and synthesis

Serum inhibin levels are elevated in women with GCT and have been associated with suppressed FSH levels, indicating a biologically active form of inhibin (6, 63). In contrast, in an inhibin-deficient mouse model, SCSTs developed in 100% of animals (64). Matzuk *et al* (64, 65) explained this apparent contradiction by the development of resistance to inhibin in the human disease, whose normal role may be tumor suppression specifically in the gonads.

2.1.2.5. WNT/β-catenin signaling pathway

Another pathway that has been implicated in several cancer types is the WNT/β-catenin signaling pathway (52). Boerboom *et al* (52, 53) evaluated the WNT/β-catenin pathway that is normally expressed in ovarian granulosa cells and found hyperactivation in a subset of human and equine GCTs. In order to confirm whether activation of the WNT/β-catenin signaling pathway was in fact involved in GCT development, this group developed a mouse model expressing a dominant stable β-catenin mutant (52). The majority of animals did

in fact develop ovarian lesions at an advanced age that were confirmed as GCTs (52). These findings were the first to demonstrate a causal link between altered WNT/β-catenin signaling and GCT tumorigenesis (53), and later served to develop the mouse model that is the focus of this paper.

2.1.2.6. Oncogenes and tumor-suppressors genes

Several oncogenes that have been implicated in a range of tumors have also been examined for their potential role in GCT pathogenesis however, these investigations have yielded few novel insights. No mutation or overexpression was found in the following oncogenes: *c-myc, p21-ras, c-erbB2, K-ras, N-ras, H-ras, B-raf,* or *WT1* (6, 12, 54, 55). Neither mutation nor loss of tumor-suppressor p53 was found to be implicated in GCT (27, 56, 57). The inability to identify oncogenes and tumor-suppressor genes that might be responsible for initiating the development of GCT represents a further delay in the development of effective treatment agents, as many current cancer drugs specifically target the proteins encoded by oncogenes (66).

2.1.2.7. *FOXL2* mutation

The most significant breakthrough in GCT pathogenesis came in 2009 when Shah *et al* (67) identified a somatic missense point mutation in *FOXL2* 402C→G (C134W) in 97% of adult GCTs. The *FOXL2* gene encodes a member of the forkhead domain/winged-helix family of transcription factors that is specifically expressed in ovarian follicular cells and is crucial for granulosa cell function and follicle development (6). This mutation is almost entirely absent in other cancer types, such as epithelial ovarian, breast, and even juvenile

GCTs (67, 68). Several subsequent studies confirmed the mutation in adult-type GCT with an overall occurrence of 94% (6). These findings strongly suggest that *FOXL2* mutation is pathognomonic for adult-type GCT and that it constitutes either an activating or a gain-of-function mutation (6, 67, 69).

In an attempt to explain the mechanism of tumorigenesis driven by the FOXL2 mutation, studies have evaluated FOXL2 targets, such as the aromatase gene *CYP19A1* (69). FOXL2 induces *CYP19A1* which leads to increased estrogen conversion by granulosa cells (69). Fleming *et al* (70) confirmed that aromatase is a direct target of FOXL2 (C134W) in adult-type GCT and more specifically, that the mutation altered the regulation of the aromatase promoter (6). As hyperestrogenism is seen in approximately 70% of adult-type GCTs, deregulated activity of FOXL2 via altered aromatase activity might contribute to the development of this condition (6, 69).

In a FOXL2 knockout mouse model, increased follicular loss and oocyte atresia were found, which suggests that FOXL2 plays an anti-apoptotic role (69, 71). This finding was supported by Kim *et al* (72), who demonstrated that mutant FOXL2 exhibited minimal cell death in comparison to the wild-type. These findings suggest a tumor suppressor role for FOXL2 in normal granulosa cells, which in the mutant form, is impaired and incapable of mediating death ligand-induced apoptosis (72).

In juvenile-type GCT, FOXL2 expression is decreased or absent and has been associated with aggressive or advanced stage disease (6, 68).

2.2. Angiogenesis

In 1971, Judah Folkman (73) hypothesized that tumor growth depends on the formation of new blood vessels from pre-existing ones, a concept coined as angiogenesis, and proposed angiogenesis as a therapeutic target for cancer treatment. Angiogenesis is now known to be crucial for tumor growth, as small tumor masses are unable to grow beyond 1-2 mm without a blood vessel supply to support their growth (74). Early during tumor development, an angiogenic switch occurs in which quiescent tumor endothelial cells are activated (75, 76). Tumor cells release pro-angiogenic mediators that bind to adjacent vascular endothelial cells and stimulate the formation of new blood vessel capillaries that infiltrate the tumor mass (74). This infiltration allows the microscopic tumor mass to expand because tumors, just like normal tissues, require oxygen, nutrients, and the evacuation of carbon dioxide and metabolic waste (3). This neovascularization also allows the tumor to eventually form metastases (74).

Tumor blood vessels tend to be aberrant, characterized by precocious capillary sprouting, convoluted and excessive vessel branching, distorted and enlarged vessels, erratic blood flow, micro-hemorrhaging, leakiness, and abnormal levels of endothelial cell proliferation and apoptosis (3, 77, 78).

2.2.1. Regulation of angiogenesis

The angiogenic process depends on interactions between vascular endothelial cells, tumor cells, inflammatory cells, the surrounding micro-environment, and many auxiliary factors (79). Numerous environmental factors stimulate VEGF expression, a major mediator of angiogenesis, which include hypoxia, low pH, hormones, growth factors, and cytokines (74,

75, 80-84). Mutations in oncogenes and/or tumor suppressor genes also contribute to upregulating VEGF (74, 85). Negative regulators play essential roles in keeping angiogenesis under control, and include: thrombospondin, endostatin, tumstatin, vasostatin, and vasohibin (84). For the purposes of this study, the remainder of this review will focus on the VEGF pathway, as it represents the major regulatory pathway involved in tumor angiogenesis (83, 84).

2.2.2. Vascular endothelial growth factor

In 1989, Napoleone Ferrara (86) purified VEGF, a key mediator in angiogenesis, vasculogenesis, and lymphangiogenesis during embryonic and post-natal development (82). The VEGF family is made up of five members, with VEGFA, being the best-characterized and the most important activator of vascular endothelial cells (76, 79, 80, 84, 87). VEGFA is a homodimeric glycoprotein made up of two identical 23 kDa subunits (82). Alternative exon splicing of the VEGFA gene generates various isoforms, with $VEGF_{165}$ being the predominant and most mitogenic isoform, that is frequently overexpressed in many tumor types, including colon, renal cell, and ovarian cancer (79, 80, 82, 83).

VEGFA signals via two cell surface receptor tyrosine kinases (RTK), VEGFR1 (Flt-1) and VEGFR2 (Flk-1 in mice/KDR in humans), which are chiefly expressed in endothelial cells (Figure 2) (13, 14, 76, 80, 82, 83, 88). The receptors possess seven extracellular immunoglobulin-like domains and an intracellular tyrosine kinase domain (88). VEGFR2 is the dominant pro-angiogenic receptor and upon VEGF binding, VEGFR2 undergoes dimerization and tyrosine kinase auto-phosphorylation, which activates downstream signaling pathways involved in vascular endothelial permeability, proliferation, migration, and survival

(14, 79, 82, 83, 87). Some of these pathways include ERK1/2 and PI3K/AKT (80). VEGFR2 is also strongly expressed in some tumor cells (e.g. granulosa) (13). VEGFR1, on the other hand, is suspected to be a decoy receptor that sequesters VEGF and prevents binding to VEGFR2 (83, 84, 89).

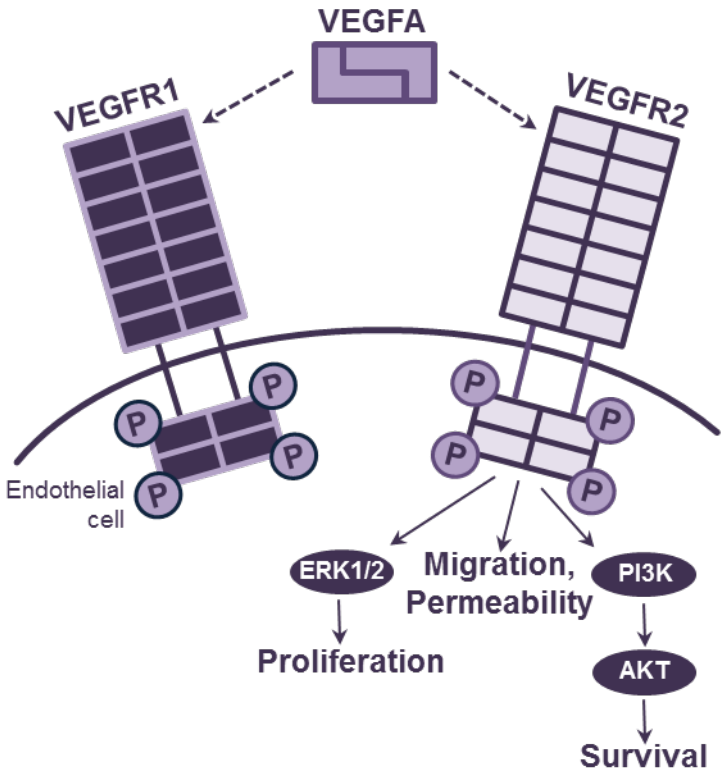


Figure 2. VEGFR2 signaling pathway in endothelial cells. VEGFA binding to its main receptor VEGFR2 activates downstream effectors involved in vascular endothelial cell proliferation, migration, permeability, and survival. VEGFR1 may be a decoy receptor that prevents VEGFA binding to VEGFR2. AKT = protein kinase B, ERK = extracellular regulated kinase, PI3K = phosphoinositide 3-kinase.

Adapted from Ferrara and Kerbel 2005.

2.2.3. Other roles for VEGF in tumorigenesis

Although VEGF's role in tumor growth and metastasis has mostly been attributed to its angiogenic activity, VEGF has recently been implicated in other tumorigenic functions as well. VEGF is suspected of playing an immunosuppressive role in cancer development (74,

90), as well as directly promoting tumor growth by binding to its receptors expressed on tumor cells (91). VEGF increases the permeability of tumor microvasculature, which contributes to reshaping the extracellular matrix (leading to cell migration and proliferation), forming ascites, and forming metastases by allowing tumor cells to disseminate through the leaky capillaries (76).

2.2.4. VEGF and GCT

Angiogenesis is suspected to play an important role in the development and progression of the highly vascularized GCTs and its key mediator, VEGF, has been identified as an important component of this disease (11, 13, 14). Schmidt *et al* (92) found VEGF expressed in 94% of GCT specimens and none in the controls. Similarly, Färkkilä *et al* (13) found VEGF expressed in 93% of GCTs and VEGFR2 highly expressed in 82% of tumors, in both granulosa and endothelial cells of primary and recurrent tumors. VEGF expression was positively correlated to tumor microvessel density and to the expression of VEGFR2 at protein and mRNA level (13). Also, high levels of VEGF were detected in the serum of patients with primary GCT, whose levels significantly dropped following tumor removal (13, 14). Together, these data demonstrate VEGF's critical role in GCT disease that extends beyond affecting a single cell population, that is implicated in primary and recurrent disease, and that is in part responsible for tumor microvessel development. This makes VEGF a logical therapeutic target for GCT.

Chapter 3: Transgenic mouse models

3.1. Importance

Several mouse models genetically engineered to develop GCT have been created in an attempt to reproduce the human disease. An animal model that is easily reproducible is especially useful for this disease as the incidence in women is quite low, which limits the possibility of organizing large-scale randomized trials. Although the extent in which a mouse model is able to imitate the human disease is unclear (and varies depending on the given model), it serves as a powerful tool in which etiology, diagnosis, treatment, recurrence and especially novel therapeutic agents can be investigated.

3.2. Existing models of GCT

3.2.1. Inhibin α-subunit knockout

Matzuk *et al* (64) created an inhibin α-subunit knockout model by using homologous recombination in mouse embryonic stem cells to delete the α-inhibin gene. Mice homozygous for the null allele (or inhibin-deficient) developed early onset (4 weeks), bilateral, multifocal, hemorrhagic, mixed or incompletely differentiated SCSTs with 100% penetrance in both sexes (6, 64). Tumor development was accompanied by a cachexia-like wasting syndrome that ultimately led to their death (6, 93). Based on these findings, α-inhibin was identified as a tumor-suppressor gene and inhibin, as a tumor-suppressor protein with gonadal specificity (64, 93).

3.2.2. Targeted overexpression of luteinizing hormone

Risma *et al* (94) generated a transgenic model with chronically elevated serum luteinizing hormone (LH) levels. This model expressed a chimeric LHβ subunit in gonadotropes that contained the C-terminal peptide of the human chorionic gonadotropin β-subunit, linked to a bovine LHβ subunit, and driven by a bovine α-subunit promoter (94). The transgenic females showed chronically elevated LH levels (5-10 fold above controls), were infertile, and (some) developed pathologic ovarian lesions such as cyst formation, ovarian hypertrophy, and GCTs by 4-8 months of age (94). The findings in this study suggested that gonadotropin hyperstimulation may be a mechanism for GCT tumorigenesis (94).

3.2.3. Simian virus 40 T-antigen

Kananen *et al* (95) produced transgenic mice that expressed the viral oncogene, simian virus 40 T-antigen, driven by a 6-kb fragment of the murine inhibin α-subunit promoter (6, 96). Transgenic females were infertile and developed ovarian granulosa and theca cells tumors by 5-7 months of age with 100% penetrance (6, 96). Tumor growth was gonadotropin-dependant, in a manner similar to inhibin-α knockout mice (95, 97). Mice displayed many similarities to the human disease, such as rapid development of primary tumors, continued folliculogenesis (until advanced disease), depressed serum gonadotropins, elevated serum inhibin levels, and similar histopathologic features and therefore represented a useful animal model for GCT (6, 97).

3.2.4. Estrogen receptor β knockout

Krege *et al* (98) generated transgenic mice lacking estrogen receptor β (ERβ-/-) by inserting a neomycin resistance gene into exon 3 of the coding gene by homologous recombination in embryonic stem cells. ERβ(-/-) female mice developed large ovarian sex cord (poorly differentiated) and GCTs (differentiated and estrogen secreting) with 100% penetrance by 2 years of age (6, 63). Tumors had high proliferative indices and expressed high levels of nuclear phospho-SMAD3 and LH receptor (6, 63). Fan *et al* (63) suggested that in the absence of ERβ, the FSH/SMAD3 pathway is able to signal and in combination with increased ERα expression, induce proliferation, leading to the development of ovarian tumors (6).

3.2.5. SMAD knockouts

Pangas *et al* (99) developed a mouse model by genetic deletion of the bone morphogenetic protein (BMP) signaling transcription factors, SMADs 1, 5, and 8, specifically in ovarian granulosa cells (100). Both female Smad1/5 double knockouts and Smad 1/5/8 triple knockouts were infertile and developed poorly differentiated GCTs with 100% penetrance by 3 months of age (6, 99). Almost 80% of mice developed peritoneal and lymphatic metastases (6, 99). Further analyses of the Smad1/5 double knockout model showed similarities with the human juvenile-type GCT such as activation of TGFβ/SMAD2/3 pathway (6, 100). These findings demonstrated a role for the SMAD family in the malignant transformation of granulosa cells, especially in juvenile form of GCT (99, 100).

3.2.6. Bone morphogenetic protein receptor knockouts

Edson *et al* (101) generated BMP receptor 1A and 1B double-mutant mice that developed GCT. Similar to SMAD1 and SMAD 5, which showed redundancy in suppressing GCT development in mice, BMPR1A and BMPR1B were found to act in conjunction to prevent GCT tumorigenesis (101). This study in combination with the studies by Pangas and Middlebrook strongly suggest a role for the BMP pathway (including BMP receptors and SMADS) in ovarian tumor suppression (101).

3.3. Transgenic GCT models relevant to the present study

3.3.1. The *Ctnnb1*$^{tm1Mmt/+}$; *Amhr2*$^{tm3(cre)Bhr/+}$ model

In 2005, Boerboom *et al* (52) reported that misregulated WNT/β-catenin signaling leads to ovarian GCT development. β-catenin is a key effector of the WNT signal transduction pathway that is involved in cell proliferation and cell fate during embryogenesis, including embryonic development of the ovary (102-104). Altered regulation of the WNT/β-catenin pathway has previously been implicated in many types of cancer (52, 105, 106).

Subsequently, a transgenic mouse model, *Ctnnb1*$^{tm1Mmt/+}$; *Amhr2*$^{tm3(cre)Bhr/+}$ (*CA*), was generated that expressed a dominant stable β-catenin mutant specifically in ovarian granulosa cells (Figure 3) (52). By 6 weeks of age, *CA* mice developed pretumoral ovarian lesions resembling antral-size follicles, containing solid nests of disorganized and pleiomorphic granulosa cells, few proliferating cells, and no oocytes (52). The lesions were highly vascularized (52). In a subset of older mice, uni- and bilateral ovarian tumors consisting of large blood-filled cysts were discovered and revealed to be GCTs (52). Discrete histological

patterns were found, the most common composed of a solid sheet of disorganized granulosa cells with randomly distributed, fenestrated, antrum-like spaces filled with eosinophilic material (52). In a subset of tumors, well-organized areas of ossification and mineralization were found (52). No dissemination beyond the ovaries was observed (52). The prevalence of GCT increased over time with 0% of tumor formation in mice at 19 weeks of age, 44% at 6 months of age, and 57% at 7.5 months of age, however, no increase in mortality rate was observed (52). These findings suggested that constitutive activation of the WNT/β-catenin pathway in granulosa cells induces a premalignant state but requires additional factors to cause GCT formation (52,102).

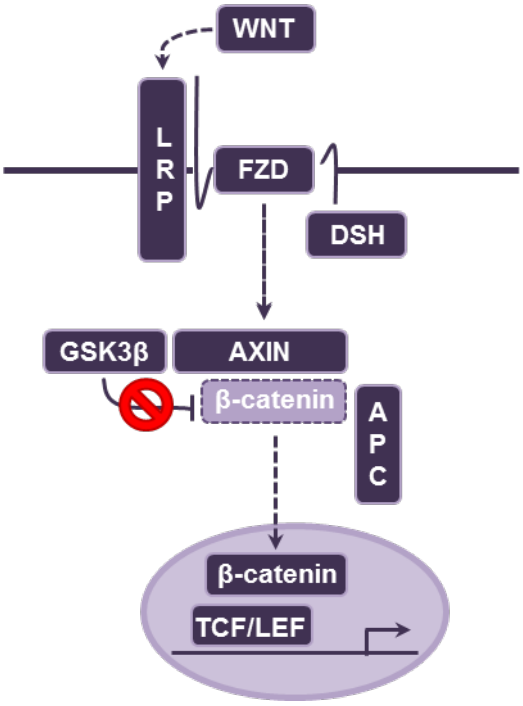


Figure 3

WNT/β-catenin signaling pathway and dominant stable β-catenin mutant. In the *CA* mouse model, GSK3β is unable to phosphorylate β-catenin (phosphorylation normally leads to degradation of β-catenin), which becomes a dominant stable mutant that translocates to the nucleus and mediates the transcription of target genes. APC = adenomatosis polyposis coli, DSH = disheveled, FZD = frizzled receptor, GSK3β = glycogen synthase kinase 3 beta, LRP = low-density lipoprotein receptor-related protein, TCF/LEF = transcription factor, T-cell specific/lymphoid enhancer factor 1.

Adapted from Laguë, *et al* 2008.

3.3.2. The *Pten*$^{tm1Hwu/tm1Hwu}$; *Amhr2*$^{tm3(cre)Bhr/+}$ model

Next, the possible involvement of the PI3K/AKT pathway in GCT development was investigated. The PI3K/AKT pathway is known to contribute to the development of many cancer types when dysregulated, as many of its components are tumor-suppressor genes or proto-oncogenes (102, 107). *PTEN* is a tumor suppressor gene that reduces PI3K/AKT activity by dephosphorylating phosphatidylinositol (3,4,5)-triphosphate and is inactivated in many types of cancer (102, 108).

In 2008, a genetically engineered mouse model, *Pten*$^{tm1Hwu/tm1Hwu}$; *Amhr2*$^{tm3(cre)Bhr/+}$ (*PA*), was created by conditional targeting of *PTEN*, which triggered the derepression of the PI3K/AKT pathway in granulosa cells (Figure 4) (102). In the *PA* model, only ≈7% of female mice developed aggressive, mostly bilateral, anaplastic GCTs with pulmonary metastases, diagnosed between 7 weeks to 7 months of age (102). GCTs showed either a solid or trabecular pattern, with two distinct tumor cell populations (differing by degrees of anaplasia), areas of osseous metaplasia, and cystic structures (102). These findings suggested that dysregulation of the PI3K/AKT pathway contributes to the pathogenesis of GCT and that the *PA* model provided the first metastatic phenotype in a transgenic mouse model of GCT (102).

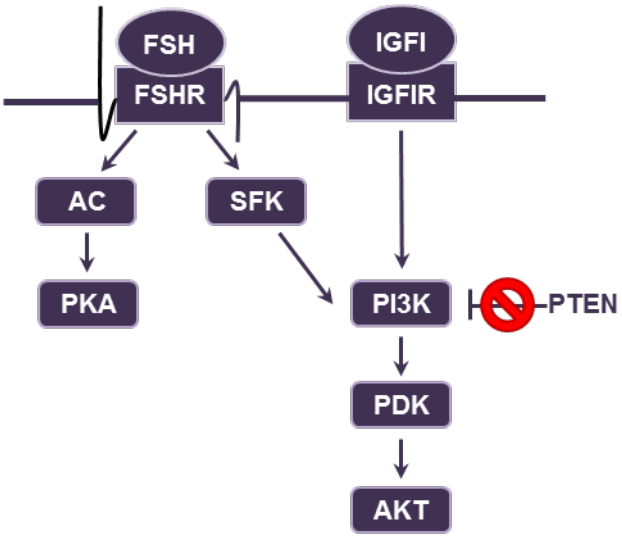


Figure 4
PI3K/AKT pathway and conditional targeting of *PTEN*. In the *PA* mouse model, inactivated *PTEN* is unable to antagonize PI3K/AKT, which consequently remains active. AC = adenyl cyclase, AKT = protein kinase B, FSH(R) = follicle stimulating hormone (receptor), IGR1(R) = insulin-like growth factor-1 (receptor), PDK = 3-phosphoinositide-dependent protein kinase, PI3K = phosphoinositide 3-kinase, PKA = protein kinase A, PTEN = phosphatase and tensin homolog, SFK = SRC family protein kinases.

Adapted from Laguë, *et al* 2008.

3.3.3. The *Pten*$^{tm1Hwu/tm1Hwu}$; *Ctnnb1*$^{tm1Mmt/+}$; *Amhr2*$^{tm3(cre)Bhr/+}$ model

A mouse model was then generated to determine whether the WNT/β-catenin and PI3K/AKT pathways would interact in GCT development (102, 109-111). In *Pten*$^{tm1Hwu/tm1Hwu}$; *Ctnnb1*$^{tm1Mmt/+}$; *Amhr2*$^{tm3(cre)Bhr/+}$ (*PCA*) mice, both PI3K/AKT and WNT/β-catenin pathways were constitutively activated in granulosa cells and resulted in bilateral GCT development with 100% penetrance, perinatal onset, very rapid growth, and pulmonary tumor cell embolisms (by 6 weeks of age) (102, 112). Abdominal distension due to large GCTs (>2cm in diameter) was severe by 7 weeks of age, typically causing death by 8 weeks of age (102, 112). This phenotype resembled that of the *PA* mice, with solid and trabecular histological patterns, different cell populations, and areas of ossification (102). In a surgical model of *PCA*, GCTs were surgically removed from 6 week-old mice that were later sacrificed 6-16 weeks postoperatively (102, 112). 100% of mice showed large lung metastases (102). In additional 6 week-old mice, tumors were removed and tumor cells suspended in saline were injected into the peritoneal cavity after closure of the abdominal wall (102). 6-9 weeks

postoperatively, the mice were sacrificed and multiple abdominal tumors invading the lungs, mesentery, peritoneum, abdominal muscles, pancreas, adrenal glands, and liver were discovered (102). This surgical mouse model showed that *PCA* mice form pulmonary metastases and have the ability to seed into the peritoneal cavity in a manner similar to the advanced disease in women (17, 102).

3.3.4. The *KRAS*^{G12D}*; Ctnnb1*$^{tm1Mmt/+}$; *Amhr2*$^{tm3(cre)Bhr/+}$ model

In a collaborative laboratory, Richards *et al* (10) generated a mouse model in which constitutive activation of β-catenin was combined with oncogenic $KRAS_{G12D}$ expression selectively in granulosa cells. The RAS pathway is known to play important roles in normal ovarian function (113) including ovulation, oocyte maturation, and the terminal differentiation of granulosa cells (114-117). Mutant $KRAS_{G12D}$ has shown anti-proliferative properties, such as inhibition of proliferation, differentiation, and apoptosis in granulosa cells, which manifests as accumulations of small abnormal follicle-like structures in the ovary leading to premature ovarian failure (10, 118, 119). Richards *et al* (10) generated a *KRAS*^{G12D}*; Ctnnb1*$^{tm1Mmt/+}$; *Amhr2*$^{tm3(cre)Bhr/+}$ mutant model in which mice developed early-onset GCTs that displayed increased granulosa cell proliferation, decreased apoptosis, and impaired differentiation, which ultimately resulted in early death. These unexpected observations resembled those found in the *PCA* model (10). The authors concluded that either KRAS activation or *PTEN* loss is able to promote GCT progression if initiated by activated β-catenin (10).

3.3.5. Conclusions from the GCT models

A synergistic interaction was found to occur between the PI3K/AKT and WNT/β-catenin pathways that resulted in GCT development in all cases; however the molecular mechanisms mediating the interaction remain unknown (102). The pretumoral ovarian lesions found in the *CA* model suggest that activation of the WNT/β-catenin pathway is involved in initiating GCT development (102). In contrast, the rarity of GCT formation combined with the aggressive phenotype in the *PA* model suggest that the PI3K/AKT pathway is more involved in GCT progression (102). Therefore, the development of GCTs with 100% penetrance obtained by combining the two pathways in the *PCA* model, might be explained by a coordinated effort by both pathways, with tumor growth initiated by one pathway (WNT/β-catenin) followed by tumor progression stimulated by the other (PI3K/AKT) (102). Also, the observed similarities in the *PCA* model compared to the advanced disease in women, such as formation of lung metastases and the ability to seed into the peritoneal cavity, suggest that similar molecular mechanisms of tumorigenesis might be involved (102, 112).

Chapter 4: Targeting angiogenesis

Angiogenesis is known to play a crucial role in tumor growth and formation of metastases in a variety of cancer types (84, 120, 121). For this reason, angiogenesis is a logical target for cancer treatment, and several agents that specifically inhibit one of the many steps of the angiogenic pathway have been generated (74, 79, 122-125). Interest in novel therapies has also been prompted due to drug resistance and disease recurrence in association with chemotherapy in ovarian and other cancer types (76). Several classes of inhibitors that

target various steps of the VEGF pathway are currently under investigation and include VEGFR antibodies, soluble VEGFRs, aptamers, and small-molecule VEGFR tyrosine kinase inhibitors (TKI) (Figure 5) (74, 79, 84, 122-125). However, the class of angiogenic inhibitors that has shown the most promise and is widely used in the clinical setting is monoclonal antibodies that bind and neutralize the VEGF ligand and consequently, prevent VEGF binding to its receptors (4, 80, 82, 123, 126, 127).

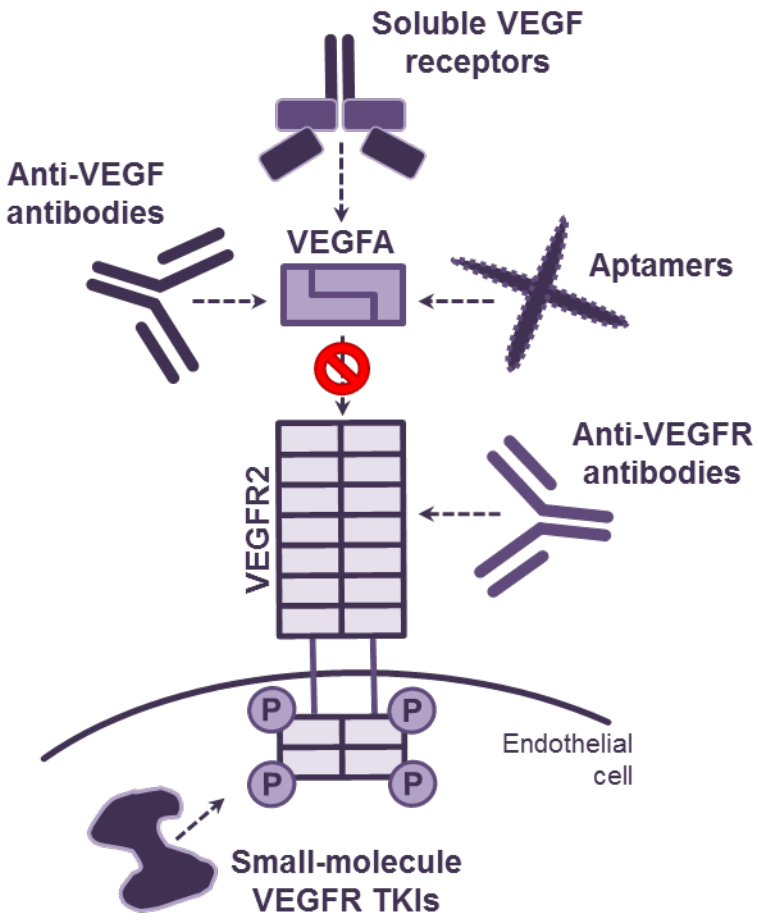


Figure 5. Classes of VEGF inhibitors. The VEGF pathway is inhibited by targeting either the VEGFA ligand or its main receptor VEGFR2. Anti-VEGF antibodies, soluble VEGF receptors, and aptamers bind VEGFA and prevent binding to its main receptor VEGFR2. Anti-VEGFR antibodies and small-molecule VEGFR TKIs bind VEGFR2 and prevent the transduction of intracellular signaling. TKI = tyrosine kinase inhibitors.

Adapted from Ferrara & Kerbel 2005.

4.1. Classes of angiogenic inhibitors

4.1.1. VEGFR antibodies

As VEGFR2 is the major receptor involved in transducing the VEGFA signaling cascade, its extracellular domain represents a logical target for the inhibition of tumor angiogenesis (79, 122). Fully human monoclonal antibody, ramucirumab (IMC-1121B), is the most clinically advanced and specific inhibitor of VEGFR2 (74, 79). Phase I and II trials have demonstrated its efficacy at causing tumor responses and stabilizing tumor size in patients with a variety of tumors who have received prior treatment, including treatment with other anti-angiogenic agents (79). Many phase II and III trials are underway evaluating its efficacy in the treatment of melanoma, gastric, and ovarian cancer, among many others (79).

4.1.2. Soluble VEGFRs

Another approach to inhibiting VEGF signaling is to prevent VEGF from binding to its normal receptors by having it bind instead to decoy-soluble VEGFRs (123). A potent high-affinity VEGF blocker, aflibercept (or VEGF-Trap), is a decoy fusion protein consisting of domain 2 from VEGFR1 fused to domain 3 from VEGFR2 attached to the Fc fragment of IgG1, that binds and neutralizes all VEGFA isoforms (74, 79). Aflibercept has been shown to inhibit tumor growth, suppress angiogenesis, induce tumor necrosis, and reduce tumor microvessel density in various *in vitro* and *in vivo* studies (79, 123, 128-131). Although it hasn't been approved for use in the clinical setting, it shows good potential, and many studies are currently underway evaluating its use in colorectal, lung cancer, and lymphoma (79, 131).

4.1.3. Aptamers

RNA oligonucleotide ligands (or aptamers) to human $VEGF_{165}$ potently inhibit VEGF binding to its receptors (124). In the clinical setting, pegaptanib sodium (Macugen), is an aptamer that binds and blocks $VEGF_{165}$ activity with high specificity and affinity, and was the first anti-VEGF agent to be approved by the FDA in 2004 for the treatment of neovascular age-related macular degeneration (124, 132).

4.1.4. Small-molecule VEGFR tyrosine kinase inhibitors

Small-molecule TKIs act by reversible competition with the ATP binding site of the catalytic (tyrosine) domain of several oncogenic tyrosine kinases, such as VEGFRs, and thus interfere with multiple signaling pathways (125). A long list of inhibitors have shown efficacy in treating various forms of cancer. Of particular interest, are the receptor TKIs that target VEGFRs, which include semaxinib (SU5416), vatalanib (PTK787/ZK222584), sorafenib (BAY 43-9006), sunitinib malate (Sutent, SU11248), cediranib (AZD2171), pazopanib (GW78603), and BIBF 1120, with sunitinib and sorafenib being the most established (84, 125). Sunitinib targets many tyrosine kinases including VEGFR, PDGFR, c-KIT, and FLT-3 (133), and has shown improved progression-free survival and response rate in phase III trials of metastatic renal cell carcinoma (134). This led to its FDA approval for treatment of advanced renal cell carcinoma as well as imatinib-resistant gastrointestinal stromal tumors (82). Sorafenib targets Raf, VEGFR, EGFR, and PDGFR (135) and was shown to inhibit tumor cell proliferation and angiogenesis (125). In a phase III trial, sorafenib improved progression-free survival and overall survival (82, 136) and was subsequently approved by the FDA for treatment of advanced/metastatic renal cell carcinoma and hepatocellular cancer (79).

Unfortunately, these two drugs demonstrated little activity and high toxicity in the treatment of ovarian cancer, so further development into its use in ovarian cancer was halted (76, 137, 138).

4.1.5. Monoclonal anti-VEGFA antibodies

Bevacizumab (Avastin) is a recombinant humanized monoclonal IgG1 antibody (mAb) that binds and neutralizes all biologically active forms of VEGFA (74, 80) and was the first anti-angiogenic drug to be approved for cancer treatment (4). Its activities include inhibiting neovascularization and inducing regression of existing microvessels, leading to inhibition of tumor growth and formation of metastasis (80, 126, 127). Bevacizumab was investigated in two separate phase III trials in combination with chemotherapy for the treatment of metastatic colorectal cancer and advanced non-squamous, non-small-cell lung cancer (NSCLC) (139, 140). Both studies showed significantly improved median survival, median duration of progression-free survival, and response rates (139, 140). In 2004, the FDA approved the use of bevacizumab combined with chemotherapy for the treatment of metastatic colorectal cancer and NSCLC (11, 141, 142). Today, bevacizumab is also approved for the treatment of metastatic kidney cancer in combination with interferon-α, and glioblastoma as second-line treatment (142).

4.1.5.1. Dosage and administration

Several trials have attempted to establish an optimal bevacizumab dosage and schedule of administration however, no ideal dosing schemes exist that may be applied for all types of cancer (143, 144). The effective and well tolerated dose of bevacizumab for the treatment of

metastatic colorectal cancer in combination with 5-fluorouracil (bolus-IFL) is 5mg/kg by intravenous (IV) injection every 2 weeks (143). For the treatment of other tumor types, recommended doses vary between 5-15mg/kg every 2-3 weeks and are often combined with other chemotherapy drugs (145). Although optimal dosing schemes vary, the same general guidelines for the administration of bevacizumab apply to all types of cancer (145). For the first infusion, bevacizumab is administered slowly over 90 minutes to watch for signs of infusion reactions and is subsequently infused over a shorter period of time (30-60 minutes for subsequent infusions) as long as it is well tolerated (146). Monitoring for signs of toxicity and adverse events (that are well described, see below) is necessary and if such signs occur, treatment is discontinued (146).

4.1.5.2. In epithelial ovarian cancer

More focus has been placed on investigating the use of bevacizumab in the treatment of the more common epithelial ovarian cancer than on GCT (11). Phase II trials evaluating either single-agent bevacizumab or in combination with chemotherapy have shown improved response rates, median progression-free survival, and median overall survival relative to controls (147-152). Recently, phase III trials have been conducted evaluating bevacizumab in combination with paclitaxel and carboplatin (in comparison to chemotherapy alone) and found significantly longer progression-free survival (153, 154). Many other phase III trials of bevacizumab in combination with chemotherapy for the treatment of recurrent disease are currently underway (80). Based on the encouraging results obtained in the treatment of epithelial ovarian tumors, bevacizumab has slowly begun to be incorporated into treatment protocols for GCT.

4.1.5.3. In GCT

To date, very few studies have investigated the use of bevacizumab in the treatment of GCT and the results have yielded little insight (11, 15, 16). Tao *et al* (11) evaluated the clinical efficacy of bevacizumab with or without chemotherapy and found a response rate of 38% and a clinical benefit rate of 63%. It must be noted that this study was limited by its retrospective nature, its small sample size, and the variation of treatments administered (11). In a case report documented by Kesterson *et al* (15), a patient with refractory GCT was treated with paclitaxel and bevacizumab. A symptomatic improvement of the patient's malignant ascites was obtained and attributed to bevacizumab (as prior monotherapy with paclitaxel offered no improvement (15). Conversely, a case report documented by Barrena *et al* (16) found no clinical improvement with bevacizumab therapy in the first-line treatment of adult-type GCT. It is therefore difficult to draw any conclusions from the limited number of small-scale studies evaluating bevacizumab for the treatment of GCT. If standardized treatment regimens incorporating bevacizumab are to be established, then large-scale randomized trials are required.

4.2. Resistance

Although inhibitors of the VEGF pathway are valid treatment options that have shown promising results in the treatment of various cancer types, their benefits can be transitory (84, 155). Many theories have attempted to explain this acquired resistance to anti-angiogenic drugs, some of which include: VEGFA replacement by other angiogenic pathways, the selection of aggressive tumor cells that are resistant to hypoxia and are less dependent on angiogenesis, and vascular remodelling and stabilization that is less responsive to angiogenic

inhibition (4, 84, 156-158). Beyond having a transitory effect, some VEGF inhibitors have even been implicated in accelerating tumor progression and worsening patient prognosis. In a study by Ebos *et al* (155), short-term treatment with sunitinib was found to accelerate metastatic tumor growth and decrease overall survival in a mouse model. Similarly, Pàez-Ribes *et al* (159) observed increased invasiveness and metastasis after treatment with a VEGFR2 inhibitor in mouse models of pancreatic neuroendocrine carcinoma and glioblastoma. These findings suggest that targeting the VEGF signaling pathway is effective in the short-term (e.g. at reducing tumor burden), but in the long-term, the tumor develops a resistance to treatment, disseminates, and forms metastases (159).

4.3. Adverse events

Although bevacizumab is generally well tolerated, monotherapy with this agent has been associated with a number of side effects, with gastro-intestinal perforation (11.4%), hypertension (9.1-9.7%), proteinuria (1.6-15.9%), and arterial thromboembolisms (1.6-6.8%) occurring most often (147-149). Other adverse events that have been reported include: hematologic, gastro-intestinal, allergic, hepatic, pain, bleeding, dyspnea, pulmonary emboli, and impaired wound-healing (147-149). In a meta-analysis including 16 randomized control trials and a total of 10,217 patients diagnosed with various advanced solid tumors, the overall incidence of fatal adverse events with bevacizumab was 2.5%, with the most common causes being hemorrhage (23.5%), neutropenia (12.2%), and gastrointestinal tract perforation (7.1%) (80, 160). These side effects and the development of resistance found with some VEGF inhibitors are critical factors that must be taken into consideration when evaluating anti-VEGFA therapy in future trials.

4.4. Combinatorial therapies

Given the complexity of signaling pathways involved in carcinogenesis, targeting a single pathway has shown limited clinical benefits. For this reason, combining different treatment modalities that target different pathways tends to be a more effective approach (16). Preclinical studies have shown synergistic effects in suppressing tumor growth by combining anti-angiogenic drugs with chemotherapy or radiation therapy (121); however the mechanisms of action remain theoretical. Browder *et al* (161) and Klement *et al* (162) suggest that metronomic chemotherapy preferentially damages endothelial tumor cells and by simultaneously blocking VEGFA, endothelial cell survival signal is reduced, which further amplifies endothelial cell targeting by chemotherapy, and consequently, more cancer cells are killed (84). Another hypothesis, by Jain *et al* (163) suggests that anti-angiogenic agents normalize the tumor vasculature and as a result, excessive endothelial and perivascular cells are pruned, tumor hypoxia and interstitial pressure in tumors are reduced, leading to improved oxygenation, perfusion, and delivery of chemotherapy (82, 84, 87, 164).

Hypothesis and objectives

Few studies have focused on investigating adjuvant treatment protocols for GCT in women as the prevalence is quite low and consequently, large-scale randomized trials are difficult to perform. However, GCTs still represent up to 5% of all ovarian tumors, are potentially malignant with a tendency toward late recurrence, require surveillance for life, and are therefore of clinical importance. Taking into consideration the role of angiogenesis and VEGF in GCT disease, we hypothesized that the VEGFA signaling pathway is a valid and useful pharmacological target for the treatment of GCT. We proposed that the *PCA* model would be a useful preclinical model for the investigation of this therapeutic approach, as it imitates the advanced disease in women, with early onset and 100% penetrance.

In order to test this hypothesis, this study's objectives were to evaluate the effect of anti-VEGFA antibody in the *PCA* model on:

1. Tumor growth and survival
2. Cell proliferation and apoptosis
3. Tumor microvessel density
4. Activation of VEGFR2 signaling pathway effectors

Publication as first author

Anti-VEGFA therapy reduces tumor growth and extends survival in a murine model of ovarian granulosa cell tumor

Mayra Tsoi[1], Marie-Noëlle Laguë[1], Alexandre Boyer[1], Marilène Paquet[2], Marie-Ève Nadeau[3], and Derek Boerboom[1]

Authors' affiliations:

1. Centre de Recherche en Reproduction Animale, Faculté de Médecine Vétérinaire, Université de Montréal, St- Hyacinthe, Québec J2S 7C6, Canada.

2. Comparative Medicine & Animal Resources Centre, McGill University, Montréal, Québec H3G 1Y6, Canada.

3. Département des Sciences Cliniques, Faculté de Médecine Vétérinaire, Université de Montréal, St Hyacinthe, Québec J2S 7C6, Canada.

Submitted manuscript to *Oncotarget* (Impact Journals, submission #816, 15 pages, 6 figures, 1 table) in January 2013.

ABSTRACT

Although angiogenesis has been proposed as a therapeutic target for the treatment of ovarian granulosa cell tumor (GCT), its potential has not been evaluated in controlled studies. To do so, we used the *Pten*$^{tm1Hwu/tm1Hwu}$;*Ctnnb1*$^{tm1Mmt/+}$;*Amhr2*$^{tm3(cre)Bhr/+}$ (*PCA*) mouse model, which develops GCTs that mimic the advanced disease in women. A monoclonal anti-vascular endothelial growth factor A (VEGFA) antibody was administered weekly to *PCA* mice beginning at 3 weeks of age. By 6 weeks of age, anti-VEGFA therapy significantly decreased tumor weights relative to controls ($P<0.05$) and increased survival, with all treated animals but none of the controls surviving to 8 weeks of age. Analyses of *PCA* tumors showed that anti-VEGFA treatment resulted in significant decreases in tumor cell proliferation and microvessel density relative to controls ($P<0.05$). However, treatment did not have a significant effect on apoptosis or tumor necrosis. The VEGFA receptor 2 (VEGFR2) signaling effector p44/p42 MAPK, whose activity is associated with cell proliferation, was significantly less phosphorylated (i.e. activated) in tumors from the treated group ($P<0.05$). Conversely, no significant difference was found in the activation of AKT, a VEGFR2 signaling effector associated with cell survival. Together, these results suggest that anti-VEGFA therapy is effective at inhibiting GCT growth in the *PCA* model, and acts by reducing microvascular density and cell proliferation via inhibition of the VEGFR2-MAPK pathway. Findings from this preclinical model therefore support the investigation of targeting VEGFA for the adjuvant treatment of GCT in women.

INTRODUCTION

The granulosa cell tumor (GCT) is the most prevalent of the sex cord/stromal subgroup of ovarian tumors in women, and is thought to represent up to 5% of all ovarian cancers [1-4]. Although GCT is often characterized as a low-grade malignancy [5, 6], approximately 80% of patients with stage III or IV tumors die from recurrent disease [7]. Furthermore, a large proportion of patients develop recurrences as late as 40 years after the initial diagnosis and treatment [8], and therefore, fastidious long-term follow-up is required [1, 3, 9]. Despite the importance and insidiousness of GCT, it has received very little attention from the cancer research community, particularly relative to the more prevalent epithelial ovarian tumors. Perhaps as a consequence of this, the development of therapeutic approaches for GCT has lagged well behind other forms of ovarian cancer. Initial management of GCTs involves cytoreductive surgery, and in cases of recurrence or advanced disease, adjuvant treatment is frequently attempted [1, 3-5, 9, 10]. These adjuvant treatments have included chemotherapy, radiotherapy, hormonal therapy, and more recently, anti-angiogenic therapy [1, 3, 4, 9, 10]. Studies aiming to evaluate current adjuvant treatment protocols for GCTs in women have been limited to retrospective studies and case reports, and no well-designed randomized studies have been conducted to determine if any such regimen actually confers a survival advantage [4, 5, 11-13].

Among the potential therapeutic targets that have been proposed for the development of novel treatments for GCT [14-16], angiogenesis would appear to be particularly promising. GCTs are highly vascularized tumors, and angiogenesis is suspected to play an important role in their development and progression [4, 17, 18]. Vascular endothelial growth factor A (VEGFA) is a

key mediator of angiogenesis and is implicated in endothelial cell proliferation, migration, survival and vascular permeability [18-21]. VEGFA is overexpressed in 94% of GCTs [2] and its main receptor, VEGFR2, is expressed at high levels in 82% of primary and recurrent GCTs in both endothelial and granulosa cells [18]. VEGF was shown to be produced by endothelial as well as granulosa tumor cells [17]. In addition, VEGFA also has well-established pro-proliferative and cytoprotective functions in normal granulosa cells [22-24], and could therefore serve to promote GCT cell proliferation and suppress apoptosis, in addition to promoting angiogenesis. Collectively, these data suggest a very strong potential for VEGFA as a therapeutic target for GCT.

Avastin® (bevacizumab) is a recombinant humanized monoclonal anti-VEGFA antibody that has received FDA approval for use in the treatment of metastatic colorectal cancer and non-squamous, non-small-cell lung cancer in combination with chemotherapy [4, 25-27], as well as metastatic renal cell carcinoma (combined with interferon alpha) and glioblastoma (as a second-line treatment) [http://www.avastin.com/patient/index.html (Accessed May 30, 2012)]. Whereas some reports have shown potential beneficial effects of bevacizumab in the treatment of epithelial ovarian cancer [28-30], very few studies have investigated its use in the treatment of GCT. Tao *et al* [4] carried out a small retrospective case series and evaluated the clinical efficacy of bevacizumab with or without concurrent chemotherapy and found a response rate of 38% and a clinical benefit rate of 63%. This study was limited, however, by its retrospective nature, its small sample size, and the variation of treatments administered [4]. One case report [31] reports symptomatic improvement with bevacizumab combined with paclitaxel for the treatment of refractory GCT while another case report [32] found no clinical improvement with bevacizumab for the first-line treatment of

adult-type GCT. No prospective trial has been conducted to determine the efficacy of single-agent bevacizumab in the treatment of GCT.

A major factor that has impeded the development of novel therapeutic approaches for ovarian cancer (including GCT) has been the dearth of relevant preclinical animal models [10, 33, 34]. We have recently developed a genetically engineered mouse model, *Pten*$^{tm1Hwu/tm1Hwu}$;*Ctnnb1*$^{tm1Mmt/+}$;*Amhr2*$^{tm3(cre)Bhr/+}$ (*PCA*), in which the PI3K/AKT is derepressed and the WNT/CTNNB1 pathway is constitutively activated specifically in granulosa cells [35]. *PCA* mice develop bilateral GCTs with 100% penetrance, peri-natal onset, rapid growth, and many histopathologic features of the human disease [35, 36]. Importantly, as for the advanced disease in women, *PCA* GCTs can form distant metastases and disseminate within the peritoneal cavity [35, 36]. In this study, we therefore used the *PCA* preclinical model to perform the first controlled study to investigate the efficacy of anti-VEGFA therapy for the treatment of GCT disease. We hypothesized that intra-peritoneal administration of anti-VEGFA antibody would effectively reduce tumor growth, reduce tumor vasculature and increase tumor cell apoptosis, thereby improving survival in this murine model of GCT.

MATERIALS AND METHODS

Animals and treatments

Pten$^{tm1Hwu/tm1Hwu}$;*Ctnnb1*$^{tm1Mmt/+}$;*Amhr2*$^{tm3(cre)Bhr/+}$ (*PCA*) mice were obtained by selective breeding of the *Pten*^{tm1Hwu}, *Ctnnb1*^{tm1Mmt}, and *Amhr2*$^{tm3(cre)Bhr}$ parental strains, and genotypes were verified as previously described [35]. Tumor development in the *PCA* model

follows a predictable course. At birth, all *PCA* mice display nests of dysplastic cells in both ovaries. Tumors are macroscopically apparent at 3 weeks of age, and grow in a very rapid and aggressive fashion with abdominal distension becoming apparent by 5 weeks of age and extreme by 7 weeks. Death due to tumor-related causes inevitably occurs before 9 weeks of age [35, 36]. *PCA* mice were administered anti-mouse VEGFA monoclonal antibody clone B20-4.1.1 (provided by Genentech, Inc., San Francisco, CA) by intraperitoneal (IP) injection at 5mg/kg (or 0.9% NaCl as a control) once a week beginning at 3 weeks of age. The mice were sacrificed at 3, 4, 5, 6, 7 and 8 weeks of age and their ovarian tumors, lungs and abdominal organs were collected for subsequent use in immunohistochemistry, immunofluorescence, TUNEL assays, Western blot analysis and histopathology. In order to evaluate the effects of variable doses, additional *PCA* mice received 2.5, 5, or 10 mg/kg of anti-VEGFA antibody weekly IP beginning at 3 weeks of age until 6 weeks of age, at which point mice were sacrificed and their tumors and organs were collected as described above (n= 8-16 mice/treatment/timepoint). Masses of tumors used in all analyses are indicated in Supplemental Table 1.

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Montreal and conformed to the Canadian Council on Animal Care Policy on Humane Care and Use of Laboratory Animals.

Immunohistochemistry and Immunofluorescence

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded, 3 μm ovarian tumor sections (n = 4 mice/treatment/timepoint) using the VectaStain Elite Avidin-Biotin Complex Kit (Vector Laboratories, Inc., Burlingame, CA) as directed by the

manufacturer. Sections were probed with Proliferating Cell Nuclear Antigen (PCNA) mouse monoclonal antibody (Cell Signaling Technology, Danvers, MA, catalog number 2586) as directed by the manufacturer, except incubation with the primary antibody (dilution 1:2000) was performed for 30 minutes, and incubation with the secondary antibody (biotinylated anti-mouse reagent, Vector Laboratories, Inc., dilution 1:250) was done for 10 minutes. Sections were also probed with phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP^{TM} rabbit mAb (Cell Signaling Technology, catalog number 4370, dilution 1:200) as directed by the manufacturer. Staining was done using 3,3'-diaminobenzidine peroxidase substrate kit (Vector Laboratories, Inc.) as directed by the manufacturer. In order to determine the number of PCNA-positive cells, 4 fields per tumor (1 or 2 tumors per animal; at a 630x magnification) were selected at random. From each field, cells were counted within a randomly-selected circular area with a 90 μm diameter. For each tumor, % of PCNA-positive cells was calculated by dividing the sum of PCNA-positive cells (from all fields) by the total number of cells. Sections of the same tumors stained with HPS were also evaluated to quantify mitotic figures per 400x field as a second measure of cell proliferative activity, as well as to estimate the extent of tumor necrosis. The area of coagulative necrosis (characterized by increased eosinophilia and glassy appearance of the area with loss of cellular details) was evaluated by light microscopy and was estimated for each tumor as a percentage of the total tumor cross section area. All slides were evaluated by a board-certified veterinary pathologist who was blinded with regard to the treatment received.

Immunofluorescence was performed on O.C.T.-embedded (Sakura Finetek U.S.A., Inc. Torrance, CA) frozen ovarian tumors (n = 4 mice/treatment/timepoint). Samples were stored at -80^{o}C until they were sectioned (4 μm) and allowed to dry for 5 hours. The slides were quick-

fixed with 100% acetone (-20°C) for 20 seconds, wrapped in aluminum foil and stored at -80°C. When ready, the slides were thawed at room temperature for 20 minutes, fixed with 100% acetone (-20°C) for 10 minutes, followed by 70% ethanol (-20°C) for 5 minutes. The slides were blocked with 10% goat serum diluted in PBS for 30 minutes and then probed with rat anti-mouse CD31 antibody (BD Biosciences, Franklin Lakes, NJ, catalog number 558736, dilution 1:300, overnight at 4°C) and/or fluorescein-labeled *Lycopersicon Esculentum* (Tomato) Lectin (Vector Laboratories, Inc. catalog number FL-1171, dilution 1:1000, 10 minutes at RT) diluted in 3% goat serum in PBS. The CD31-probed slides were incubated with the secondary antibody Alexa Fluor 594 goat anti-rat IgG (Invitrogen, Burlington, ON, catalog number A11007, dilution 1:2000) for 1 hour at room temperature, diluted in 3% goat serum. The slides were washed with 1% Triton-X-100 (Bioshop Canada, Inc., Burlington, ON) in PBS for 10 minutes and mounted with Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories, Inc.). In order to evaluate the amount of CD31 signal, 5 fields per tumor (at 200x magnification) were selected at random and ImageJ software was used to quantify the area of signal emitted in each field. The evaluator was blinded with respect to the ages and treatments received. The double-labeled (CD31 + lectin) slides were used to confirm the specificity of endothelial cell staining.

TUNEL assays

TUNEL assays were performed on formalin-fixed, paraffin-embedded, 3 μm ovarian tumor sections (n = 4 mice/treatment/timepoint) using the *In Situ* Cell Death Detection Kit, TMR red (Roche Diagnostics GmbH, Mannheim, Germany, catalog number 12156792910), according to the manufacturer's instructions. Vectashield mounting medium for fluorescence

with DAPI (Vector Laboratories, Inc.) was used to mount the slides. In order to determine the number of positive cells for TUNEL, 4 fields per tumor (1 or 2 tumors per animal; at 630x magnification) were selected at random. Within each field, a circular area with a 90 μm diameter was chosen at random in order to count the number of TUNEL-positive cells. For each tumor, % TUNEL-positive cells was calculated by dividing the sum of TUNEL-positive cells (from all fields) by the total number of cells. A normal healthy ovary containing atretic follicles was used as a positive control. The evaluator was blinded with respect to the ages and treatments received.

Western Blot Analysis

Tumor samples from 6 week-old *PCA* mice were used for Western blot analysis (n = 4 mice/treatment). Protein extracts were obtained using T-PER Tissue Protein Extraction Reagent and Halt Protease and Phosphatase Inhibitor Single-Use Cocktail (Thermo Fisher Scientific, Rockford, IL, catalog numbers 78510 and 78442, respectively) as described by the manufacturer. Protein concentrations were quantified using the Bradford method. Samples (25μg or 50μg) were resolved on 7-12% sodium dodecyl sulfate-polyacrylamide gels and transferred to Hybond-P PVDF Membrane (GE Amersham, Piscataway, NJ). Blots were probed with primary antibodies against p44/42 MAPK (Erk1/2) (137F5) Rabbit mAb, phospho-p44/42 MAPK (Erk 1/2) (Thr202/Tyr204) (D13.14.4E) XP^{TM} Rabbit mAb, AKT, phospho-AKT (Ser473) (587F11) Mouse mAb, VEGF receptor 2 (55B11) Rabbit mAb, phospho-VEGF receptor 2 (Tyr951) (15D2) Rabbit mAb, phospho-VEGF receptor 2 (Tyr 1059) (D5A6) Rabbit mAb (Cell Signaling Technology, catalog numbers 4695, 4370, 9272, 4051, 2479, 4991 and 3817 respectively), and β-Actin (C4) (Santa Cruz Biotechnology, Inc.,

Santa Cruz, CA, catalog number sc-47778) as directed by the manufacturers. The blots were incubated with ECL Peroxidase-labeled secondary antibodies (GE Amersham) and then ECL Plus Western blotting detection reagents (GE Amersham) were used to visualize the protein bands by chemiluminescence on High-Performance Chemiluminescence Film (GE Amersham). The signal strengths were quantified using Kodak 1D v.3.6.5 software (Eastman Kodak Company).

Statistical methods

Effects of antibody treatment on tumor size, cell proliferation, cell apoptosis, CD31 signal intensity and VEGFA signaling pathway protein expression were analyzed by ANOVA, followed by Newman-Keul's or Dunnett's post-test to identify differences between specific groups. $P<0.05$ was considered statistically significant. Prism 4.0a software (GraphPad Software, Inc., San Diego, CA) was used for analysis.

RESULTS

Anti-VEGFA therapy reduces tumor burden and improves survival in *PCA* mice

To study the efficacy of anti-VEGFA therapy in the *PCA* model, mice were treated with anti-VEGFA antibody (5mg/kg, IP) once a week beginning at 3 weeks of age. The mice were sacrificed at 3, 4, 5, 6, 7 and 8 weeks of age and their ovarian tumors and viscera were collected for analysis. A dose-response experiment was also conducted using 2.5, 5 and 10 mg/kg of anti-VEGFA, IP, once a week, beginning at 3 weeks of age until 6 weeks of age, at which point the mice were sacrificed.

PCA mice treated with anti-VEGFA antibody in the dose-response trial demonstrated that significant effects on tumor burden were obtained at the 5mg/kg dose, but no further benefit was obtained at 10mg/kg (Fig. 1A). In the timecourse trial, *PCA* mice showed significantly reduced ovarian tumor weights at 6 and 7 weeks of age relative to controls (Fig. 1B). Importantly, anti-VEGFA treatment also extended the survival of treated animals. Whereas all animals treated with anti-VEGFA antibody survived up to 8 weeks of age, 27,8% (n= 5 of 18) of controls died before 7 weeks of age or had to be euthanized due to deteriorating health, and no control mice survived until 8 weeks of age (Fig. 1C).

Anti-VEGFA treatment reduces cell proliferation but does not affect apoptosis in *PCA* GCTs

To determine the cause(s) of reduced GCT size in anti-VEGFA-treated *PCA* mice, cell proliferation was assessed by PCNA immunohistochemistry and by histological analysis of mitotic figures. A significant decrease in the number of PCNA-positive cells was observed in the anti-VEGFA treated group compared to the saline group at 6 weeks of age (Fig. 2A). Likewise, fewer mitoses per high-power field were observed at 6 weeks of age in the anti-VEGFA treated group (Figure 2B).

TUNEL assays were performed to determine if increased tumor cell apoptosis contributed to the decrease in tumor growth in anti-VEGFA-treated mice. No significant difference in the number of TUNEL-positive cells in the anti-VEGFA-treated group compared to the saline group was observed at any age (Fig. 3A). Likewise, although small foci of necrosis were observed in some tumors (particularly at 6 weeks), anti-VEGFA therapy did not increase the overall area of necrosis observed in histological sections (Fig. 3B).

Anti-VEGFA treatment significantly reduces tumor microvessel density in GCTs

To study the effects of anti-VEGFA antibody on angiogenesis in *PCA* GCTs, the tumor microvasculature was visualized by immunofluorescence using the endothelial cell marker CD31 (PECAM-1). Tumors from 6 week-old, anti-VEGFA-treated mice had a markedly lower microvessel abundance than saline-treated controls, with CD31 signal reduced by more than half in the 5mg/kg group (Fig. 4A-C). Specificity of the CD31 signal for endothelial cells was confirmed by double-labeling with tomato plant lectin (Fig. 4D).

Intracellular signaling is altered in tumors from *PCA* mice treated with anti-VEGFA antibody

To identify potential alterations in intracellular signaling activity downstream of VEGF receptor 2 (VEGFR2) caused by anti-VEGFA treatment, activity levels of the p44/42 MAPK (ERK1/2) and the PI3K/AKT signaling pathways were evaluated by western blotting. Levels of phospho-p44/42 MAPK (Thr202/Tyr204) were significantly lower in GCTs from 6 week-old *PCA* mice that had received as little as 2.5mg/kg of anti-VEGFA antibody, as was the ratio of phosphorylated-to-total p44/42 MAPK (Fig. 5A, B), indicative of decreased MAPK signaling activity. Immunohistochemistry was performed to determine the cell population(s) within the tumors in which this decrease occurred, which showed that granulosa, stromal and endothelial cells (identified based on morphological characteristics) all appeared to be affected (Figure 5C).

Contrary to the MAPK pathway, no significant difference was found in the expression of AKT or phospho-AKT between the various anti-VEGFA-treated groups and the saline group (Figure 6A, B), suggesting that activity of the PI3K/AKT pathway was not altered by

treatment. Activation of VEGFR2 was also studied, but tyrosine phosphorylation of the receptor at sites 951 and 1059 could not be detected in either the anti-VEGFA treated or the control groups (not shown).

DISCUSSION

Few therapeutic options exist for advanced-stage and recurrent GCT, and none of the widely-used chemotherapeutic regimens has been rigorously evaluated with regards to its effectiveness [11, 13, 37, 38]. The development of validated adjuvant chemotherapies for GCT is therefore of paramount importance. Whereas angiogenesis (i.e., VEGFA) been proposed as a therapeutic target for GCT [4, 17, 18, 31, 32], clinical investigations of anti-VEGFA therapies have so far been limited to small-scale retrospective studies and case reports, and have yielded limited insight [4, 31, 32]. In this report, we used the recently-developed *PCA* mouse model, which develops an ovarian cancer that mimics many of the histological and biological aspects of advanced human GCT [35, 36], to investigate the efficacy of an anti-VEGFA antibody analogous to bevacizumab. Our results in this preclinical model clearly show that anti-VEGFA therapy extends survival and significantly slows tumor growth. These findings therefore support the prospective investigation of anti-VEGFA therapy for the adjuvant treatment of GCT in women.

Our findings indicate that the main mechanism by which the anti-VEGFA antibody slowed tumor growth was inhibition of tumor cell proliferation, rather than induction of apoptosis. Proliferation was presumably inhibited, at least in part, by reduced microvessel density, resulting in decreased delivery of nutrients and growth factors to tumor cells.

However, our results also suggest that the anti-VEGFA therapy may have acted directly upon the tumor cells themselves to inhibit proliferation. Indeed, apparent lower levels of phosphorylation (i.e., activity) of the VEGFA receptor signaling effector MAPK were observed not only in endothelial cells but also in tumor cells from anti-VEGFA-treated mice. As MAPK signaling is thought to mediate the pro-proliferative actions of VEGFA [39, 40], this suggests that the anti-VEGFA treatment acted to sequester pro-proliferative VEGFA from the tumor cells in the *PCA* model. This would be entirely consistent with the well-established role of VEGFA as a granulosa cell growth factor in the context of normal ovarian follicle development [41, 42], and would indicate that GCT cells retain a certain dependence on VEGFA as a proliferative signal even after oncogenic transformation. VEGFA also signals via the PI3K/AKT pathway, whose activity is associated with the anti-apoptotic effects of VEGFA [43, 44]. Anti-VEGFA therapy had no effect on tumor cell apoptosis in the *PCA* model, and did not alter AKT phosphorylation (i.e., activity). VEGFA/AKT cytoprotective signaling would therefore not appear to be relevant to the pathogenesis of GCT, at least in the *PCA* model.

The effect of the anti-VEGFA antibody on GCT microvessel density in the *PCA* model concurs with findings from other studies that evaluated the effect of analogous anti-VEGFA antibodies on vascular growth. Korsisaari *et al* [27] found a significant reduction in vessel density after 3 weeks of administration of anti-VEGFA mAb G6-31 in a murine model of intestinal adenoma. Likewise, Borgström *et al* [45] found complete inhibition of angiogenesis in micro tumors with administration of anti-VEGF antibody A4.6.1 in a tumor xenograft study of human prostate carcinoma. In past studies, anti-VEGFA therapy has been found to reduce tumor microvessel density, decrease permeability, increase tumor pericyte coverage and

stabilize the basement membrane, which all contribute to forming a more normalized tumor vasculature [46, 47]. In consequence, tumor hypoxia and interstitial fluid pressure are reduced, which allows improved delivery of chemotherapy to the tumor, as observed in clinical trials [19, 47, 48]. If this holds true for GCT as well, another benefit of anti-VEGFA therapy may be to enhance the effects of other chemotherapeutic agents when used in the context of combinatorial therapy. The *PCA* mouse model may prove useful to test this hypothesis, as well as for the subsequent development of combinatorial treatment schemes.

In summary, this study shows that monotherapy with anti-VEGFA antibody is effective at suppressing tumor growth and extending survival in the *PCA* model of GCT. Targeting VEGFA reduced tumor cell proliferation and microvascular density, which could sensitize GCTs to the effects of other chemotherapeutic agents. Based on our results, we conclude that anti-VEGFA therapy shows great potential in the adjuvant treatment of GCT.

ACKNOWLEDGEMENTS

We thank Céline Forget, Meggie Girard, Evelyne Lapointe, and Aurore Dodelet-Devillers for their assistance. Anti-VEGFA antibody was provided by Genentech.

Mayra Tsoi, performed research, collected and analyzed data, and wrote the paper. Marie-Noëlle Laguë collected data. Alexandre Boyer and Marilène Paquet analyzed data. Marie-Ève Nadeau designed and performed research. Derek Boerboom designed research, analyzed data and wrote the paper.

GRANT SUPPORT

Financial support provided by the Canada Research Chair in Ovarian Molecular Biology and Functional Genomics and the Canadian Institutes of Health Research.

CONFLICTS OF INTEREST

The authors disclose no potential conflicts of interest.

REFERENCES

1. Koukourakis GV, Kouloulias VE, Koukourakis MJ, Zacharias GA, Papadimitriou C, Mystakidou K, et al. Granulosa cell tumor of the ovary: tumor review. Integr Cancer Ther. 2008;7:204-15.
2. Schmidt M, Kammerer U, Segerer S, Cramer A, Kohrenhagen N, Dietl J, et al. Glucose metabolism and angiogenesis in granulosa cell tumors of the ovary: activation of Akt, expression of M2PK, TKTL1 and VEGF. Eur J Obstet Gynecol Reprod Biol. 2008;139:72-8.
3. Schumer ST, Cannistra SA. Granulosa cell tumor of the ovary. J Clin Oncol. 2003;21:1180-9.
4. Tao X, Sood AK, Deavers MT, Schmeler KM, Nick AM, Coleman RL, et al. Anti-angiogenesis therapy with bevacizumab for patients with ovarian granulosa cell tumors. Gynecol Oncol. 2009;114:431-6.
5. Ayhan A, Salman MC, Velipasaoglu M, Sakinci M, Yuce K. Prognostic factors in adult granulosa cell tumors of the ovary: a retrospective analysis of 80 cases. J Gynecol Oncol. 2009;20:158-63.
6. King LA, Okagaki T, Gallup DG, Twiggs LB, Messing MJ, Carson LF. Mitotic count, nuclear atypia, and immunohistochemical determination of Ki-67, c-myc, p21-ras, c-erbB2, and p53 expression in granulosa cell tumors of the ovary: mitotic count and Ki-67 are indicators of poor prognosis. Gynecol Oncol. 1996;61:227-32.
7. Amsterdam A, Selvaraj N. Control of differentiation, transformation, and apoptosis in granulosa cells by oncogenes, oncoviruses, and tumor suppressor genes. Endocr Rev. 1997;18:435-61.

8. Hines JF, Khalifa MA, Moore JL, Fine KP, Lage JM, Barnes WA. Recurrent granulosa cell tumor of the ovary 37 years after initial diagnosis: a case report and review of the literature. Gynecol Oncol. 1996;60:484-8.
9. Geetha P, Nair MK. Granulosa cell tumours of the ovary. Aust N Z J Obstet Gynaecol. 2010;50:216-20.
10. Jamieson S, Fuller PJ. Molecular pathogenesis of granulosa cell tumors of the ovary. Endocr Rev. 2012;33:109-44.
11. Sun HD, Lin H, Jao MS, Wang KL, Liou WS, Hung YC, et al. A long-term follow-up study of 176 cases with adult-type ovarian granulosa cell tumors. Gynecol Oncol. 2012;124:244-9.
12. Malmstrom H, Hogberg T, Risberg B, Simonsen E. Granulosa cell tumors of the ovary: prognostic factors and outcome. Gynecol Oncol. 1994;52:50-5.
13. Park JY, Jin KL, Kim DY, Kim JH, Kim YM, Kim KR, et al. Surgical staging and adjuvant chemotherapy in the management of patients with adult granulosa cell tumors of the ovary. Gynecol Oncol. 2012;125:80-6.
14. Fishman A, Kudelka AP, Tresukosol D, Edwards CL, Freedman RS, Kaplan AL, et al. Leuprolide acetate for treating refractory or persistent ovarian granulosa cell tumor. J Reprod Med. 1996;41:393-6.
15. Alhilli MM, Long HJ, Podratz KC, Bakkum-Gamez JN. Aromatase inhibitors in the treatment of recurrent ovarian granulosa cell tumors: brief report and review of the literature. J Obstet Gynaecol Res. 2012;38:340-4.

16. Raspagliesi F, Martinelli F, Grijuela B, Guadalupi V. Third-line chemotherapy with tyrosine kinase inhibitor (imatinib mesylate) in recurrent ovarian granulosa cell tumor: case report. J Obstet Gynaecol Res. 2011;37:1864-7.
17. Farkkila A, Anttonen M, Pociuviene J, Leminen A, Butzow R, Heikinheimo M, et al. Vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 are highly expressed in ovarian granulosa cell tumors. Eur J Endocrinol. 2011;164:115-22.
18. Farkkila A, Pihlajoki M, Tauriala H, Butzow R, Leminen A, Unkila-Kallio L, et al. Serum vascular endothelial growth factor A (VEGF) is elevated in patients with ovarian granulosa cell tumor (GCT), and VEGF inhibition by bevacizumab induces apoptosis in GCT in vitro. J Clin Endocrinol Metab. 2011;96:E1973-81.
19. Ho QT, Kuo CJ. Vascular endothelial growth factor: biology and therapeutic applications. Int J Biochem Cell Biol. 2007;39:1349-57.
20. Greenberg JI, Cheresh DA. VEGF as an inhibitor of tumor vessel maturation: implications for cancer therapy. Expert Opin Biol Ther. 2009;9:1347-56.
21. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003;9:669-76.
22. Irusta G, Abramovich D, Parborell F, Tesone M. Direct survival role of vascular endothelial growth factor (VEGF) on rat ovarian follicular cells. Mol Cell Endocrinol. 2010;325:93-100.
23. Kosaka N, Sudo N, Miyamoto A, Shimizu T. Vascular endothelial growth factor (VEGF) suppresses ovarian granulosa cell apoptosis in vitro. Biochem Biophys Res Commun. 2007;363:733-7.

24. Greenaway J, Connor K, Pedersen HG, Coomber BL, LaMarre J, Petrik J. Vascular endothelial growth factor and its receptor, Flk-1/KDR, are cytoprotective in the extravascular compartment of the ovarian follicle. Endocrinology. 2004;145:2896-905.
25. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med. 2004;350:2335-42.
26. Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med. 2006;355:2542-50.
27. Korsisaari N, Kasman IM, Forrest WF, Pal N, Bai W, Fuh G, et al. Inhibition of VEGF-A prevents the angiogenic switch and results in increased survival of Apc+/min mice. Proc Natl Acad Sci U S A. 2007;104:10625-30.
28. Burger RA, Brady MF, Bookman MA, Fleming GF, Monk BJ, Huang H, et al. Incorporation of bevacizumab in the primary treatment of ovarian cancer. N Engl J Med. 2011;365:2473-83.
29. Monk BJ, Han E, Josephs-Cowan CA, Pugmire G, Burger RA. Salvage bevacizumab (rhuMAB VEGF)-based therapy after multiple prior cytotoxic regimens in advanced refractory epithelial ovarian cancer. Gynecol Oncol. 2006;102:140-4.
30. Richardson DL, Backes FJ, Seamon LG, Zanagnolo V, O'Malley DM, Cohn DE, et al. Combination gemcitabine, platinum, and bevacizumab for the treatment of recurrent ovarian cancer. Gynecol Oncol. 2008;111:461-6.

31. Kesterson JP, Mhawech-Fauceglia P, Lele S. The use of bevacizumab in refractory ovarian granulosa-cell carcinoma with symptomatic relief of ascites: a case report. Gynecol Oncol. 2008;111:527-9.
32. Barrena Medel NI, Herzog TJ, Wright JD, Lewin SN. Neoadjuvant bevacizumab in a granulosa cell tumor of the ovary: a case report. Anticancer Res. 2010;30:4767-8.
33. Vanderhyden BC, Shaw TJ, Ethier JF. Animal models of ovarian cancer. Reprod Biol Endocrinol. 2003;1:67.
34. Mullany LK, Richards JS. Minireview: animal models and mechanisms of ovarian cancer development. Endocrinology. 2012;153:1585-92.
35. Lague MN, Paquet M, Fan HY, Kaartinen MJ, Chu S, Jamin SP, et al. Synergistic effects of Pten loss and WNT/CTNNB1 signaling pathway activation in ovarian granulosa cell tumor development and progression. Carcinogenesis. 2008;29:2062-72.
36. Nadeau ME, Kaartinen MJ, Lague MN, Paquet M, Huneault LM, Boerboom D. A mouse surgical model for metastatic ovarian granulosa cell tumor. Comp Med. 2009;59:553-6.
37. Pautier P, Gutierrez-Bonnaire M, Rey A, Sillet-Bach I, Chevreau C, Kerbrat P, et al. Combination of bleomycin, etoposide, and cisplatin for the treatment of advanced ovarian granulosa cell tumors. Int J Gynecol Cancer. 2008;18:446-52.
38. Brown J, Shvartsman HS, Deavers MT, Ramondetta LM, Burke TW, Munsell MF, et al. The activity of taxanes compared with bleomycin, etoposide, and cisplatin in the treatment of sex cord-stromal ovarian tumors. Gynecol Oncol. 2005;97:489-96.
39. Pages G, Milanini J, Richard DE, Berra E, Gothie E, Vinals F, et al. Signaling angiogenesis via p42/p44 MAP kinase cascade. Ann N Y Acad Sci. 2000;902:187-200.

40. Berra E, Pages G, Pouyssegur J. MAP kinases and hypoxia in the control of VEGF expression. Cancer Metastasis Rev. 2000;19:139-45.
41. Qiu Y, Seager M, Osman A, Castle-Miller J, Bevan H, Tortonese DJ, et al. Ovarian VEGF(165)b expression regulates follicular development, corpus luteum function and fertility. Reproduction. 2012;143:501-11.
42. Kaczmarek MM, Schams D, Ziecik AJ. Role of vascular endothelial growth factor in ovarian physiology - an overview. Reprod Biol. 2005;5:111-36.
43. Jiang BH, Liu LZ. PI3K/PTEN signaling in angiogenesis and tumorigenesis. Adv Cancer Res. 2009;102:19-65.
44. Ma J, Sawai H, Ochi N, Matsuo Y, Xu D, Yasuda A, et al. PTEN regulates angiogenesis through PI3K/Akt/VEGF signaling pathway in human pancreatic cancer cells. Mol Cell Biochem. 2009;331:161-71.
45. Borgstrom P, Bourdon MA, Hillan KJ, Sriramarao P, Ferrara N. Neutralizing anti-vascular endothelial growth factor antibody completely inhibits angiogenesis and growth of human prostate carcinoma micro tumors in vivo. Prostate. 1998;35:1-10.
46. Inai T, Mancuso M, Hashizume H, Baffert F, Haskell A, Baluk P, et al. Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. Am J Pathol. 2004;165:35-52.
47. Goel S, Duda DG, Xu L, Munn LL, Boucher Y, et al. Normalization of the vasculature for treatment of cancer and other diseases. Physiol Rev. 2011;91:1071-121.
48. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science. 2005;307:58-62.

FIGURE LEGENDS

Figure 1. Anti-VEGFA antibody reduces tumor burden and improves survival of *PCA* mice with GCTs. A, Effects of different doses of anti-VEGFA on tumor mass in 6 week-old *PCA* mice (n= 8-16 mice/treatment/time). Data are shown as means (columns) ± SEM (error bars). Significant difference from control (saline) is indicated with one (*: $P < 0.05$) or two asterisks (**: $P < 0.01$). Photographs of representative tumors are shown below the graph. B, Timecourse of GCT mass with or without weekly anti-VEGFA treatment in *PCA* mice (n=10-26 mice/treatment/time). C, Survival curves indicating the proportion of experimental mice surviving at the indicated times (n= 4-18 mice/treatment/time). Anti-VEGFA-treated mice were sacrificed for humane reasons at 3 days past the 8 week timepoint.

Figure 2. Anti-VEGFA antibody reduces cell proliferation in GCTs from *PCA* mice. A, Graph depicting the proportion (as %) of PCNA-positive cells in *PCA* mice administered anti-VEGFA antibody or saline (control), n = 4 mice/treatment/timepoint. Representative photomicrographs of PCNA-stained tumors are shown below the graph at a 630x magnification. B, Evaluation of mitotic indices in the tumors described in panel A. Error bars = SEM. Significant difference from control ($P < 0.05$) is indicated with an asterisk (*).

Figure 3. Anti-VEGFA antibody has no significant effect on apoptosis or tumor necrosis in GCTs from *PCA* mice. A, Graph depicting the proportion (as %) of TUNEL-positive cells in *PCA* mice administered anti-VEGFA antibody or saline (control), n = 4

mice/treatment/timepoint. B, Evaluation of extent of tumor necrosis (as % of total surface in histological sections) in the tumors described in panel A. Error bars = SEM.

Figure 4. Anti-VEGFA antibody significantly reduces microvessel density in GCTs from 6 week-old *PCA* mice. A, Graph depicting CD31 immunofluorescence signal strengths in GCTs from 6 week-old *PCA* mice that received the indicated treatments (n = 4 animals/treatment). Data are shown as means (columns) ± SEM (error bars). Significant difference from control (saline) is indicated with one (*: $P < 0.05$) or two asterisks (**: $P < 0.01$). B, Representative photomicrograph (200x magnification) depicting CD31 fluorescent signal in the GCT of a 6 week-old *PCA* mouse. CD31-specific signal is red, nuclei are counterstained with DAPI (blue). C, As per panel B, showing a tumor from an anti-VEGFA treated mouse. D, As per panel B, except endothelial cells were labeled with tomato plant lectin (green) in addition to CD31 immunolabeling; 630x magnification. Overlap in lectin and CD31 signals appears yellow, and confirms the specific labeling of endothelial cells by CD31.

Figure 5. Anti-VEGFA antibody significantly reduces MAPK activation in GCTs from 6 week-old *PCA* mice. A, Graph depicting expression of MAPK, phospho-MAPK and phospho-MAPK:total MAPK ratio in tumors from 6 week-old *PCA* mice that received the indicated treatments. Data are densitometric quantification of signals obtained by western blotting (n=4 per treatment). Data are shown as means (columns) ± SEM (error bars). Significant difference from control (saline) is indicated with one (*: $P < 0.05$) or two asterisks (**: $P < 0.01$). B, Representative western blot images from the analyses shown in A (n = 2 samples/treatment). β-Actin was used as a loading control. C, Immunohistochemical analysis

of phospho-MAPK expression in tumors from 6 week-old *PCA* mice treated with anti-VEGFA (5mg/kg) or saline control; 400x magnification.

Figure 6. Anti-VEGFA antibody has no significant effect on AKT activation in GCTs from 6 week-old *PCA* mice. A, Graph depicting expression of AKT, phospho-AKT and phospho-AKT:total AKT ratio in tumors from 6 week-old *PCA* mice that received the indicated treatments. Data are densitometric quantification of signals obtained by western blotting (n=4 per treatment). Data are shown as means (columns) ± SEM (error bars). B, Representative western blot images from the analyses shown in A (n = 2 samples/treatment). β-Actin was used as a loading control.

SUPPLEMENTARY MATERIAL

Supplemental Table 1. Masses of granulosa cell tumors used for A, immunohistochemistry, mitotic figures, TUNEL assay, % necrosis. B, Tumors from 6 week-old mice used for immunofluorescence. C, Tumors from 6 week-old mice used for Western blot.

FIGURE 1

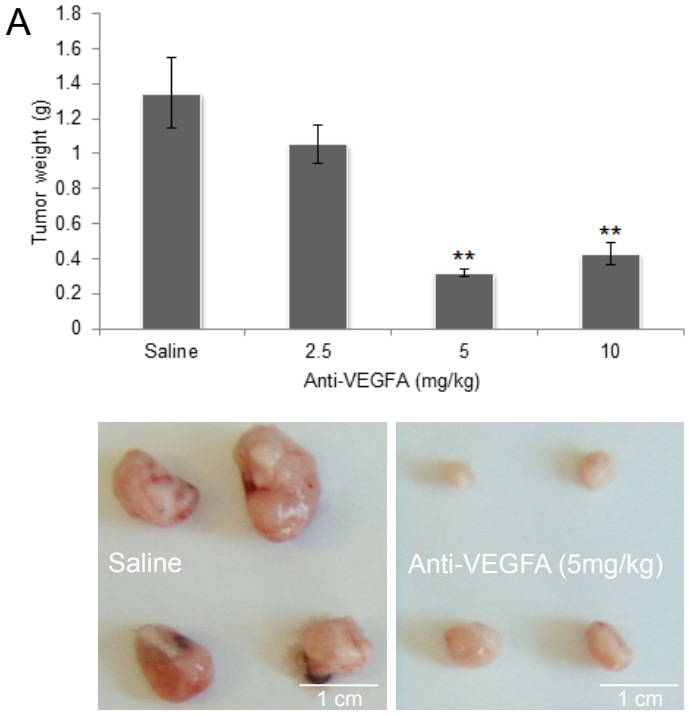

A
Tumor weight (g)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
**
Saline
2.5
5
10
Anti-VEGFA (mg/kg)
Saline
Anti-VEGFA (5mg/kg)
1 cm
1 cm
B
Anti-VEGFA (5mg/kg)
Saline
Tumor weight (g)
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
*
3
4
5
6
7
8
Age of mice (weeks)
C
Survival (%)
100
90
80
70
60
50
40
30
20
10
0
Anti-VEGFA (5mg/kg)
Saline
3
4
5
6
7
8
Age of mice (weeks)

FIGURE 2

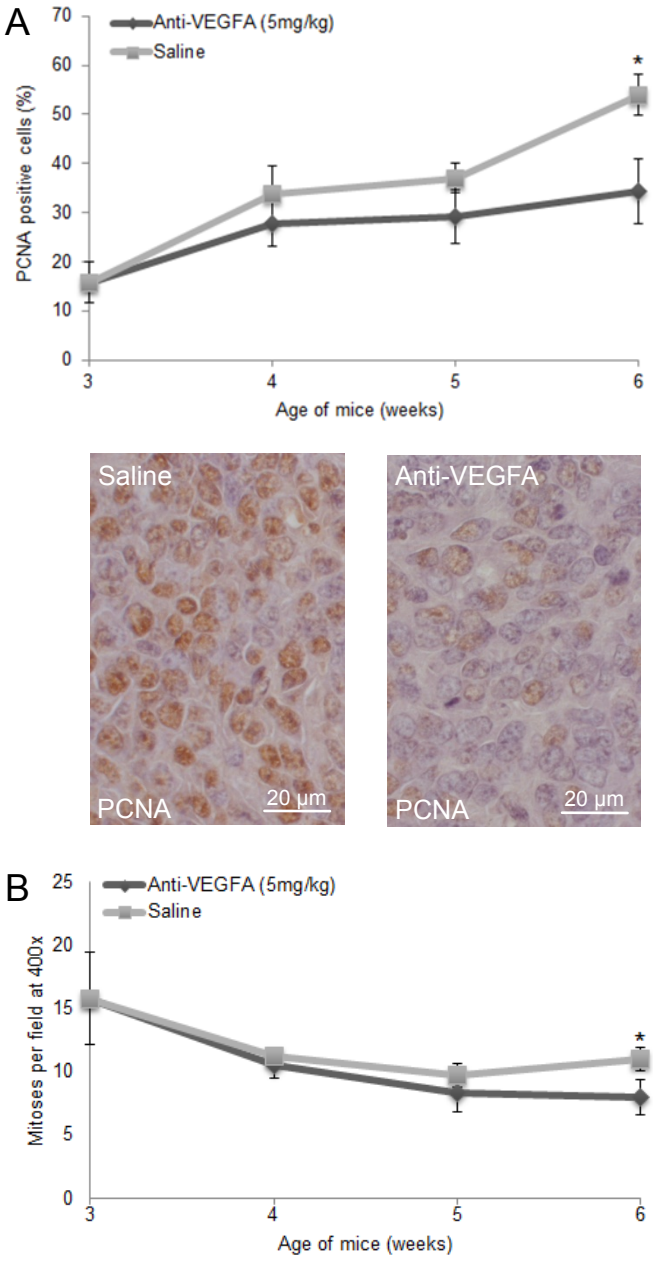

A
Anti-VEGFA (5mg/kg)
Saline
PCNA positive cells (%)
70
60
50
40
30
20
10
0
3
4
5
6
Age of mice (weeks)
*
Saline
PCNA
20 µm
Anti-VEGFA
PCNA
20 µm
B
Anti-VEGFA (5mg/kg)
Saline
Mitoses per field at 400x
25
20
15
10
5
0
3
4
5
6
Age of mice (weeks)
*

FIGURE 3

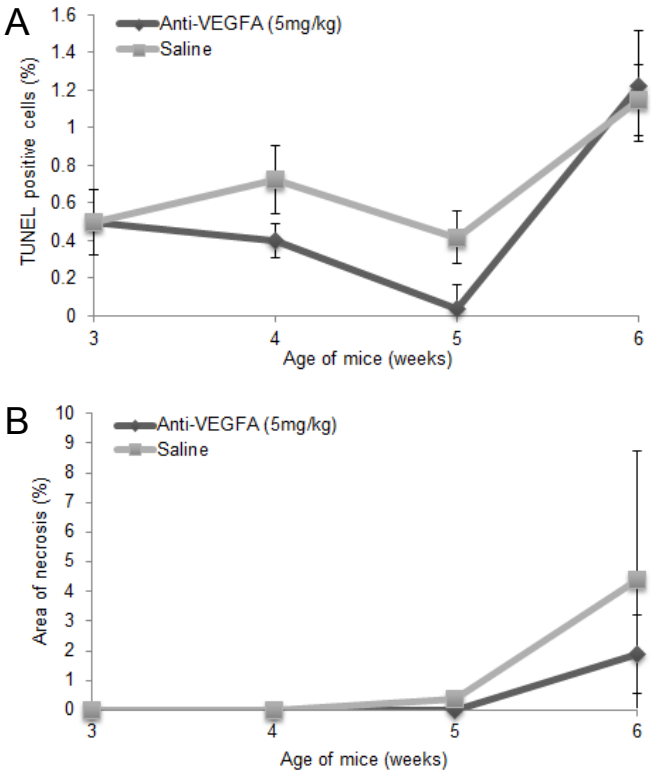

A
Anti-VEGFA (5mg/kg)
Saline
TUNEL positive cells (%)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
3
4
5
6
Age of mice (weeks)
B
Anti-VEGFA (5mg/kg)
Saline
Area of necrosis (%)
10
9
8
7
6
5
4
3
2
1
0
3
4
5
6
Age of mice (weeks)

FIGURE 4

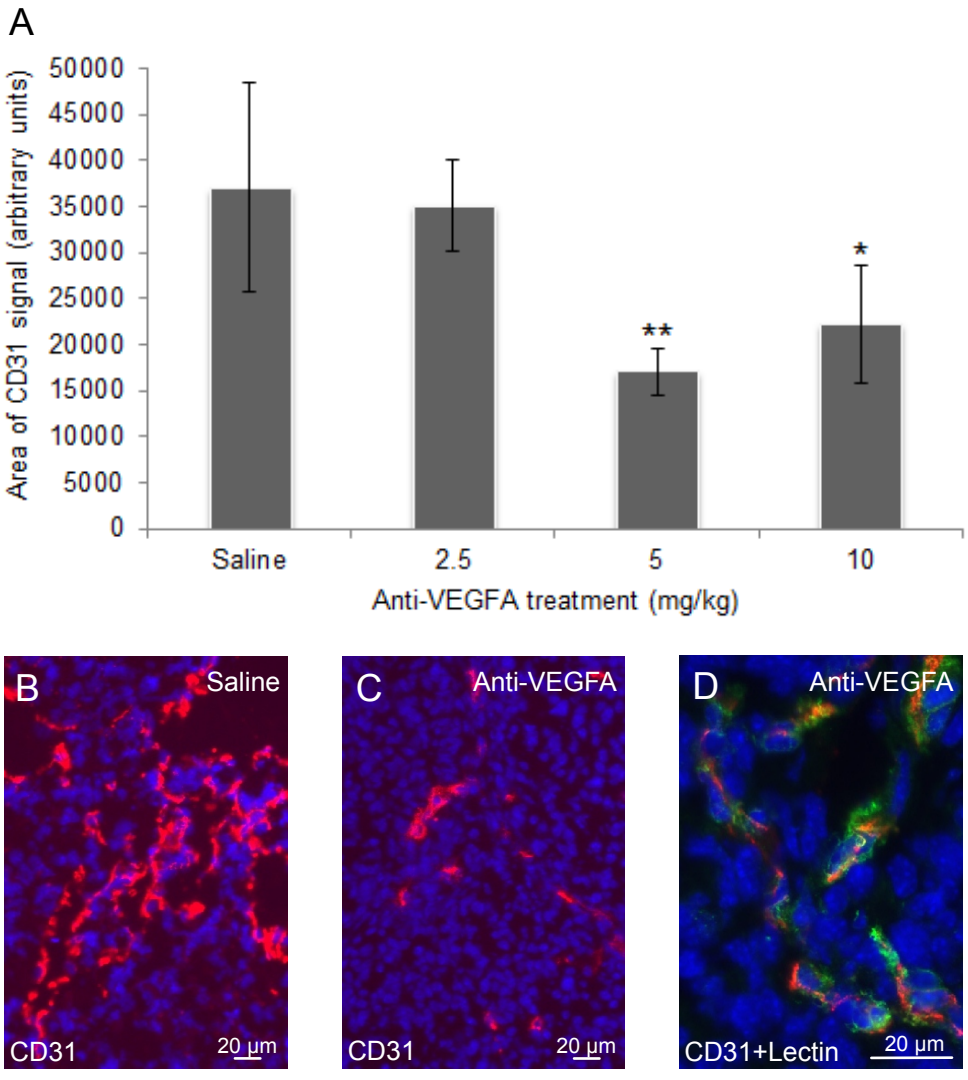

A
Area of CD31 signal (arbitrary units)
50000
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
**
*
Saline
2.5
5
10
Anti-VEGFA treatment (mg/kg)
B
Saline
CD31
20 µm
C
Anti-VEGFA
CD31
20 µm
D
Anti-VEGFA
CD31+Lectin
20 µm

FIGURE 5

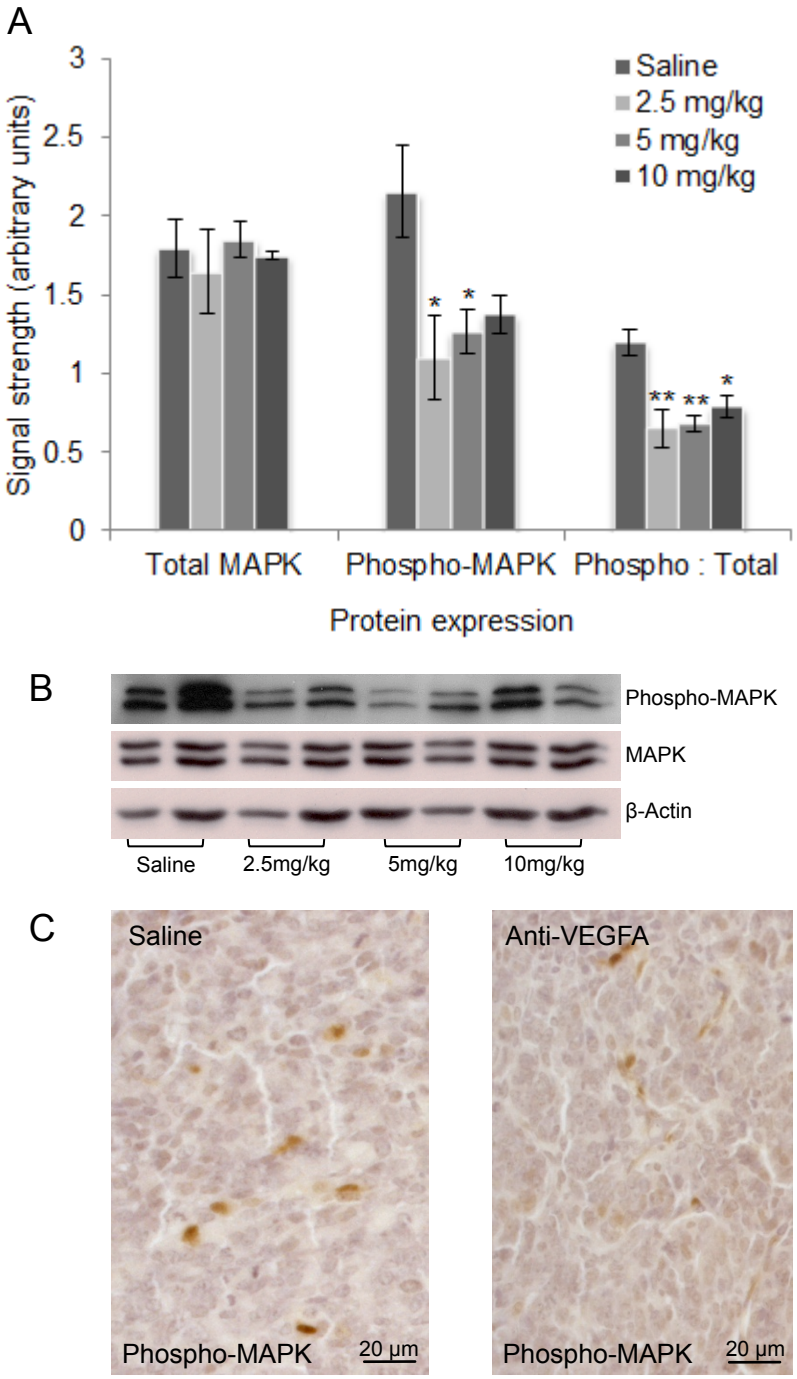
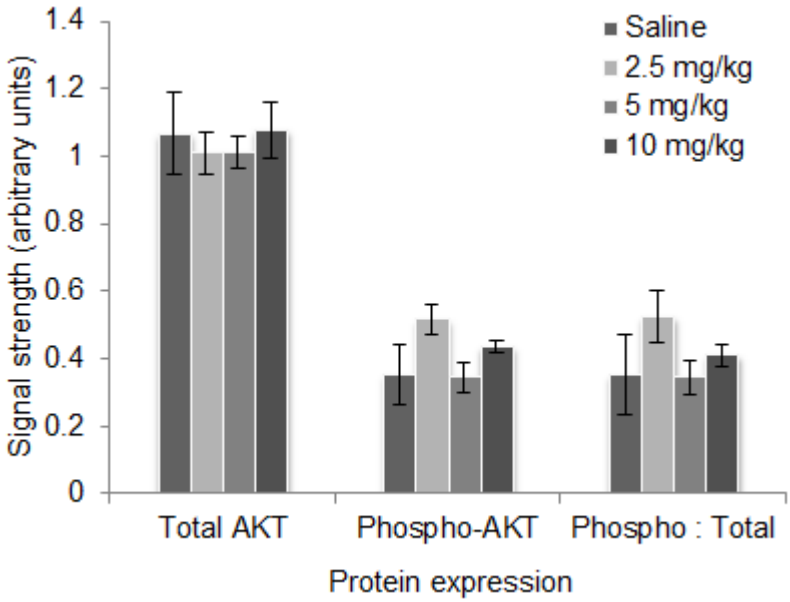
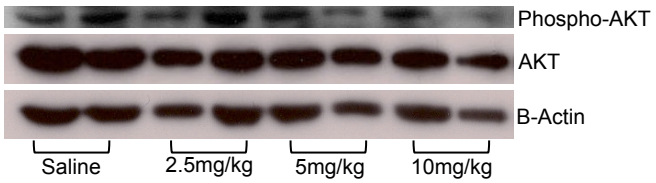

A
Signal strength (arbitrary units)
3
2.5
2
1.5
1
0.5
0
Saline
2.5 mg/kg
5 mg/kg
10 mg/kg
*
*
**
**
*
Total MAPK
Phospho-MAPK
Phospho : Total
Protein expression
B
Phospho-MAPK
MAPK
β-Actin
Saline
2.5mg/kg
5mg/kg
10mg/kg
C
Saline
Phospho-MAPK
20 µm
Anti-VEGFA
Phospho-MAPK
20 µm

FIGURE 6

A


1.4
1.2
1
0.8
0.6
0.4
0.2
0
Signal strength (arbitrary units)
Saline
2.5 mg/kg
5 mg/kg
10 mg/kg
Total AKT
Phospho-AKT
Phospho : Total
Protein expression

B


Phospho-AKT
AKT
B-Actin
Saline
2.5mg/kg
5mg/kg
10mg/kg

Supplemental Table I

A

Age (weeks)	Treatment received	Tumor mass (g, mean±SEM)
3	-	0.00466±0.000444
4	0.9% NaCl	0.0515±0.00414
4	5 mg/kg anti-VEGFA	0.0618±0.00936
5	0.9% NaCl	0.178±0.0564
5	5 mg/kg anti-VEGFA	0.0830±0.0163
6	0.9% NaCl	0.723±0.258
6	2.5 mg/kg anti-VEGFA	1.08±0.127
6	5 mg/kg anti-VEGFA	0.307±0.151
6	10 mg/kg anti-VEGFA	0.223±0.0485

B

Treatment received	Tumor mass (g, mean±SEM)
0.9% NaCl	1.39±0.238
2.5 mg/kg anti-VEGFA	1.08±0.123
5 mg/kg anti-VEGFA	0.322±0.0223
10 mg/kg anti-VEGFA	0.223±0.0485

C

Treatment received	Tumor mass (g, mean±SEM)
0.9% NaCl	1.39±0.238
2.5 mg/kg anti-VEGFA	1.09±0.127
5 mg/kg anti-VEGFA	0.322±0.0223
10 mg/kg anti-VEGFA	0.223±0.0485

General Discussion

Although ovarian GCTs generally have a good prognosis particularly in comparison to epithelial ovarian tumors, 80% of women with advanced-stage GCT die from recurrent disease (45), which can occur up to 40 years after initial diagnosis and treatment (46). Despite this, few studies have focused on investigating GCT disease and consequently, the pathogenesis of GCT remains unclear. In order to improve the prognosis for women with advanced-stage or recurrent disease, the first step would be to better understand its molecular pathogenesis by identifying oncogenic pathways and/or genetic alterations that lead to GCT development.

An important advance in the study of GCT pathogenesis came with the discovery of a somatic mutation in *FOXL2* 402C→G (C134W) in nearly all adult-type GCTs that is absent in other ovarian and SCSTs (6, 67, 69). Although FOXL2 is a transcription factor that is known to play an important role in normal granulosa cell development, the molecular implications of this mutation and its role in GCT pathogenesis have yet to be revealed (6, 67, 69).

One limitation of this study is the lack of *FOXL2* mutation in the *PCA* model of GCT. Why this seemingly pathognomonic mutation for adult-type GCT is not exhibited in the *PCA* GCT model is not clear. Initially, we wanted to validate *PCA* as a genuine model for GCT. A veterinary pathologist confirmed by histological analyses (based on tissue and cell morphologies and immunohistochemistry markers for GCT) that *PCA* mice did in fact develop GCTs. It was not possible however to further categorize GCTs as either adult or juvenile-type because the criteria established for the diagnosis of human GCTs did not apply to the mouse model. Despite the many similarities between the *PCA* mouse model and the advanced disease in women at biological and molecular levels (112), perhaps GCT in the *PCA* model remains a separate disease from the disease in humans and therefore does not express the

FOXL2 mutation. The next logical step would then be to create an animal model that exhibits the *FOXL2* mutation in granulosa cells and determine whether the mutation alone is sufficient for GCT development (6). If we are able to experimentally induce this mutation (alone or in combination with other mutations) and it leads to GCT development, then we would be in possession of a more genetically accurate animal model. We would also be one step closer to understanding the pathogenesis of GCT and ultimately identifying novel therapeutic targets.

Animal models can serve as powerful tools in which new pharmacological agents and targets can be tested. Although several mouse models of GCT have been generated, including inhibin-deficient mice or mice overexpresssing LH, few models have been able to replicate the disease in women at cellular and biological levels. For example, while the inhibin-deficient mouse model developed invasive SCSTs with 100% penetrance (64), women with GCTs have very high serum inhibin levels that are used as tumor markers for recurrent disease (6). As such, this represents an important biological difference between the mouse model and the human disease and puts into question the relevance of this particular model.

The novel *PCA* model presents an interesting alternative to the previously described mouse models. 100% of *PCA* mice developed bilateral GCTs perinatally that rapidly grew causing abdominal distension by 5 weeks of age. This high level of penetrance facilitated the acquisition of test subjects with the desired phenotype and the early onset of disease reduced the delay to obtain tissue samples. On the other hand, due to the high level of tumor aggressivity, by 8 weeks of age all animals receiving saline had died from tumor-related causes, which greatly limited the timeframe in which we could study the effects of treatment on tumor growth in comparison to control animals.

An important feature of GCT disease is tumor vascularization (11, 13, 14). VEGF, a key mediator of angiogenesis, is highly expressed in nearly all GCTs in endothelial and granulosa cells in primary and recurrent GCTs and is positively correlated with tumor microvessel density and the expression of VEGFR2 (13). Also, elevated serum levels of VEGF detected in patients with GCT were found to drop significantly after tumor removal (14). These results strongly suggest that VEGF contributes to the developed tumor vasculature in GCTs and that it could represent a valid therapeutic target for the treatment of GCT. Although numerous angiogenic inhibitors have been created to target either VEGF or VEGFR2 and have shown varying degrees of efficacy in the treatment of different tumor types, monoclonal anti-VEGFA antibodies have overall shown the most potential (4, 80, 82, 123, 126, 127). Bevacizumab is the most popular anti-VEGFA antibody that has been approved for the treatment of various cancers that is commonly administered by IV injection (145). For this project, anti-VEGFA antibody was administered by IP injection. This route of administration was chosen based on a handful of pharmacological studies that demonstrated effective absorption and determined effective doses of anti-VEGF antibodies after IP administration in mice (165, 166). Shah *et al* (165) studied the pharmacokinetics of bevacizumab following IP and IV administration using a murine model of ovarian cancer and found that bevacizumab was rapidly absorbed after IP injection with bioavailability reaching 92.8%. This group also demonstrated that both routes of administration significantly increased survival time (with no significant differences in survival between the two groups treated IV or IP)(165). Mordenti *et al* (166) evaluated the relationship between concentration and response after IP injection of a murine anti-VEGF mAb in tumor-bearing nude mice. Tumor suppression was achieved at doses equal to or greater than 2.5mg/kg and the average

trough plasma concentration was found to be 30ug/ml (166). Both of these studies served to provide the groundwork for future studies that could focus on the clinical outcomes of anti-VEGF antibody administration by IP injection.

A second reason for choosing to administer anti-VEGFA antibody by IP administration was based on the biological behavior of ovarian tumors (167). Although in human medicine the standard approach for the treatment of ovarian cancer is IV infusion of chemotherapy drugs repeatedly over 5-8 cycles, recent studies have investigated the potential of IP administration based on the fact that these tumors tend to confine themselves to the surface of the peritoneal cavity (167). IP infusion of a drug directly into the peritoneal cavity should theoretically increase the concentration of drug reaching the target tumor cells and therefore exert a more important effect. Jaaback *et al* (167) performed a review of randomized trials involving women who were being treated for newly-diagnosed primary epithelial ovarian cancer with IV alone or IV and IP chemotherapy following primary cytoreductive surgery. They discovered that IP combined with IV chemotherapy increased overall survival and progression-free survival in comparison to IV alone (167). Although not entirely conclusive, these results suggested that IP injection promotes a more efficient drug delivery to the target organ and consequently delays disease progression and improves survival. However, only future studies that are able to quantify the concentration of drug that is found within the target cells after IP administration relative to the administered dose and the effect on tumor growth would be able to provide more definitive answers.

This project aimed to investigate the potential of a monoclonal anti-VEGFA antibody for the treatment of GCT in the *PCA* preclinical model. This study's objectives were to

evaluate the effect of anti-VEGFA antibody on tumor growth, survival, cell proliferation and apoptosis, tumor microvessel density, and activation of VEGFR2 signaling pathway effectors.

Anti-VEGFA antibody significantly reduced tumor growth and improved survival in treated mice in comparison to controls. A likely explanation for the reduction in tumor growth came from analyses of GCT tumors in which significant decreases in cell proliferation and tumor microvessel density were found in treated tumors relative to controls. Given the well-established role of VEGF in endothelial and granulosa cell proliferation, these findings did not necessarily come as a surprise (82, 83, 87, 168-170). Preventing VEGF binding to its receptor would then logically prevent the activation of downstream signaling effectors involved in cell proliferation (such as MAPK). This was confirmed by an immunoblotting experiment in which significantly reduced MAPK activation was found. By immunohistochemistry, MAPK activation seemed to occur in both endothelial and granulosa cells. In addition, the reduction in microvessel density suggests that anti-VEGFA treatment prevented development of the tumor microvasculature thereby limiting the delivery of nutrients and oxygen to tumor cells, resulting in decreased cell proliferation.

Unexpectedly, anti-VEGFA treatment did not have a significant effect on apoptosis despite the fact that VEGF signaling is involved in cell survival (14, 79, 82, 83, 87). This was further confirmed by immunoblotting, where no significant reduction in AKT activation, a VEGFR2 signaling effector involved in cell survival, was found, relative to controls. It is possible that tumor cells acquired a resistance to apoptosis by any number of mechanisms (such as overexpression of anti-apoptotic molecules, downregulation/mutation of pro-apoptotic genes, etc.) (171) and that despite the absence of an important survival factor such as VEGFA, they still found the means to survive.

One limitation of the present study was the absence of phospho-VEGFR2 signal at two different phosphorylation sites in neither the control nor the treated group. These results would have served to confirm the inhibition of VEGF binding to its receptor represented as a reduction in VEGFR2 phosphorylation in treated tumors compared to controls. The failure to detect any signal might be on account of the short duration of receptor phosphorylation (i.e. perhaps VEGFA binding to VEGFR2 induces an immediate spike in receptor phosphorylation that tapers off after a few minutes), which limits the timeframe for detection by immunoblotting. Another possibility might be that the antibodies used were simply not sensitive enough to detect the activation levels of VEGFR2 in this tumor model.

Given the effectiveness of anti-VEGFA antibody at reducing tumor growth and extending survival in the *PCA* model, future experiments might look at combining anti-VEGFA antibody with chemotherapy in *PCA* mice in order to establish optimal treatment schedules. A hypothesis proposed by Jain *et al* (163) states that anti-VEGF therapy normalizes the tumor vasculature, resulting in improved oxygenation and delivery of cytotoxic agents. By combining the two therapies, both endothelial and granulosa tumor cells are targeted and destroyed, which should theoretically improve treatment efficacy. However, no studies to date have evaluated whether priming tumor blood vessels with anti-VEGFA antibody does in fact improve chemotherapy delivery to the tumor.

Although we still have a long way to go before establishing standardized treatment regimens for advanced or recurrent GCT disease, the present study has provided many reasons to be optimistic and serves as a starting point for future studies.

Conclusion

These experiments revealed that anti-VEGFA therapy is effective for the treatment of GCT in the *PCA* preclinical model. Administration of anti-VEGFA antibody significantly reduced tumor size and extended survival in treated animals relative to controls. The reduction in tumor weight was attributed to a reduction in cell proliferation and microvessel density via inhibition of the VEGFR2-MAPK signaling pathway in treated tumors in comparison to controls. Anti-VEGFA therapy did not have a significant effect on apoptosis. These findings represent important advances in the field of GCT therapy that could eventually apply to the development of standardized treatment protocols for recurrent or advanced disease in women.

Bibliography

1. American Cancer Society. What are the key statistics about ovarian cancer? 2012. http://www.cancer.org/Cancer/OvarianCancer/DetailedGuide/ovarian-cancer-key-statistics. Accessed June 11, 2012.
2. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100:57-70.
3. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646-74.
4. Ruegg C, Mutter N. Anti-angiogenic therapies in cancer: achievements and open questions. Bull Cancer. 2007;94:753-62.
5. Mullany LK, Richards JS. Minireview: animal models and mechanisms of ovarian cancer development. Endocrinology. 2012;153:1585-92.
6. Jamieson S, Fuller PJ. Molecular pathogenesis of granulosa cell tumors of the ovary. Endocr Rev. 2012;33:109-44.
7. Scully RE. Classification of human ovarian tumors. Environ Health Perspect. 1987;73:15-24.
8. Geetha P, Nair MK. Granulosa cell tumours of the ovary. Aust N Z J Obstet Gynaecol. 2010;50:216-20.
9. Thrall MM, Paley P, Pizer E, Garcia R, Goff BA. Patterns of spread and recurrence of sex cord-stromal tumors of the ovary. Gynecol Oncol. 2011.
10. Richards JS, Fan HY, Liu Z, Tsoi M, Lague MN, Boyer A, et al. Either Kras activation or Pten loss similarly enhance the dominant-stable CTNNB1-induced genetic program to promote granulosa cell tumor development in the ovary and testis. Oncogene. 2011.

11. Tao X, Sood AK, Deavers MT, Schmeler KM, Nick AM, Coleman RL, et al. Anti-angiogenesis therapy with bevacizumab for patients with ovarian granulosa cell tumors. Gynecol Oncol. 2009;114:431-6.
12. King LA, Okagaki T, Gallup DG, Twiggs LB, Messing MJ, Carson LF. Mitotic count, nuclear atypia, and immunohistochemical determination of Ki-67, c-myc, p21-ras, c-erbB2, and p53 expression in granulosa cell tumors of the ovary: mitotic count and Ki-67 are indicators of poor prognosis. Gynecol Oncol. 1996;61:227-32.
13. Farkkila A, Anttonen M, Pociuviene J, Leminen A, Butzow R, Heikinheimo M, et al. Vascular endothelial growth factor (VEGF) and its receptor VEGFR-2 are highly expressed in ovarian granulosa cell tumors. Eur J Endocrinol. 2011;164:115-22.
14. Farkkila A, Pihlajoki M, Tauriala H, Butzow R, Leminen A, Unkila-Kallio L, et al. Serum vascular endothelial growth factor A (VEGF) is elevated in patients with ovarian granulosa cell tumor (GCT), and VEGF inhibition by bevacizumab induces apoptosis in GCT in vitro. J Clin Endocrinol Metab. 2011;96:E1973-81.
15. Kesterson JP, Mhawech-Fauceglia P, Lele S. The use of bevacizumab in refractory ovarian granulosa-cell carcinoma with symptomatic relief of ascites: a case report. Gynecol Oncol. 2008;111:527-9.
16. Barrena Medel NI, Herzog TJ, Wright JD, Lewin SN. Neoadjuvant bevacizumab in a granulosa cell tumor of the ovary: a case report. Anticancer Res. 2010;30:4767-8.
17. Schumer ST, Cannistra SA. Granulosa cell tumor of the ovary. J Clin Oncol. 2003;21:1180-9.

18. Koukourakis GV, Kouloulias VE, Koukourakis MJ, Zacharias GA, Papadimitriou C, Mystakidou K, et al. Granulosa cell tumor of the ovary: tumor review. Integr Cancer Ther. 2008;7:204-15.

19. Park JY, Jin KL, Kim DY, Kim JH, Kim YM, Kim KR, et al. Surgical staging and adjuvant chemotherapy in the management of patients with adult granulosa cell tumors of the ovary. Gynecol Oncol. 2012;125:80-6.

20. Fuller PJ, Chu S, Fikret S, Burger HG. Molecular pathogenesis of granulosa cell tumours. Mol Cell Endocrinol. 2002;191:89-96.

21. Sun HD, Lin H, Jao MS, Wang KL, Liou WS, Hung YC, et al. A long-term follow-up study of 176 cases with adult-type ovarian granulosa cell tumors. Gynecol Oncol. 2012;124:244-9.

22. Ayhan A, Salman MC, Velipasaoglu M, Sakinci M, Yuce K. Prognostic factors in adult granulosa cell tumors of the ovary: a retrospective analysis of 80 cases. J Gynecol Oncol. 2009;20:158-63.

23. JL Benedet SP, Hextan YS Ngan, Neville F Hacker. Staging Classifications and Clinical Practice Guidelines for Gynaecologic Cancers. International Journal of Gynecology and Obstetrics. 2000;70:207-312.

24. Ko SF, Wan YL, Ng SH, Lee TY, Lin JW, Chen WJ, et al. Adult ovarian granulosa cell tumors: spectrum of sonographic and CT findings with pathologic correlation. AJR Am J Roentgenol. 1999;172:1227-33.

25. Mohr U. International classification of rodent tumors; the mouse. Berlin: Springer-Verlag; 2001.

26. Lappohn RE, Burger HG, Bouma J, Bangah M, Krans M. Inhibin as a marker for granulosa cell tumor. Acta Obstet Gynecol Scand Suppl. 1992;155:61-5.

27. Fuller PJ, Chu S. Signalling pathways in the molecular pathogenesis of ovarian granulosa cell tumours. Trends Endocrinol Metab. 2004;15:122-8.

28. Jamieson S, Fuller PJ. Management of granulosa cell tumour of the ovary. Curr Opin Oncol. 2008;20:560-4.

29. Lappohn RE, Burger HG, Bouma J, Bangah M, Krans M, de Bruijn HW. Inhibin as a marker for granulosa-cell tumors. N Engl J Med. 1989;321:790-3.

30. Rey RA, Lhomme C, Marcillac I, Lahlou N, Duvillard P, Josso N, et al. Antimullerian hormone as a serum marker of granulosa cell tumorsof the ovary: comparative study with serum alpha-inhibin and estradiol. Am J Obstet Gynecol. 1996;174:958-65.

31. Lane AH, Lee MM, Fuller AF, Jr., Kehas DJ, Donahoe PK, MacLaughlin DT. Diagnostic utility of Mullerian inhibiting substance determination in patients with primary and recurrent granulosa cell tumors. Gynecol Oncol. 1999;73:51-5.

32. Geerts I, Vergote I, Neven P, Billen J. The role of inhibins B and antimullerian hormone for diagnosis and follow-up of granulosa cell tumors. Int J Gynecol Cancer. 2009;19:847-55.

33. Savage P, Constenla D, Fisher C, Shepherd JH, Barton DP, Blake P, et al. Granulosa cell tumours of the ovary: demographics, survival and the management of advanced disease. Clin Oncol (R Coll Radiol). 1998;10:242-5.

34. Hauspy J, Beiner ME, Harley I, Rosen B, Murphy J, Chapman W, et al. Role of adjuvant radiotherapy in granulosa cell tumors of the ovary. Int J Radiat Oncol Biol Phys. 2011;79:770-4.

35. Wolf JK, Mullen J, Eifel PJ, Burke TW, Levenback C, Gershenson DM. Radiation treatment of advanced or recurrent granulosa cell tumor of the ovary. Gynecol Oncol. 1999;73:35-41.

36. Malmstrom H, Hogberg T, Risberg B, Simonsen E. Granulosa cell tumors of the ovary: prognostic factors and outcome. Gynecol Oncol. 1994;52:50-5.

37. Evans AT, 3rd, Gaffey TA, Malkasian GD, Jr., Annegers JF. Clinicopathologic review of 118 granulosa and 82 theca cell tumors. Obstet Gynecol. 1980;55:231-8.

38. Ohel G, Kaneti H, Schenker JG. Granulosa cell tumors in Israel: a study of 172 cases. Gynecol Oncol. 1983;15:278-86.

39. Schwartz PE, MacLusky N, Sakamoto H, Eisenfeld A. Steroid-receptor proteins in nonepithelial malignancies of the ovary. Gynecol Oncol. 1983;15:305-15.

40. Malik ST, Slevin ML. Medroxyprogesterone acetate (MPA) in advanced granulosa cell tumours of the ovary--a new therapeutic approach? Br J Cancer. 1991;63:410-1.

41. Fishman A, Kudelka AP, Tresukosol D, Edwards CL, Freedman RS, Kaplan AL, et al. Leuprolide acetate for treating refractory or persistent ovarian granulosa cell tumor. J Reprod Med. 1996;41:393-6.

42. Hardy RD, Bell JG, Nicely CJ, Reid GC. Hormonal treatment of a recurrent granulosa cell tumor of the ovary: case report and review of the literature. Gynecol Oncol. 2005;96:865-9.

43. Kauppila A, Bangah M, Burger H, Martikainen H. GnRH agonist analog therapy in advanced/recurrent granulosa cell tumors: further evidence of a role of inhibin in monitoring response to treatment. Gynecol Endocrinol. 1992;6:271-4.

44. Maxwell GL, Soisson AP, Miles P. Failure of gonadotropin releasing hormone therapy in patients with metastatic ovarian sex cord stromal tumors. Oncology. 1994;51:356-9.
45. Amsterdam A, Selvaraj N. Control of differentiation, transformation, and apoptosis in granulosa cells by oncogenes, oncoviruses, and tumor suppressor genes. Endocr Rev. 1997;18:435-61.
46. East N, Alobaid A, Goffin F, Ouallouche K, Gauthier P. Granulosa cell tumour: a recurrence 40 years after initial diagnosis. J Obstet Gynaecol Can. 2005;27:363-4.
47. Zhang M, Cheung MK, Shin JY, Kapp DS, Husain A, Teng NN, et al. Prognostic factors responsible for survival in sex cord stromal tumors of the ovary--an analysis of 376 women. Gynecol Oncol. 2007;104:396-400.
48. Brown J DM, Nick AM, Milojevic L, Gershenson DM, AKS. Vascular endothelial growth factor overexpression and angiogenesis are prevalent and predict clinical outcome in sex cord-stromal ovarian tumors. Gynecologic Oncology. 2009;112.
49. Mayr D, Kaltz-Wittmer C, Arbogast S, Amann G, Aust DE, Diebold J. Characteristic pattern of genetic aberrations in ovarian granulosa cell tumors. Mod Pathol. 2002;15:951-7.
50. Van den Berghe I, Dal Cin P, De Groef K, Michielssen P, Van den Berghe H. Monosomy 22 and trisomy 14 may be early events in the tumorigenesis of adult granulosa cell tumor. Cancer Genet Cytogenet. 1999;112:46-8.
51. Lin YS, Eng HL, Jan YJ, Lee HS, Ho WL, Liou CP, et al. Molecular cytogenetics of ovarian granulosa cell tumors by comparative genomic hybridization. Gynecol Oncol. 2005;97:68-73.

52. Boerboom D, Paquet M, Hsieh M, Liu J, Jamin SP, Behringer RR, et al. Misregulated Wnt/beta-catenin signaling leads to ovarian granulosa cell tumor development. Cancer Res. 2005;65:9206-15.

53. Boerboom D, White LD, Dalle S, Courty J, Richards JS. Dominant-stable beta-catenin expression causes cell fate alterations and Wnt signaling antagonist expression in a murine granulosa cell tumor model. Cancer Res. 2006;66:1964-73.

54. Jamieson S, Alexiadis M, Fuller PJ. Expression status and mutational analysis of the ras and B-raf genes in ovarian granulosa cell and epithelial tumors. Gynecol Oncol. 2004;95:603-9.

55. Coppes MJ, Ye Y, Rackley R, Zhao XL, Liefers GJ, Casey G, et al. Analysis of WT1 in granulosa cell and other sex cord-stromal tumors. Cancer Res. 1993;53:2712-4.

56. Liu FS, Ho ES, Lai CR, Chen JT, Shih RT, Yang CH, et al. Overexpression of p53 is not a feature of ovarian granulosa cell tumors. Gynecol Oncol. 1996;61:50-3.

57. Kappes S, Milde-Langosch K, Kressin P, Passlack B, Dockhorn-Dworniczak B, Rohlke P, et al. p53 mutations in ovarian tumors, detected by temperature-gradient gel electrophoresis, direct sequencing and immunohistochemistry. Int J Cancer. 1995;64:52-9.

58. Hunzicker-Dunn M, Maizels ET. FSH signaling pathways in immature granulosa cells that regulate target gene expression: branching out from protein kinase A. Cell Signal. 2006;18:1351-9.

59. Bittinger S, Alexiadis M, Fuller PJ. Expression status and mutational analysis of the PTEN and P13K subunit genes in ovarian granulosa cell tumors. Int J Gynecol Cancer. 2009;19:339-42.

60. Chu S, Mamers P, Burger HG, Fuller PJ. Estrogen receptor isoform gene expression in ovarian stromal and epithelial tumors. J Clin Endocrinol Metab. 2000;85:1200-5.

61. Anttonen M, Unkila-Kallio L, Leminen A, Butzow R, Heikinheimo M. High GATA-4 expression associates with aggressive behavior, whereas low anti-Mullerian hormone expression associates with growth potential of ovarian granulosa cell tumors. J Clin Endocrinol Metab. 2005;90:6529-35.

62. Kyronlahti A, Ramo M, Tamminen M, Unkila-Kallio L, Butzow R, Leminen A, et al. GATA-4 regulates Bcl-2 expression in ovarian granulosa cell tumors. Endocrinology. 2008;149:5635-42.

63. Fan X, Gabbi C, Kim HJ, Cheng G, Andersson LC, Warner M, et al. Gonadotropin-positive pituitary tumors accompanied by ovarian tumors in aging female ERbeta-/- mice. Proc Natl Acad Sci U S A. 2010;107:6453-8.

64. Matzuk MM, Finegold MJ, Su JG, Hsueh AJ, Bradley A. Alpha-inhibin is a tumour-suppressor gene with gonadal specificity in mice. Nature. 1992;360:313-9.

65. Matzuk MM, Kumar TR, Shou W, Coerver KA, Lau AL, Behringer RR, et al. Transgenic models to study the roles of inhibins and activins in reproduction, oncogenesis, and development. Recent Prog Horm Res. 1996;51:123-54; discussion 55-7.

66. Croce CM. Oncogenes and cancer. N Engl J Med. 2008;358:502-11.

67. Shah SP, Kobel M, Senz J, Morin RD, Clarke BA, Wiegand KC, et al. Mutation of FOXL2 in granulosa-cell tumors of the ovary. N Engl J Med. 2009;360:2719-29.

68. Kalfa N, Philibert P, Patte C, Ecochard A, Duvillard P, Baldet P, et al. Extinction of FOXL2 expression in aggressive ovarian granulosa cell tumors in children. Fertil Steril. 2007;87:896-901.

69. Kobel M, Gilks CB, Huntsman DG. Adult-type granulosa cell tumors and FOXL2 mutation. Cancer Res. 2009;69:9160-2.

70. Fleming NI, Knower KC, Lazarus KA, Fuller PJ, Simpson ER, Clyne CD. Aromatase is a direct target of FOXL2: C134W in granulosa cell tumors via a single highly conserved binding site in the ovarian specific promoter. PLoS One. 2010;5:e14389.

71. Schmidt D, Ovitt CE, Anlag K, Fehsenfeld S, Gredsted L, Treier AC, et al. The murine winged-helix transcription factor Foxl2 is required for granulosa cell differentiation and ovary maintenance. Development. 2004;131:933-42.

72. Kim JH, Yoon S, Park M, Park HO, Ko JJ, Lee K, et al. Differential apoptotic activities of wild-type FOXL2 and the adult-type granulosa cell tumor-associated mutant FOXL2 (C134W). Oncogene. 2011;30:1653-63.

73. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971;285:1182-6.

74. Bamias A, Pignata S, Pujade-Lauraine E. Angiogenesis: A promising therapeutic target for ovarian cancer. Crit Rev Oncol Hematol. 2012.

75. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell. 1996;86:353-64.

76. Schmitt J, Matei D. Targeting angiogenesis in ovarian cancer. Cancer Treat Rev. 2012;38:272-83.

77. Nagy JA, Chang SH, Shih SC, Dvorak AM, Dvorak HF. Heterogeneity of the tumor vasculature. Semin Thromb Hemost. 2010;36:321-31.

78. Baluk P, Hashizume H, McDonald DM. Cellular abnormalities of blood vessels as targets in cancer. Curr Opin Genet Dev. 2005;15:102-11.

79. Spratlin J. Ramucirumab (IMC-1121B): Monoclonal antibody inhibition of vascular endothelial growth factor receptor-2. Curr Oncol Rep. 2011;13:97-102.

80. Sato S, Itamochi H. Bevacizumab and ovarian cancer. Curr Opin Obstet Gynecol. 2012;24:8-13.

81. Martin L, Schilder R. Novel approaches in advancing the treatment of epithelial ovarian cancer: the role of angiogenesis inhibition. J Clin Oncol. 2007;25:2894-901.

82. Ho QT, Kuo CJ. Vascular endothelial growth factor: biology and therapeutic applications. Int J Biochem Cell Biol. 2007;39:1349-57.

83. Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003;9:669-76.

84. Ferrara N, Kerbel RS. Angiogenesis as a therapeutic target. Nature. 2005;438:967-74.

85. Burger RA. Overview of anti-angiogenic agents in development for ovarian cancer. Gynecol Oncol. 2011;121:230-8.

86. Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun. 1989;161:851-8.

87. Greenberg JI, Cheresh DA. VEGF as an inhibitor of tumor vessel maturation: implications for cancer therapy. Expert Opin Biol Ther. 2009;9:1347-56.

88. Takahashi S. Vascular endothelial growth factor (VEGF), VEGF receptors and their inhibitors for antiangiogenic tumor therapy. Biol Pharm Bull. 2011;34:1785-8.

89. Park JE, Chen HH, Winer J, Houck KA, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J Biol Chem. 1994;269:25646-54.

90. Bellati F, Napoletano C, Gasparri ML, Ruscito I, Marchetti C, Pignata S, et al. Current knowledge and open issues regarding Bevacizumab in gynaecological neoplasms. Crit Rev Oncol Hematol. 2011.

91. Chen H, Ye D, Xie X, Chen B, Lu W. VEGF, VEGFRs expressions and activated STATs in ovarian epithelial carcinoma. Gynecol Oncol. 2004;94:630-5.

92. Schmidt M, Kammerer U, Segerer S, Cramer A, Kohrenhagen N, Dietl J, et al. Glucose metabolism and angiogenesis in granulosa cell tumors of the ovary: activation of Akt, expression of M2PK, TKTL1 and VEGF. Eur J Obstet Gynecol Reprod Biol. 2008;139:72-8.

93. Matzuk MM, Finegold MJ, Mather JP, Krummen L, Lu H, Bradley A. Development of cancer cachexia-like syndrome and adrenal tumors in inhibin-deficient mice. Proc Natl Acad Sci U S A. 1994;91:8817-21.

94. Risma KA, Clay CM, Nett TM, Wagner T, Yun J, Nilson JH. Targeted overexpression of luteinizing hormone in transgenic mice leads to infertility, polycystic ovaries, and ovarian tumors. Proc Natl Acad Sci U S A. 1995;92:1322-6.

95. Kananen K, Markkula M, Rainio E, Su JG, Hsueh AJ, Huhtaniemi IT. Gonadal tumorigenesis in transgenic mice bearing the mouse inhibin alpha-subunit promoter/simian virus T-antigen fusion gene: characterization of ovarian tumors and

establishment of gonadotropin-responsive granulosa cell lines. Mol Endocrinol. 1995;9:616-27.

96. Rahman NA, Kananen Rilianawati K, Paukku T, Mikola M, Markkula M, Hamalainen T, et al. Transgenic mouse models for gonadal tumorigenesis. Mol Cell Endocrinol. 1998;145:167-74.

97. Rahman NA, Huhtaniemi IT. Ovarian tumorigenesis in mice transgenic for murine inhibin alpha subunit promoter-driven Simian Virus 40 T-antigen: ontogeny, functional characteristics, and endocrine effects. Biol Reprod. 2001;64:1122-30.

98. Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, et al. Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci U S A. 1998;95:15677-82.

99. Pangas SA, Li X, Umans L, Zwijsen A, Huylebroeck D, Gutierrez C, et al. Conditional deletion of Smad1 and Smad5 in somatic cells of male and female gonads leads to metastatic tumor development in mice. Mol Cell Biol. 2008;28:248-57.

100. Middlebrook BS, Eldin K, Li X, Shivasankaran S, Pangas SA. Smad1-Smad5 ovarian conditional knockout mice develop a disease profile similar to the juvenile form of human granulosa cell tumors. Endocrinology. 2009;150:5208-17.

101. Edson MA, Nalam RL, Clementi C, Franco HL, Demayo FJ, Lyons KM, et al. Granulosa cell-expressed BMPR1A and BMPR1B have unique functions in regulating fertility but act redundantly to suppress ovarian tumor development. Mol Endocrinol. 2010;24:1251-66.

102. Lague MN, Paquet M, Fan HY, Kaartinen MJ, Chu S, Jamin SP, et al. Synergistic effects of Pten loss and WNT/CTNNB1 signaling pathway activation in

ovarian granulosa cell tumor development and progression. Carcinogenesis. 2008;29:2062-72.

103. Vainio S, Heikkila M, Kispert A, Chin N, McMahon AP. Female development in mammals is regulated by Wnt-4 signalling. Nature. 1999;397:405-9.

104. Jeays-Ward K, Hoyle C, Brennan J, Dandonneau M, Alldus G, Capel B, et al. Endothelial and steroidogenic cell migration are regulated by WNT4 in the developing mammalian gonad. Development. 2003;130:3663-70.

105. Lustig B, Behrens J. The Wnt signaling pathway and its role in tumor development. J Cancer Res Clin Oncol. 2003;129:199-221.

106. Giles RH, van Es JH, Clevers H. Caught up in a Wnt storm: Wnt signaling in cancer. Biochim Biophys Acta. 2003;1653:1-24.

107. Dillon RL, White DE, Muller WJ. The phosphatidyl inositol 3-kinase signaling network: implications for human breast cancer. Oncogene. 2007;26:1338-45.

108. Chow LM, Baker SJ. PTEN function in normal and neoplastic growth. Cancer Lett. 2006;241:184-96.

109. Desbois-Mouthon C, Cadoret A, Blivet-Van Eggelpoel MJ, Bertrand F, Cherqui G, Perret C, et al. Insulin and IGF-1 stimulate the beta-catenin pathway through two signalling cascades involving GSK-3beta inhibition and Ras activation. Oncogene. 2001;20:252-9.

110. Larue L, Bellacosa A. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. Oncogene. 2005;24:7443-54.

111. Katoh M. Cross-talk of WNT and FGF signaling pathways at GSK3beta to regulate beta-catenin and SNAIL signaling cascades. Cancer Biol Ther. 2006;5:1059-64.

112. Nadeau ME, Kaartinen MJ, Lague MN, Paquet M, Huneault LM, Boerboom D. A mouse surgical model for metastatic ovarian granulosa cell tumor. Comp Med. 2009;59:553-6.

113. Fan HY, Richards JS. Minireview: physiological and pathological actions of RAS in the ovary. Mol Endocrinol. 2010;24:286-98.

114. Park JY, Su YQ, Ariga M, Law E, Jin SL, Conti M. EGF-like growth factors as mediators of LH action in the ovulatory follicle. Science. 2004;303:682-4.

115. Ashkenazi H, Cao X, Motola S, Popliker M, Conti M, Tsafriri A. Epidermal growth factor family members: endogenous mediators of the ovulatory response. Endocrinology. 2005;146:77-84.

116. Hsieh M, Conti M. G-protein-coupled receptor signaling and the EGF network in endocrine systems. Trends Endocrinol Metab. 2005;16:320-6.

117. Fan HY, Liu Z, Shimada M, Sterneck E, Johnson PF, Hedrick SM, et al. MAPK3/1 (ERK1/2) in ovarian granulosa cells are essential for female fertility. Science. 2009;324:938-41.

118. Fan HY, Liu Z, Cahill N, Richards JS. Targeted disruption of Pten in ovarian granulosa cells enhances ovulation and extends the life span of luteal cells. Mol Endocrinol. 2008;22:2128-40.

119. Fan HY, Shimada M, Liu Z, Cahill N, Noma N, Wu Y, et al. Selective expression of KrasG12D in granulosa cells of the mouse ovary causes defects in follicle development and ovulation. Development. 2008;135:2127-37.

120. Ferrara N. Pathways mediating VEGF-independent tumor angiogenesis. Cytokine Growth Factor Rev. 2010;21:21-6.

121. Gasparini G, Longo R, Fanelli M, Teicher BA. Combination of antiangiogenic therapy with other anticancer therapies: results, challenges, and open questions. J Clin Oncol. 2005;23:1295-311.

122. Eskens FA. Angiogenesis inhibitors in clinical development; where are we now and where are we going? Br J Cancer. 2004;90:1-7.

123. Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, et al. VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci U S A. 2002;99:11393-8.

124. Ruckman J, Green LS, Beeson J, Waugh S, Gillette WL, Henninger DD, et al. 2'-Fluoropyrimidine RNA-based aptamers to the 165-amino acid form of vascular endothelial growth factor (VEGF165). Inhibition of receptor binding and VEGF-induced vascular permeability through interactions requiring the exon 7-encoded domain. J Biol Chem. 1998;273:20556-67.

125. Arora A, Scholar EM. Role of tyrosine kinase inhibitors in cancer therapy. J Pharmacol Exp Ther. 2005;315:971-9.

126. Kim KJ, Li B, Houck K, Winer J, Ferrara N. The vascular endothelial growth factor proteins: identification of biologically relevant regions by neutralizing monoclonal antibodies. Growth Factors. 1992;7:53-64.

127. Kim KJ, Li B, Winer J, Armanini M, Gillett N, Phillips HS, et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. Nature. 1993;362:841-4.

128. Byrne AT, Ross L, Holash J, Nakanishi M, Hu L, Hofmann JI, et al. Vascular endothelial growth factor-trap decreases tumor burden, inhibits ascites, and causes dramatic vascular remodeling in an ovarian cancer model. Clin Cancer Res. 2003;9:5721-8.

129. Huang J, Frischer JS, Serur A, Kadenhe A, Yokoi A, McCrudden KW, et al. Regression of established tumors and metastases by potent vascular endothelial growth factor blockade. Proc Natl Acad Sci U S A. 2003;100:7785-90.

130. Fukasawa M, Korc M. Vascular endothelial growth factor-trap suppresses tumorigenicity of multiple pancreatic cancer cell lines. Clin Cancer Res. 2004;10:3327-32.

131. Chu QS. Aflibercept (AVE0005): an alternative strategy for inhibiting tumour angiogenesis by vascular endothelial growth factors. Expert Opin Biol Ther. 2009;9:263-71.

132. Veritti D, Sarao V, Lanzetta P. Neovascular age-related macular degeneration. Ophthalmologica. 2012;227 Suppl 1:11-20.

133. Mendel DB, Laird AD, Xin X, Louie SG, Christensen JG, Li G, et al. In vivo antitumor activity of SU11248, a novel tyrosine kinase inhibitor targeting vascular endothelial growth factor and platelet-derived growth factor receptors: determination of a pharmacokinetic/pharmacodynamic relationship. Clin Cancer Res. 2003;9:327-37.

134. Motzer RJ, Hutson TE, Tomczak P, Michaelson MD, Bukowski RM, Rixe O, et al. Sunitinib versus interferon alfa in metastatic renal-cell carcinoma. N Engl J Med. 2007;356:115-24.

135. Wilhelm SM, Carter C, Tang L, Wilkie D, McNabola A, Rong H, et al. BAY 43-9006 exhibits broad spectrum oral antitumor activity and targets the RAF/MEK/ERK pathway and receptor tyrosine kinases involved in tumor progression and angiogenesis. Cancer Res. 2004;64:7099-109.

136. Escudier B, Eisen T, Stadler WM, Szczylik C, Oudard S, Siebels M, et al. Sorafenib in advanced clear-cell renal-cell carcinoma. N Engl J Med. 2007;356:125-34.

137. Matei D, Sill MW, Lankes HA, DeGeest K, Bristow RE, Mutch D, et al. Activity of sorafenib in recurrent ovarian cancer and primary peritoneal carcinomatosis: a gynecologic oncology group trial. J Clin Oncol. 2011;29:69-75.

138. Biagi JJ, Oza AM, Chalchal HI, Grimshaw R, Ellard SL, Lee U, et al. A phase II study of sunitinib in patients with recurrent epithelial ovarian and primary peritoneal carcinoma: an NCIC Clinical Trials Group Study. Ann Oncol. 2011;22:335-40.

139. Hurwitz H, Fehrenbacher L, Novotny W, Cartwright T, Hainsworth J, Heim W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. N Engl J Med. 2004;350:2335-42.

140. Sandler A, Gray R, Perry MC, Brahmer J, Schiller JH, Dowlati A, et al. Paclitaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med. 2006;355:2542-50.

141. Korsisaari N, Kasman IM, Forrest WF, Pal N, Bai W, Fuh G, et al. Inhibition of VEGF-A prevents the angiogenic switch and results in increased survival of Apc+/min mice. Proc Natl Acad Sci U S A. 2007;104:10625-30.

142. Avastin® (bevacizumab) Treatment for mCRC, NSCLC, GBM and mRCC In. 2012. URL: www.avastin.com/patient/index.html. Consulted May 30, 2012.

143. Shih T, Lindley C. Bevacizumab: an angiogenesis inhibitor for the treatment of solid malignancies. Clin Ther. 2006;28:1779-802.

144. Bergsland E, Dickler MN. Maximizing the potential of bevacizumab in cancer treatment. Oncologist. 2004;9 Suppl 1:36-42.

145. http://www.gene.com/gene/products/information/pdf/avastin-prescribing.pdf. Consulted January 10 2013.

146. Nagane M, Nishikawa R, Narita Y, Kobayashi H, Takano S, Shinoura N, et al. Phase II study of single-agent bevacizumab in Japanese patients with recurrent malignant glioma. Jpn J Clin Oncol. 2012;42:887-95.

147. Burger RA, Sill MW, Monk BJ, Greer BE, Sorosky JI. Phase II trial of bevacizumab in persistent or recurrent epithelial ovarian cancer or primary peritoneal cancer: a Gynecologic Oncology Group Study. J Clin Oncol. 2007;25:5165-71.

148. Monk BJ, Han E, Josephs-Cowan CA, Pugmire G, Burger RA. Salvage bevacizumab (rhuMAB VEGF)-based therapy after multiple prior cytotoxic regimens in advanced refractory epithelial ovarian cancer. Gynecol Oncol. 2006;102:140-4.

149. Cannistra SA, Matulonis UA, Penson RT, Hambleton J, Dupont J, Mackey H, et al. Phase II study of bevacizumab in patients with platinum-resistant ovarian cancer or peritoneal serous cancer. J Clin Oncol. 2007;25:5180-6.

150. Chura JC, Van Iseghem K, Downs LS, Jr., Carson LF, Judson PL. Bevacizumab plus cyclophosphamide in heavily pretreated patients with recurrent ovarian cancer. Gynecol Oncol. 2007;107:326-30.

151. Garcia AA, Hirte H, Fleming G, Yang D, Tsao-Wei DD, Roman L, et al. Phase II clinical trial of bevacizumab and low-dose metronomic oral cyclophosphamide in recurrent ovarian cancer: a trial of the California, Chicago, and Princess Margaret Hospital phase II consortia. J Clin Oncol. 2008;26:76-82.

152. McGonigle KF, Muntz HG, Vuky J, Paley PJ, Veljovich DS, Greer BE, et al. Combined weekly topotecan and biweekly bevacizumab in women with platinum-resistant ovarian, peritoneal, or fallopian tube cancer: results of a phase 2 study. Cancer. 2011;117:3731-40.

153. Burger RA, Brady MF, Bookman MA, Fleming GF, Monk BJ, Huang H, et al. Incorporation of bevacizumab in the primary treatment of ovarian cancer. N Engl J Med. 2011;365:2473-83.

154. Perren TJ, Swart AM, Pfisterer J, Ledermann JA, Pujade-Lauraine E, Kristensen G, et al. A phase 3 trial of bevacizumab in ovarian cancer. N Engl J Med. 2011;365:2484-96.

155. Ebos JM, Lee CR, Cruz-Munoz W, Bjarnason GA, Christensen JG, Kerbel RS. Accelerated metastasis after short-term treatment with a potent inhibitor of tumor angiogenesis. Cancer Cell. 2009;15:232-9.

156. Alitalo K, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. Nature. 2005;438:946-53.

157. Yu L, Liang XH, Ferrara N. Comparing protein VEGF inhibitors: In vitro biological studies. Biochem Biophys Res Commun. 2011;408:276-81.

158. Glade Bender J, Cooney EM, Kandel JJ, Yamashiro DJ. Vascular remodeling and clinical resistance to antiangiogenic cancer therapy. Drug Resist Updat. 2004;7:289-300.

159. Paez-Ribes M, Allen E, Hudock J, Takeda T, Okuyama H, Vinals F, et al. Antiangiogenic therapy elicits malignant progression of tumors to increased local invasion and distant metastasis. Cancer Cell. 2009;15:220-31.

160. Ranpura V, Hapani S, Wu S. Treatment-related mortality with bevacizumab in cancer patients: a meta-analysis. JAMA. 2011;305:487-94.

161. Browder T, Butterfield CE, Kraling BM, Shi B, Marshall B, O'Reilly MS, et al. Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer. Cancer Res. 2000;60:1878-86.

162. Klement G, Baruchel S, Rak J, Man S, Clark K, Hicklin DJ, et al. Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. J Clin Invest. 2000;105:R15-24.

163. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. Science. 2005;307:58-62.

164. Goel S, Duda DG, Xu L, Munn LL, Boucher Y, Fukumura D, et al. Normalization of the vasculature for treatment of cancer and other diseases. Physiol Rev. 2011;91:1071-121.

165. Shah DK, Veith J, Bernacki RJ, Balthasar JP. Evaluation of combined bevacizumab and intraperitoneal carboplatin or paclitaxel therapy in a mouse model of ovarian cancer. Cancer Chemother Pharmacol. 2011;68:951-8.

166. Mordenti J, Thomsen K, Licko V, Chen H, Meng YG, Ferrara N. Efficacy and concentration-response of murine anti-VEGF monoclonal antibody in tumor-bearing mice and extrapolation to humans. Toxicol Pathol. 1999;27:14-21.

167. Jaaback K, Johnson N, Lawrie TA. Intraperitoneal chemotherapy for the initial management of primary epithelial ovarian cancer. Cochrane Database Syst Rev. 2011:CD005340.

168. Irusta G, Abramovich D, Parborell F, Tesone M. Direct survival role of vascular endothelial growth factor (VEGF) on rat ovarian follicular cells. Mol Cell Endocrinol. 2010;325:93-100.

169. Kosaka N, Sudo N, Miyamoto A, Shimizu T. Vascular endothelial growth factor (VEGF) suppresses ovarian granulosa cell apoptosis in vitro. Biochem Biophys Res Commun. 2007;363:733-7.

170. Greenaway J, Connor K, Pedersen HG, Coomber BL, LaMarre J, Petrik J. Vascular endothelial growth factor and its receptor, Flk-1/KDR, are cytoprotective in the extravascular compartment of the ovarian follicle. Endocrinology. 2004;145:2896-905.

171. Igney FH, Krammer PH. Death and anti-death: tumour resistance to apoptosis. Nat Rev Cancer. 2002;2:277-88.