# Molecular Pathogenesis of Granulosa Cell Tumors of the Ovary

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Granulosa cell tumors of the ovary (GCT) comprise a distinct subset of ovarian cancers that account for approximately 5% of all ovarian malignancies. They are thought to arise from normal proliferating granulosa cells of the late preovulatory follicle and exhibit many morphological and biochemical features of these cells. GCT are distinct from other ovarian carcinomas in their hormonal activity; their ability to secrete estrogen, inhibin, and Müllerian inhibiting substance accounts for some of the clinical manifestations of the disease and also provides useful tumor markers for disease surveillance. Although considered to be of low malignant potential, GCT are commonly associated with slow, indolent disease progression, and frequent yet long delays to tumor recurrence are characteristic of this disease. Unlike the more intensely investigated epithelial ovarian tumors, relatively little is known about the molecular and genetic changes that give rise to GCT. To date, many investigations have centered around pathways known to be involved in normal granulosa cell proliferation, including those activated by FSH receptor stimulation. Most recently, the finding that approximately 97% of adult GCT harbor a somatic missense mutation in the *FOXL2* gene (c.402C→G; p.C134W) represents an exciting advancement in the field of GCT research. The high frequency with which the mutation occurs in adult GCT, along with its absence from juvenile GCT and other human malignancies is suggestive of an oncogenic or gain-of-function mutation and, indeed, that the mutation is pathognomonic for adult GCT. In this review, we explore the implications of this finding and the most recent work characterizing molecular pathways of potential pathogenetic significance in GCT. **(*Endocrine Reviews* 33: 109–144, 2012)**



## I. Introduction

Granulosa cell tumors of the ovary (GCT) represent a specific subset of malignant ovarian tumors and can be further categorized into two distinct subtypes, the ju-

Abbreviations: ActR, Activin receptor; AP-1, activator protein-1; ArKO, aromatase knockout; BMP, bone morphogenetic protein; CREB, cAMP response element-binding protein; CSF1R, colony stimulating factor 1 receptor; CTP, carboxyl-terminal peptide; EGF, epidermal growth factor; EGFR, EGF receptor; $\alpha$ERKO, ER$\alpha$ knockout; $\beta$ERKO, ER$\beta$ knockout; FDA, Food and Drug Administration; FLT3, fms-like tyrosine kinase 3; FOXL2, forkhead box L2; GCT, granulosa cell tumor of the ovary; GR, glucocorticoid receptor; I$\kappa$B$\alpha$, inhibitor of $\kappa$B$\alpha$; IGFBP, IGF-binding protein; MEN, multiple endocrine neoplasia; MIS, Müllerian inhibiting substance; NF$\kappa$B, nuclear factor $\kappa$B; NR, nuclear receptor; PAPP-A, pregnancy-associated plasma protein-A; PI3K, phosphatidylinositol 3-kinase; PJS, Peutz-Jeghers syndrome; PKA, protein kinase A; PPAR$\gamma$, peroxisome proliferator-activated receptor $\gamma$; rhTRAIL, recombinant human TRAIL; RTK, receptor tyrosine kinase; SERM, selective estrogen receptor modulator; SF-1, steroidogenic factor-1; SMAD, Sma and Mad-related protein; StAR, steroidogenic acute regulatory protein; SV40 TAg, simian virus 40 T-antigen; TKI, tyrosine kinase inhibitor; TLR4, toll-like receptor 4; TNFR1, TNF receptor 1; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R1, TRAIL receptor 1; VEGF, vascular endothelial growth factor; VEGFR, VEGF receptor.

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venile and the adult form. GCT exhibit a molecular profile that is consistent with FSH-responsive granulosa cells of the late preovulatoy follicle (1, 2). Their unique hormonal activity commonly results in early-stage detection due to the clinical endocrine manifestations of the disease, and as a result, GCT are generally considered to have a better prognosis than epithelial ovarian tumors. However, due to their characteristic slow, indolent pattern of progression and high rate of tumor recurrence, approximately 80% of patients with advanced stage or recurrent tumors succumb to their disease (3).

In accordance with their cellular origin, many studies into the molecular pathogenesis of GCT have focused on examination of the signaling pathways associated with FSH-stimulated cellular proliferation and those associated with normal granulosa cell development and differentiation. Despite these extensive efforts, until recently, relatively little was known about the molecular changes that give rise to GCT. In 2009, a study using whole-transcriptome sequencing technology uncovered a novel somatic missense mutation in the *FOXL2* gene (c.402C→G; p.C134W) in approximately 97% of adult-type GCT (4). Subsequent studies have confirmed the presence of the mutation in adult GCT and absence from juvenile GCT and other human malignancies (5–11). Despite their many clinical and molecular similarities, the lack of the mutation in juvenile GCT strongly suggests a distinct molecular etiology for this subtype. The high-frequency single-nucleotide mutation is suggestive of an oncogenic or gain-of-function mutation, and emerging publications are beginning to shed some light on the possible mechanism by which mutant *FOXL2* may be promoting tumorigenesis in granulosa cells (12–14). The finding of the *FOXL2* C134W mutation as an almost universal feature of adult GCT is an exciting advance in clinical molecular oncology. In this review, we will summarize the current state of knowledge regarding the clinical manifestation and management of GCT. We will then systematically review studies on the molecular pathogenesis of GCT as well as detail recent advances in the field.

## II. Clinical Information

Globally, invasive ovarian tumors are the most common fatal gynecological malignancy with an estimated 21,880 new cases and 13,850 deaths reported in the United States in 2010 (15).

### A. Ovarian tumor classification

Ovarian tumors are a heterogeneous group of neoplasms, which are classified based primarily on their histopathological patterns, reflecting the various cell types comprising the ovary (16). According to the World Health Organization guidelines, they can be divided into three major categories: epithelial ovarian tumors (common epithelial tumors), sex cord-stromal tumors (*e.g.* granulosa cell tumors), and germ cell tumors (17). A fourth group are secondary tumors in the ovary that arise from a primary lesion elsewhere, commonly the gastrointestinal tract or breast, and metastasize to the ovary (Table 1).

**TABLE 1.** Summary of the World Health Organization histological classification of tumors of the ovary

1. Common epithelial tumors
   - A. Serous tumor
   - B. Mucinous tumor, endocervical-like and intestinal types
   - C. Endometrioid tumor
   - D. Clear cell (mesonephroid) tumor
   - E. Transitional cell tumor
   - F. Squamous cell tumor
   - G. Mixed epithelial tumor (specific types)
   - H. Undifferentiated carcinoma
2. Sex cord-stromal tumors
   - A. Granulosa cell tumor
     - i. Adult granulosa cell tumor
     - ii. Juvenile granulosa cell tumor
   - B. Theca-fibroma group
     - i. Thecoma
     - ii. Fibroma
   - C. Sertoli-stromal cell tumor
     - i. Sertoli-Leydig cell tumor
     - ii. Sertoli cell tumor
     - iii. Stromal-Leydig cell tumor
   - D. Sex cord-stromal tumor of mixed or unclassified cell type
     - i. Sex cord tumor with annular tubules
     - ii. Gynandroblastoma
     - iii. Sex cord-stromal tumor, unclassified
   - E. Steroid cell tumor
3. Germ cell tumors
   - A. Dysgerminoma
   - B. Endodermal sinus tumor
   - C. Embryonal carcinoma
   - D. Polyembryoma
   - E. Choriocarcinoma
   - F. Teratoma
   - G. Mixed forms
4. Secondary (metastatic) tumors

#### *1. Epithelial ovarian tumors*

The majority of ovarian tumors are thought to be derived from the relatively pluripotent cells of the surface epithelium and typically represent 80–90% of all ovarian malignancies (18). The ovarian surface epithelium is not a true epithelium, but instead is a derivative of a common embryological precursor, the coelomic mesothelium, which has taken on an epithelial appearance and has both epithelial and mesenchymal characteristics (19). Therefore, epithelial ovarian tumors have the potential to differentiate into a variety of subtypes, each of which takes on a histopathological appearance that closely resembles the normal cells lining other organs of the female genital tract (20) (Table 1). For example, cells of serous tumors resemble those of the Fallopian tube, mucinous tumors the endocervix, and endometrioid tumors take on an endome-

trial appearance (21). Epithelial ovarian tumors can be further subclassified according to their degree of differentiation (tumor grade) (20).

#### 2. Sex cord-stromal tumors

Sex cord-stromal tumors represent approximately 8% of all ovarian tumors and are believed to arise from and/or to contain combinations of the sex cord and stromal components of the developing gonad (21). In females, the embryonic sex cords develop into granulosa cells, whereas the stroma develops into the theca and stromal lutein cells of the ovary; hence, ovarian tumors within this group may comprise a varying combination of one or more of these cell types (21). The most frequently diagnosed tumor type within the sex cord-stromal category is the GCT, accounting for 90% of tumors within this group and approximately 5% of malignant ovarian tumors overall (22). Other less common sex cord-stromal tumors include thecoma-fibromas, Sertoli-Leydig cell tumors, gynandroblastomas, and sex cord tumors with annular tubules (Table 1).

*Granulosa cell tumors of the ovary.* GCT are thought to arise from the granulosa cells of the ovary (23). Granulosa cells constitute the somatic component of the ovarian follicle and function to produce sex steroids and other growth factors required for folliculogenesis and ovulation. Two subtypes of GCT have been described based primarily on clinical behavior and histopathological characteristics, the juvenile and the adult form, of which the latter is much more common and accounts for 95% of GCT (22, 24, 25) (Table 2). The annual incidence of GCT in developed countries varies from 0.4–1.7 cases per 100,000 women (23). Unless otherwise specifically stated, this review will focus on the adult subtype of GCT.

#### 3. Germ cell tumors

The germ cell tumor group, which accounts for approximately 1–2% of all ovarian malignancies, occurs much more frequently among children and young adults and comprises all of the neoplasms thought to be derived from the primordial germ cells of the embryonic gonad (26). Varying widely in their histopathological appearance, the germ cell tumor group comprises dysgerminomatous and nondysgerminomatous tumors, including endodermal sinus tumors (yolk sac tumors), embryonal carcinomas, polyembryomas, choriocarcinomas, immature and mature teratomas, and mixed germ cell tumors (17) (Table 1).

### B. Clinical presentation and diagnosis

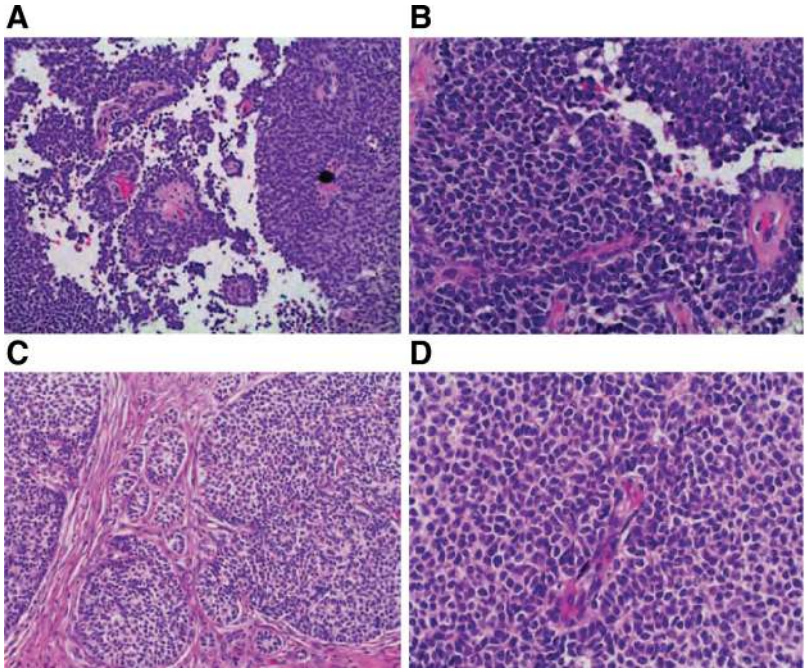
Adult GCT most commonly occur in the perimenopausal or early postmenopausal female with a median age at presentation reported to be 50–54 yr of age (23). Juvenile GCT are diagnosed in patients 10 yr of age or younger in 44% of cases, between 11 and 20 yr in 34% of cases, between 21 and 30 yr in 18% of cases, and over the age of 30 yr in 3% of cases (27). In two series of patients under the age of 18 yr, the median age at diagnosis was 7–8 yr (28, 29). Although extremely uncommon, adult GCT can occasionally be found in children (22). Similarly, juvenile GCT can occur in young postpubertal women, and cases of juvenile GCT in postmenopausal women have also been reported (22, 27, 30). It should be noted that in all of these cases, the distinction, juvenile *vs.* adult GCT, was based on morphological criteria; a more robust classification is likely to result from establishing the *FOXL2* mutation status of GCT (see *Section I.F.1*).

Most patients present with nonspecific symptoms of abdominal pain, distension, or bloating due to a large ovarian mass (22). In addition, a majority of patients exhibit endocrine manifestations as a result of tumor-derived estrogen secretion. In prepubertal females these effects may manifest as isosexual precocious pseudopuberty, including precocious breast development, increased pubic hair, vaginal bleeding, and advanced growth and bone age (28, 29, 31). In the reproductive age group patients may have menstrual irregularities such as menorrhagia, inter-

**TABLE 2.** Differences in adult *vs.* juvenile GCT

| | Adult | Juvenile | Ref. |
|---|---|---|---|
| Median age at presentation (yr) | 50–54 | 7–8 | 23, 28, 29 |
| Patients presenting with stage I disease | 50–90% | 97% | 27, 35, 38–40, 42–44 |
| 5-yr survival rate for stage I disease | 75–95% | 93% | 27, 45 |
| Histology | Well and poorly differentiated patterns of histology with characteristic coffee-bean grooved nuclei | Larger luteinized cells containing hyperchromatic nuclei that lack nuclear grooves | 22, 23, 47–49 |
| G protein mutations | No | *gsp* mutations in 30% of cases | 178, 179, 182–185 |
| *FOXL2* C124W mutation | Yes | No | 5, 6 |
| *FOXL2* expression | Yes | Reduced expression in aggressive/advanced stage tumors | 6, 91 |

**Figure 1.**




Histology of two representative adult GCT. The sections are stained with hematoxylin and eosin. The first tumor (A and B) shows partly solid, partly cystic with macrofollicles and pseudopapillae formation with a Call-Exner body seen in the center at higher magnification (B). The second tumor (C and D) is composed of variably sized, relatively circumscribed solid nests. At higher magnification, cells are seen with coffee-bean-shaped hyperchromatic nuclei and few distinct nuclear grooves (D). The sections are at ×20 (A and C) and ×40 (B and D) magnification.

menstrual bleeding, or secondary amenorrhea, and in postmenopausal women vaginal bleeding is the most frequent presenting symptom (24). Due to the high vascularization of GCT, approximately 10% of patients present with abdominal pain and acute hemoperitoneum caused by tumor rupture (32). In addition, prolonged exposure of the endometrium to tumor-derived estradiol has been associated with both endometrial hyperplasia and concomitant endometrial adenocarcinoma (23, 33–35).

### C. Prognostic factors

In contrast to ovarian tumors of epithelial origin, GCT are considered to be of low malignant potential and are characterized by their slow, indolent growth with tendency toward late recurrence. Despite several retrospective analyses that involved relatively large series of patients (considering the rarity with which GCT present), tumor stage at time of diagnosis remains the only prognostic factor that is explicitly related to survival in GCT patients (36–40).

The International Federation of Gynecology and Obstetrics staging system is used for GCT (41). Patients most commonly present with stage I disease (50–90%), which is considered to be a low-grade malignancy and is associated with a relatively favorable prognosis (35, 38, 39, 40, 42–44). The overall 5-yr survival rate for patients diagnosed with stage I disease has been variously reported to range from 75–95%, but the majority of studies demonstrated a survival rate of greater than 90% (45). These rates decline significantly in patients with more advanced-stage disease, with the 5-yr survival rate for stage II and stages III or IV reported to be from 55–75% and 22–50%, respectively (23). However, because the clinical course of GCT is characterized by indolent growth with late recurrence, often the recurrent and/or metastatic lesion appears many years after removal of the primary tumor, with periods in excess of 10 or even 20 yr not uncommon (46). The longest reported lapse from primary tumor resection to tumor recurrence is 40 yr (47). Furthermore, when the tumor recurs, 80% of patients will succumb to their disease (3, 42), highlighting the need for a reliable prognostic tool.

Other parameters inconsistently reported and providing overall less convincing evidence as being important for prognosis include patient age, primary tumor size and rupture of tumor, mitotic activity, and nuclear atypia (35).

### D. Pathology

At presentation GCT may vary in size from nonpalpable lesions to large abdominal masses, although the average diameter is approximately 12 cm. The single most common presentation is a solid and cystic tumor mass in which the cyst may contain hemorrhagic fluid (22).

Histological examination of adult GCT reveals a distinctive appearance (Fig. 1). Granulosa cells assume a variety of patterns including both well and poorly differentiated histologies, appearing alone or more commonly with a theca cell or fibroma-like component or both. Well-differentiated forms are further subdivided into microfollicular, macrofollicular, trabecular, insular, solid-tubular, and hollow-tubular patterns (22). Call-Exner bodies are a characteristic feature of the microfollicular form, the most common histological subtype, and consist of small rings of well-differentiated granulosa cells, often with shrunken nuclei surrounding a cavity of eosinophilic material (Fig. 1B). The less well-differentiated subtypes are characterized by undulating parallel (watered-silk) or zigzag (gyriform) rows of gran-

ulosa cells, usually in single file, and a diffuse (sarcomatoid) pattern, characterized by a monotonous cellular growth (22). Although in some cases one pattern is exclusive or predominates, in many adult GCT a mixture of patterns is found. Both the well-differentiated and the less well-differentiated adult GCT contain round to oval, pale cells with characteristic coffee-bean grooved nuclei (Fig. 1D). Few mitotic figures, mild nuclear atypia, and little cytoplasm are usually found; however, luteinization is sometimes evident (23, 48, 49).

Although juvenile GCT share a similar gross appearance with the adult subtype, comprising a mixture of solid and cystic components with hemorrhagic areas, at the histological level the two types differ greatly. Juvenile GCT are distinguished by a follicular or diffuse pattern of larger luteinized cells that contain hyperchromatic or markedly bizarre nuclei, lacking the characteristic nuclear grooves of adult GCT (48, 50).

### E. Etiology and risk factors

#### *1. Cytogenetic abnormalities*

Compared with other ovarian tumor types, GCT exhibit a relatively stable karyotype. Although limited in number, cytogenetic studies have revealed a distinctive pattern of chromosomal aberrations with trisomy 12, trisomy 14, and monosomy 22 observed in 14–33, 25–33, and 35–40% of cases, respectively (51, 52). Between 5 and 20% of GCT are aneuploid; however, neither karyotype nor ploidy are of prognostic significance (51, 53–55).

Although the etiology of GCT had until very recently been obscure, there are several rare tumor predisposition syndromes associated with the development of GCT.

#### *2. Genetic syndromes*

***Peutz-Jeghers syndrome (PJS).*** PJS, a rare autosomal dominantly inherited disorder, is associated with germline mutations in the STK11/LKB1 tumor-suppressor gene (chromosome 19p13.3) (56, 57). PJS is characterized by gastrointestinal hamartomatous polyposis, increased risk of benign and malignant tumors of various organs, and mucocutaneous pigmentation of the lips, buccal mucosa, and digits (58). Peutz-Jeghers females also display increased susceptibility to a specific type of ovarian sex cord-stromal tumor, which shows a unique morphology intermediate between those of GCT and Sertoli cell tumors (59–70). Approximately 30% of patients with these lesions have PJS, and it has been suggested that this association, along with the distinctive features of the tumor should warrant a separate classification (49). Indeed, neither loss of heterozygosity at 19p13.3 nor mutations in the *LKB1* gene are associated with sporadic GCT (70, 71).

***Ollier disease/Maffucci syndrome.*** The literature contains a handful of case studies reporting juvenile GCT in association with Ollier disease and Maffucci syndrome (27, 72–81). These rare disorders are characterized by the presence of enchondroma, a type of benign cartilage tumor found in the bone marrow, which may present as an individual tumor or less commonly as multiple tumors. Ollier disease is defined by the distribution of multiple enchondromas throughout multiple sites in the body, whereas Maffucci syndrome is characterized by the presence of multiple enchondromas in association with multiple hemangiomas of soft tissue (82).

#### *3. Hyperstimulation*

Continuous exposure of the ovary to ovulation-inducing drugs such as the selective estrogen receptor modulator (SERM), clomiphene citrate, or to high concentrations of pituitary gonadotropins in the context of treatment for infertility has been reported to increase the risk of developing GCT (83, 84). However, it has also been argued that several confounding variables and biases weakened these reports. For example, patients undergoing treatment for infertility are subjected to far more surveillance with ultrasound than would be the general population (85), consistent with the fact that in one study, five of 11 neoplasms identified were of borderline malignancy (84), a proportion that is significantly higher than that usually found in the general population (approximately one in 10) (85). It is suggested that because tumors of borderline malignancy are often asymptomatic, the detection of those in patients undergoing fertility treatment is likely to be due to the rigorous screening procedures that are required during this treatment and therefore indicate evidence of an ascertainment bias in these studies (85, 86). Furthermore, in another study of 10,358 women of which approximately half had undergone ovarian stimulation to induce superovulation and in which duration of follow-up ranged from 1–15 yr, no significant increased risk of ovarian cancer was identified (87). It has also been suggested that infertility itself may constitute a risk factor for developing ovarian cancer, independent of exposure to fertility drugs (88–91).

### F. *FOXL2*

#### *1. C134W mutation in adult GCT*

Using whole-transcriptome paired-end RNA sequencing technology, Shah *et al.* (4) identified a single somatic missense mutation in *FOXL2* (402C→G) in four GCT with the predicted consequence to be the substitution of a tryptophan residue for a highly conserved cystine residue at amino acid position 134 (C134W). Subsequent direct sequencing of DNA from additional GCT samples re-

**TABLE 3.** Summary of the published data on the occurrence of the FOXL2 (c.402C→G; p.C134W) mutation in human sex cord-stromal tumors

| Ref. | Adult GCT | Juvenile GCT | Thecoma | SLCT | SCST (unclassified) |
|---|---|---|---|---|---|
| 4 | 90/93 | 1/10 | 3/14 | 0/15 | |
| 7 | 53/56 | 0/5 | 2/16 | 0/4 | |
| 8 | 18/20 | 0/3 | | | |
| 6 | 52/56 | 0/3 | | | |
| 11 | 3/3 | | | | |
| 9 | 39/42 | 0/9 | 2/5 | 3/40 | 1/4 |
| 10 | 17/19 | 1/1 | | | |
| | 272/289 (94%) | 2/31 (6%) | 7/35 (20%) | 3/59 (5%) | 1/4 (25%) |

SCST, Sex cord-stromal tumor; SLCT, Sertoli-Leydig cell tumor.

vealed the mutation to be present in 86 of 89 (97%) morphologically identified adult GCT, in one of 10 (10%) juvenile GCT, and in three of 14 (21%) thecomas (4). Subsequently, six independent studies reported the mutation in 53 of 56 (7), 18 of 20 (8), 52 of 56 (6), three of three (11), 39 of 42 (9), and 17 of 19 (10) adult GCT, respectively (Table 3). The authors suggest that the majority of the 17 tumors histologically classified as adult GCT but found to be *FOXL2* mutation negative were diagnostically challenging in that they exhibited immunohistochemical profiles that were distinct from those of most adult GCT (4) or were of mixed cellular origin with the predominant contribution being from other ovarian stromal components, *i.e.* thecoma or cellular fibroma (6, 7, 10). The high frequency with which the mutation occurs in adult GCT and its absence from juvenile GCT (4, 6–11) and other tumor types (5, 7, 9–11) suggests this mutation is pathognomonic for adult-type GCT. In a clinical setting, screening for the mutation is likely to be a useful diagnostic tool to differentiate between adult and juvenile GCT and other sex cord-stromal tumors.

#### *2. Loss of protein expression in juvenile GCT*

Paradoxically, a previous study reported extinction of forkhead box L2 (FOXL2) protein expression in aggressive juvenile GCT (92). Kalfa *et al.* (92) found the expression of FOXL2 to be absent or decreased in juvenile GCT with an aggressive pattern of progression. Patients with no or reduced expression of FOXL2 more frequently had a higher mitotic activity in the tumor hemorrhagic ascites and significantly more advanced disease at diagnosis (92). Thus, loss of FOXL2 protein expression may be an adverse prognostic factor for juvenile GCT (92).

### G. Human GCT-derived cell lines

Two human GCT-derived cell lines, COV434 and KGN, have proven to be useful *in vitro* model systems to investigate granulosa cell tumorigenesis (Table 4). The COV434 cell line, established in 1984 from a metastatic GCT obtained from a 27-yr-old patient (93), has been shown to produce estradiol in response to FSH, indicating the presence of a functional FSH receptor (94, 95). Similarly, the KGN cell line, established in 1994 from a recurrent, metastatic GCT removed from a 73-yr-old patient also expresses a functional FSH receptor (96).

In light of the identification of the FOXL2 C134W mutation in adult GCT, the presence of the mutation in the KGN cell line (5, 12) and absence from the COV434 cell line (6) suggests that these cell lines are derived from adult and juvenile GCT, respectively. Moreover, as well as being FOXL2 mutation negative, the COV434 cell line does not express the *FOXL2* gene, providing further evidence that it represents a juvenile GCT of an advanced tumor stage (6).

### H. Tumor markers

The identification of specific tumor markers is used to facilitate early detection of recurrent disease. The characteristic hormonal activity of GCT suggests a role for the secreted hormones as tumor markers for postoperative patient management.

#### *1. Estradiol*

Estradiol ($E_2$ or 17$\beta$-estradiol) is the principal sex hormone in females. It is irreversibly converted from the precursor steroid hormone androstenedione by the enzyme

**TABLE 4.** Characteristics of the human GCT-derived cell lines KGN and COV434

| | KGN | COV434 | Ref. |
|---|---|---|---|
| Patient age (yr) | 73 | 27 | 94, 96 |
| Tumor classification | Stage III primary tumor (1984) metastatic recurrence in pelvic region (1994) | Solid primary metastatic tumor (1984) | 94, 96 |
| Karyotype | 45,XX,7q−,−22 | 47,XX,+5,22q+ | 93, 96 |
| Functional FSH receptor | Yes | Yes | 94, 96 |
| Aromatase activity | Yes | Yes | 94, 96 |
| Constitutive NF$\kappa$B and AP-1 activity | Yes | Yes | 203 |
| *ER*$\alpha$ mRNA expression | No | No | 203 |
| *ER*$\beta$ mRNA expression | Yes | Yes | 203 |
| *FOXL2* C124W mutation | Heterozygous | Wild type | 5, 6 |
| *FOXL2* mRNA expression | Yes | No | 5, 6 |

cytochrome P450 aromatase. During the reproductive years, the major site of estradiol production is the granulosa cells. GCT generally secrete increased levels of estradiol as a result of abundant, unregulated aromatase expression (97, 98). Elevated serum estradiol is responsible for some of the clinical manifestations of the disease, which suggests that it may serve as a useful tumor marker for GCT (99). Although this has been true for some cases, in others, no correlation between estradiol levels and disease progression or recurrence was observed (100, 101). One possible explanation for this disparity may be due to a lack of theca cells in the tumor stroma of certain GCT, given that it is the theca cells that produce androstenedione (23). Therefore, although estradiol measurement may be useful in the postoperative management of some patients, its accuracy is not consistent enough to be relied upon as a tumor marker in GCT (23).

#### *2. Inhibin*

The inhibins, members of the TGF$\beta$ superfamily of pleiotropic growth factors, are glycoprotein hormones synthesized predominantly by the granulosa cells in females. The inhibins consist of a dimer of two partially homologous subunits, an $\alpha$-subunit (made up of three regions: Pro, $\alpha_N$, and $\alpha_C$) covalently linked to either a $\beta$A or $\beta$B subunit to form inhibin A and inhibin B, respectively. The genes encoding the $\beta$A (*INHBA*) and $\beta$B (*INHBB*) subunits display spatiotemporally distinct expression patterns in the normal ovary (reviewed in Refs. 102 and 103). The $\beta$-subunits are localized primarily to the granulosa cells (104, 105), although $\beta$A-subunit expression has been observed in the theca cells of human dominant follicles (106). $\beta$A-subunit mRNA has been reported in all follicle stages, including the dominant follicle and the corpus luteum, whereas $\beta$B-subunit expression appears to be restricted to small primary follicles (106). In addition to being an important autocrine and paracrine granulosa cell growth factor within the ovary (107, 108), inhibins also act in an endocrine manner to regulate the synthesis and/or secretion of FSH by pituitary gonadotropes via a negative feedback loop within the hypothalamic-pituitary-gonadal axis (109). With the depletion of ovarian follicles at menopause, serum inhibin A and B decrease to undetectable levels (110, 111), providing a baseline with which to compare serum levels in postmenopausal women with GCT.

Lappöhn *et al.* (100) were the first to report the production of inhibin by GCT and identify a correlation between elevated serum inhibin levels and tumor size, thereby demonstrating its potential usefulness as a marker for both primary and recurrent disease. Further prospective studies confirmed this finding and also showed that serum inhibin correlated negatively with serum FSH concentrations, suggesting that the inhibin secreted by GCT is biologically active (112, 113). These early and subsequent (114) studies identifying elevated serum inhibin in GCT patients were performed using the Monash assay, a RIA that detects all serum $\alpha$-subunits including those in biologically active inhibin dimers as well as bioinactive free $\alpha$-subunits that have undergone differing degrees of glycosylation and processing (115, 116). The subsequent development of ELISA capable of distinguishing between the individual forms of inhibin A and B (111, 117–119) revealed that they were increased to varying extents in women with GCT (110, 120, 121). Using these inhibin subunit-specific ELISA, it was revealed that inhibin B is the major form of inhibin secreted by GCT and that measurement of serum inhibin B concentration is a more accurate test than that of inhibin A in detecting GCT, and also reflects tumor burden (120–122). However, the increased specificity (and arguably sophistication) of these assays does not necessarily translate into a clinical advantage when compared with the original Monash assay (123–125). In reviewing their experiences with the inhibin assays, Robertson *et al.* (126) showed that the more specific assays for detecting serum inhibin A and B were less accurate for discriminating between normal and cancer samples when compared with the Pro-$\alpha$C (free $\alpha$-subunit) assays, although the total inhibin assay that detects all forms that contain the carboxyl-terminal ($\alpha$C) region of the $\alpha$-subunit both gave the best differentiation and was the preferred method (127, 128).

To further confound this ambiguity, examination of inhibin $\alpha$-subunit expression by immunohistochemistry in 30 GCT revealed 26 (87%) stained positively for the $\alpha$-subunit, all of which were stage I and II tumors (129). Of the four remaining tumors, all of which were classified as either stage III or IV, three were $\alpha$-subunit immunonegative, whereas one exhibited slight staining for the $\alpha$-subunit (129). This study reveals that 1) not all GCT may express inhibin and 2) loss of inhibin $\alpha$-subunit expression may be associated with a poor prognosis. This finding could be seen to contradict the dogma that increased postmenopausal serum inhibin levels are a marker for GCT and indeed can often precede a clinical recurrence in those cases.

#### *3. Müllerian inhibiting substance*

Several studies investigating the dynamics of serum Müllerian inhibiting substance (MIS), also referred to as anti-Müllerian hormone, in patients with GCT show it to be a reliable marker for tumor activity with a sensitivity ranging between 76 and 100% (101, 130–134). MIS, a TGF$\beta$ superfamily member, is expressed by granulosa cells during the reproductive period and controls the formation of primary follicles by inhibiting excessive follic-

ular recruitment by FSH. Anttonen *et al.* (135) found that MIS gene expression correlated inversely with GCT size, with reduced expression in 87% of tumors greater than 10 cm in size. This finding may suggest MIS is a less useful marker than the inhibin assay in more advanced disease; however; there has been no direct comparison.

Although it is commonly accepted that MIS and inhibin display a higher degree of sensitivity and are more reliable than estradiol in detecting primary and/or recurrent GCT (136), additional studies are required to confirm whether total inhibin, inhibin B, or MIS constitutes the more reliable marker in the detection and management of patients with GCT. A recent review evaluating the usefulness of serum levels of inhibin B and MIS in the diagnosis and follow-up of GCT suggests MIS is the more sensitive and reliable marker of the two (137); however, this is based on a retrospective compilation of previously published data and not a direct comparison between MIS and inhibin B levels in a single cohort of patients.

## I. Treatment and disease management

The rarity with which GCT occur as well as their prolonged natural history and high rate of recurrence make it difficult to form clear conclusions regarding clinical behavior and to carry out prospective randomized studies for the purpose of developing standard treatment guidelines. Conventional treatment for GCT has predominantly been surgery followed by chemotherapy where recurrent and/or advanced disease has occurred.

### *1. Surgery*

It is suggested that in approximately 78–91% of cases, GCT are stage I and unilaterally confined to the ovary at diagnosis (23). In these patients, more conservative fertility-sparing surgery with unilateral salpingo-oophorectomy appears to be the most appropriate course of action. This recommendation is borne out by several retrospective analyses in which the results with conservative surgery did not differ from those using standard surgery with hysterectomy (38, 39). An endometrial biopsy is also recommended to ensure concomitant endometrial pathology has been excluded. For women with a stage I tumor that is confined to the ovary, adjuvant therapy is not recommended (138). In postmenopausal women and patients with more advanced-stage disease or bilateral ovarian involvement, total abdominal hysterectomy and bilateral salpingo-oophorectomy with removal of all visible disease is thought to be the most appropriate initial treatment (23, 24, 45).

### *2. Adjuvant therapies (radiation and conventional chemotherapy)*

For patients with metastatic disease or for those whose disease has recurred after primary surgical resection, adjuvant therapy is an option; however, the efficacy of radiotherapy or systemic chemotherapy for patients with GCT remains inconclusive. The existing literature is comprised of case reports and retrospective reviews of archival patient data. Additionally, due to the often long interval to recurrence, evaluations of the efficacy of adjuvant treatment on overall or disease-free survival rates in GCT are often difficult to interpret (32).

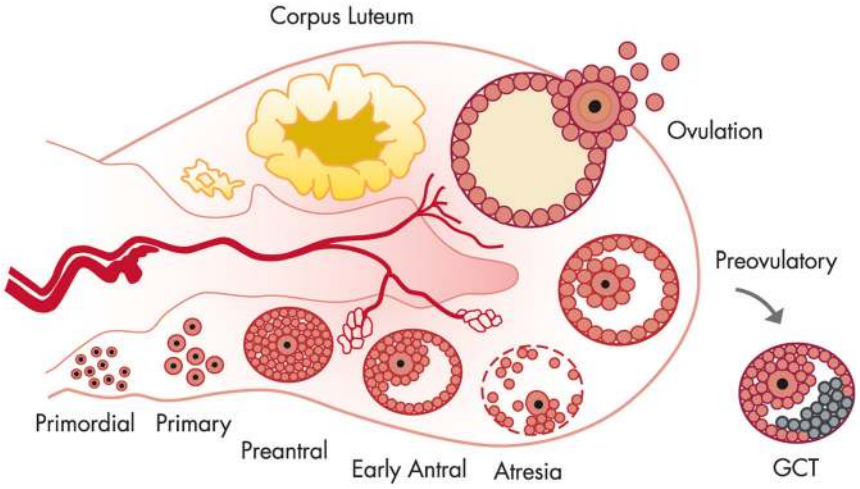
Current chemotherapy is based on platinum-containing protocols derived largely from experience with ovarian epithelial tumors (45). Studies and case reports using cisplatin in combination with either doxorubicin and cyclophosphamide, vinblastine and bleomycin, or etoposide and bleomycin have been reported with response rates varying from 60–83% and duration of response 5.2–58.6 months (99, 139–153).

### *3. Hormonal treatment (GnRH antagonists, tamoxifen, and aromatase inhibitors)*

As previously described, GCT are often distinguished by their ability to secrete estrogen, and thus, there is interest in examining the benefits of hormonal manipulation as a potential therapeutic strategy in these neoplasms. Although a limited number of studies have reported recurrent GCT responding to hormonal treatments such as the progestin medroxyprogesterone 17-acetate (154–156), the aromatase inhibitor anastrozole (157–159), the ER antagonist tamoxifen (160), and several GnRH agonists (161–163), in most cases, these treatments were used as a last resort, and results suggest only transient responses of a few months duration. Conversely, there are also reports of patients showing no response to the same hormonal therapies (162, 164–166). Due to the typically late recurrence of GCT and the relatively short disease-free periods described in many of these studies (*i.e.* less than 12 months), the true success of hormonal interventions in the treatment of GCT remains questionable at best. These reports do, however, highlight the inadequacy of current nonsurgical therapeutic options for patients with GCT.

Due to the typically high rate of late recurrence of this disease, long-term follow up throughout the patients' life is needed, even in the case of early-stage diagnosis. Any abdominal or pelvic mass in these patients should be considered recurrent GCT until proven otherwise regardless of the time from initial diagnosis. Lifetime surveillance is critically important in the management of these patients (147).

**Figure 2.**




Schematic representation of ovarian folliculogenesis. Folliculogenesis is a cyclical process by which quiescent primordial follicles are recruited to enter the growth phase, proceeding through multiple stages of development that culminate in the release of the ovum from the dominant follicle and the terminal differentiation of the remnant follicle into corpora lutea. The nondominant follicles undergo atresia. GCT exhibit a molecular profile that suggests they arise from proliferating granulosa cells of the preovulatory follicle.

## III. The Molecular Genetics of GCT

It is commonly accepted that tumor formation occurs due to a succession of genetic changes, each contributing a growth advantage, which ultimately results in the transformation of normal cells into malignant cells (167). The molecular changes that give rise to GCT are likely to involve disruption of signaling pathways that function during normal folliculogenesis to regulate granulosa cell proliferation. Indeed, GCT exhibit many features of proliferating granulosa cells of the preovulatory follicle, including expression of the FSH receptor (168), FSH binding (169, 170), estrogen synthesis (3), *GATA-4* expression (135, 171), and expression of the inhibin subunit genes with synthesis of biologically active inhibin (100, 172, 173). This molecular phenotype is consistent with activation of the FSH receptor signaling pathway (2, 174). Concordantly, the gene expression profile of GCT is also consistent with FSH-stimulated granulosa cells, which includes high expression of the gene encoding the FSH receptor as well as high expression of FSH early-response genes such as the regulatory subunit of protein kinase A (RII-$\beta$; *PRKAR2B*), cyclin D2 (*CCND2*), and the late-response marker cyclooxygenase-2 (COX-2; *PTGS2*) (1); this was observed in association with low expression of both the early-response gene serum/glucocorticoid-regulated kinase 1 (*SGK1*) and the late-response marker LH receptor (LHR; *LHCGR*) (1).

### A. FSH-mediated signaling pathways

In the normal ovary, granulosa cell proliferation enters its most rapid phase between the preantral and preovulatory stages of follicle development (175). Although the FSH receptor is expressed on the surface of preantral granulosa cells, preantral folliculogenesis is dependent on the autocrine and paracrine actions of intraovarian factors and is able to proceed independently of gonadotropin stimulation. After puberty, additional growth to the stage at which follicles have the potential to undergo ovulation is absolutely dependent on the pituitary gonadotropins. This phase coincides with the cells' receptivity to FSH in that the presence of the FSH receptor enhances follicle growth (Fig. 2).

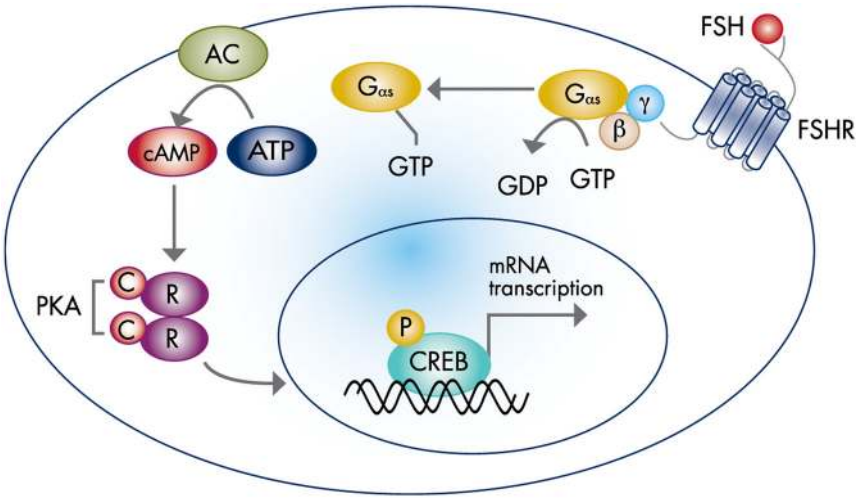
#### *1. cAMP/PKA*

FSH binding to its receptor triggers activation of the classical, linear adenylyl cyclase/cAMP/protein kinase A (PKA) signaling pathway, which in turn promotes the phosphorylation and activation of the transcription factor cAMP response element-binding protein (CREB). Phosphorylated CREB regulates the expression of a variety of target genes required for cellular proliferation and differentiation (Fig. 3).

Given the obligatory role of FSH receptor expression and FSH binding during the rapidly proliferative phase of granulosa cell development, it has been hypothesized that activating mutations of the FSH receptor gene may lead to an altered protein product capable of initiating uninhibited proliferation in granulosa cells. Although Kotlar *et al.* (176) initially reported the presence of a single-nucleotide missense mutation (F591S) in the *FSHR* gene in seven of nine juvenile GCT and two of three adult GCT, subsequent studies did not identify the F591S or any other *FSHR* mutations in a combined total of 46 GCT (168, 177–179). The analysis of additional GCT samples and reanalysis of the original GCT samples by Kotlar *et al.* (180) failed to reproduce their initial finding, which they suggested must have been due to DNA contamination.

Heterotrimeric G proteins, consisting of $\alpha$-, $\beta$-, and $\gamma$-subunits, couple seven-transmembrane domain receptors (also known as G protein-coupled receptors) to intracellular second messenger systems, including the FSH receptor in granulosa cells (Fig. 3). The first evidence of the oncogenic potential of G proteins was reported in a subset of GH-secreting pituitary adenomas in which a somatic

**Figure 3.**




FSH receptor activation of cAMP-dependent PKA. FSH binding to the FSHR on granulosa cells triggers the exchange of *GDP* for *GTP* on the α-subunit of the G protein complex. Gα-GTP dissociates from the β- and γ-subunits to activate adenylyl cyclase (AC), which in turn catalyzes the conversion of ATP to cAMP. The increase in cAMP causes it to bind to the regulatory (R) subunit of PKA, thereby promoting the dissociation and activation of the catalytic (C) subunit. The activated C subunit is free to translocate to the nucleus where it phosphorylates transcription factors such as the prototypic CREB protein, thus controlling gene expression. FSHR, FSH receptor.

mutation in the gene encoding the $G\alpha_s$ protein was found in 18 of the 42 (42%) adenomas examined, causing inhibition of the proteins intrinsic GTPase activity (181). The mutations in this oncogene, termed *gsp*, result in amino acid substitution at either of two residues (201 and 227), both of which are completely conserved in all known Gα proteins (181). The possibility that similar mutations may activate other G proteins prompted Lyons *et al.* (182) to investigate other human endocrine tumors for mutations that replace either of these two amino acids in genes encoding other Gα proteins. The gene encoding the $G\alpha_{i\text{-}2}$ protein was reported to harbor mutations that result in the substitution of arginine-179 for either a cysteine or histidine in three of 11 (27%) tumors of the adrenal cortex and three of 10 (30%) sex cord-stromal tumors of the ovary, of which seven were GCT (182). This $G\alpha_{i\text{-}2}$ oncogene was termed *gip2* (182). In the same study, no *gsp* mutations were observed in six sex-cord stromal tumors (182). Subsequent studies examining GCT for the presence of *gsp* and *gip2* mutations have been reported in the literature. Shen *et al.* (183) failed to identify the previously reported *gip2* R179C/H mutation in 13 GCT. Similarly, Ligtenberg *et al.* (178) observed no known *gsp* or *gip2* mutations in 22 GCT, nor did Fragoso *et al.* (184) in two GCT. Hannon *et al.* (179) did not find the *gsp* mutation in 17 adult GCT.

Interestingly the *gsp* mutations, R201C or R201H, were observed in nine of 30 (30%) juvenile GCT samples with laser microdissection, confirming that they were localized exclusively to the tumor-derived granulosa cells and were absent in the ovarian stroma (185). When compared with patients with normal $G\alpha_s$ the clinical symptoms and age of occurrence were not significantly different, and patients carrying the mutation did not exhibit more severe hyperestrogenic symptoms (185). They did, however, exhibit a significantly more aggressive pattern of behavior with seven of the nine (77%) oncogenic *gsp*-positive patients presenting with stage Ic disease or developing recurrence, whereas most of the mutation-negative patients had a tumor strictly limited to the ovary without recurrence (76%), suggesting the *gsp* mutation status of juvenile GCT patients may be a prognostic factor for these tumors (185). Hannon *et al.* (179) did not, however, observe the *gsp* mutations in 10 juvenile GCT; however, information regarding tumor stage and/or recurrence was not provided.

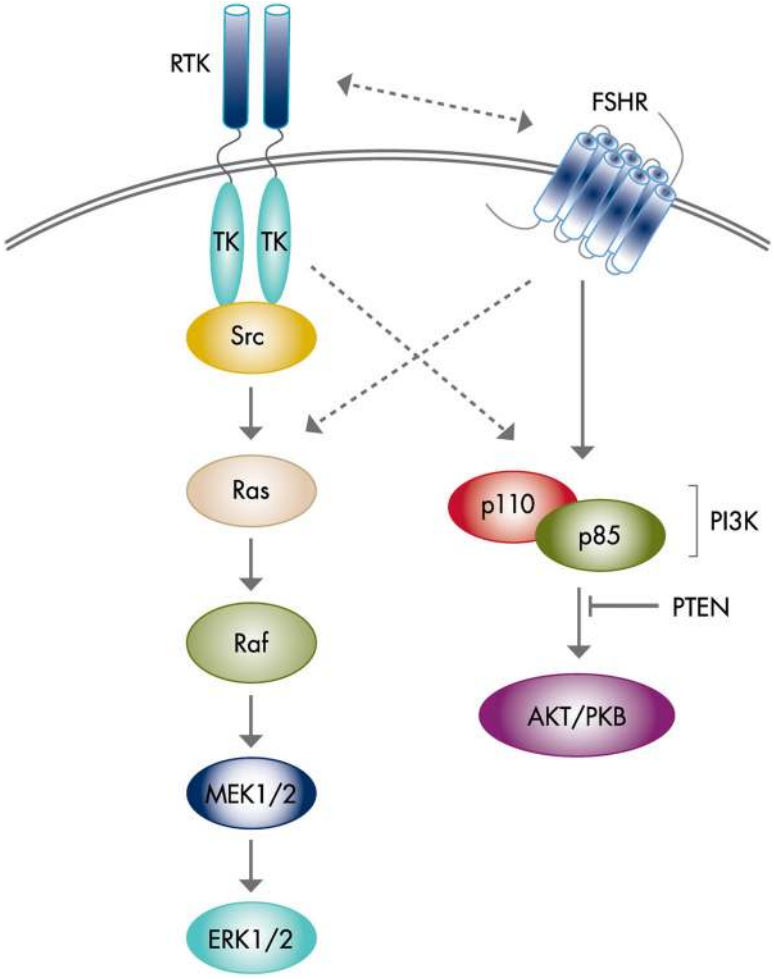
#### *2. Phosphatidylinositol 3-kinase (PI3K)/AKT*

After activation of the FSH receptor in granulosa cells, the classical adenylate cyclase/cAMP/PKA pathway is capable of diverging at PKA to activate distinct downstream signaling cascades, including the PI3K/AKT pathway (186–188) (Fig. 4). Aberrant activation of this pathway has been implicated in many solid tumors including breast, prostate, colon, and epithelial ovarian tumors (189, 190). In epithelial ovarian tumors, the pathway is commonly activated through mutation or increased copy number of the genes encoding the PI3K subunits, *PIK3R1* (p85) (191) and *PIK3CA* (p110) (192), or inactivation of the tumor suppressor gene *PTEN* (193). Despite these findings, however, neither mutation nor overexpression of *PIK3CA* and *PIK3R1* nor loss of expression of *PTEN* have been observed in adult GCT (194).

#### *3. Epidermal growth factor (EGF)*

The EGF/ErbB (erythroblastic leukemia viral oncogene homolog) family of receptor tyrosine kinases (RTK) includes four members: EGF receptor (EGFR)/ErbB1/heregulin-1 (Her1), ErbB2/Her2, ErbB3/Her3, and ErbB4/Her4. Typically for RTK these transmembrane receptors contain a common extracellular ligand-binding domain, a single membrane-spanning region and an intracellular domain with

**Figure 4.**




Receptor tyrosine kinases (RTK) and the FSH receptor (FSHR) both activate the MAPK and PI3K intracellular signaling pathways in granulosa cells. This activation may be achieved via direct downstream signaling and/or via multiple cross talk mechanisms between the receptors themselves and/or intracellular components of the pathways.

intrinsic protein tyrosine kinase activity. A family of ligands known as the EGF-related peptide growth factors bind the extracellular domain, triggering receptor homo- or heterodimerization. Receptor dimerization causes the autophosphorylation of specific tyrosine residues within the cytoplasmic domain which in turn activate an array of intracellular signaling pathways (195). Of the four family members no specific ligand has been identified for ErbB2. It can however be transactivated via heterodimerization with other ErbB family members, thereby acting as a co-receptor to enhance ligand binding and ligand-induced biological responses (195). Like other RTK, overexpression and/or mutation of EGF/ErbB receptor family members, in particular EGFR and ErbB2, has been shown to contribute to the etiology and progression of several forms of human cancer including those of the brain, breast, and ovarian epithelium (195, 196). To date, three small- molecule tyrosine kinase inhibitors (TKI) designed to target the EGFR have been approved by the U.S. Food and Drug Administration (FDA) for use as cancer therapeutic agents: gefitinib (*ZD1839*, Iressa), erlotinib (OSI 774, Tarceva), and lapatinib (GW572016, Tykerb).

In granulosa cells, it is known that the EGF/ErbB receptor family members activate several intracellular signaling pathways, including the MAPK pathway to promote cell proliferation and the PI3K/AKT pathway to promote cell survival (196, 197) (Fig. 4). Wayne *et al.* (198) demonstrated that FSH-stimulated activation of the MAPK pathway requires EGFR tyrosine kinase activity and that dominant-negative rat sarcoma as well as EGFR tyrosine kinase inhibition blocks FSH-induced phosphorylation of ERK1/2 (198). Furthermore, there is evidence that EGF signaling through EGFR is required for normal gonadotropin-induced steroidogenesis in granulosa cells (199).

Immunohistochemical analysis has shown all four EGF/ErbB receptor family members to be expressed in GCT (200, 201) as well as EGFR in the KGN cell line (202) and erbB2 and erbB4 in the COV434 cell line (200). Moreover, Furger *et al.* (200) showed that heregulin-$\beta$2, a ligand for erbB3 and erbB4, increased cell proliferation in COV434 cells by activation of the ERK1/2 transcription factors via the MAPK pathway. In addition, treatment with heregulin/pseudomonas exotoxin 40, a ligand toxin shown to display selective cytotoxicity against erbB4-positive breast cancer cell line, exerted a strong and irreversible cytotoxic activity toward COV434 cells (200).

The activator protein-1 (AP-1) transcription factor is constitutively activated in KGN and COV434 cells with targeted chemical inhibition of ERK fully abrogating the constitutive activity of AP-1 in both cell lines (203). Similarly, constitutive activation of ERK1/2 signaling has also been observed in the KGN cell line, with small interfering RNA silencing of ERK1/2 protein expression resulting in the complete suppression of cell proliferation (204).

Taken together, these findings provide strong, albeit circumstantial evidence that the constitutive activation of AP-1 transcription is mediated by constitutive ERK phosphorylation via a RTK/MAPK/ERK signaling cascade (Fig. 4). Moreover, given that similarities were observed in the expression profiles of genes involved in AP-1 activation be-

tween the two cell lines and a panel of human GCT samples (Chu, S., Prince Henry's Institute of Medical Research, unpublished data), constitutive ERK activation resulting in activation of AP-1 target genes may play a role in the pathogenesis of these tumors.

#### *4. Vascular endothelial growth factor (VEGF)*

VEGF and its receptors, VEGFR-1 (FLT1), VEGFR-2 (KDR), and VEGFR-3 (FLT4), are key regulators of tumor angiogenesis and are the primary targets of the TKI sunitinib (BAY 43-9006, Sutent) and pazopanib (GW786034, Votrient), which are in clinical use for the treatment of renal cell carcinoma. Several studies have observed VEGF protein and/or mRNA expression in human GCT (205–207). Examining a panel of 106 GCT, Färkkilä *et al.* (207) found VEGF and VEGFR-2 to be highly expressed in primary and recurrent tumors, in comparison with normal granulosa-lutein cells. Moreover, the expression of VEGF correlated positively with tumor microvessel density and with VEGFR-2 expression at the protein and mRNA levels (207). VEGF protein was not prognostic for tumor recurrence; however, patients with primary GCT had high serum VEGF levels (207). A number of case reports have examined the clinical efficacy of bevacizumab, a monoclonal antibody to VEGF, which has been approved by the FDA for use as an adjuvant therapy in colorectal cancer. Of the 10 patients reportedly treated with bevacizumab, nine with adult and one with juvenile GCT, only one patient had a complete clinical response (206, 208, 209). Although a much larger cohort of patients would be required to draw any clear conclusions, these initial case reports indicate adjuvant treatment with bevacizumab may provide limited efficacy at best in patients with GCT.

#### *5. Other tyrosine kinases*

In a case report of an 87-yr-old patient with recurrent, metastatic GCT, immunohistological examination of the tumor revealed overexpression of mast/stem cell growth factor receptor (SCFR; KIT; CD117), prompting treatment with the TKI imatinib (STI571, Gleevec) (210). That the patient showed a significant response (210) prompted Chu *et al.* (211) to further investigate a more general role for imatinib in GCT. The expression profiles of the genes encoding the imatinib-sensitive tyrosine kinases (*KIT*, *ABL*, and *PDGFR-α* and *-β*) were characterized in a panel of human GCT samples, and the effect of imatinib, and subsequently nilotinib (a second-generation related TKI; AMN107, Tasigna), on the KGN and COV434 cell lines was examined (211). All four kinases were expressed but at levels lower than those observed in premenopausal ovarian samples. Known activating mutations in *KIT* (exons 9–11) and *PDGFRA* (exon 18) were not found by direct sequencing (211). Both cell lines responded to imatinib and nilotinib, showing dose-dependent decreases in cell proliferation and viability. These responses paralleled those observed in an imatinib-sensitive chronic myeloid leukemia cell line (K562) but at approximately 240- and approximately 1000-fold higher concentrations of imatinib and nilotinib, respectively, suggesting that GCT in general are unlikely to respond to imatinib and/or nilotinib therapy (211). The response of the cell lines implies an off-target effect (*i.e.* imatinib inhibition of a tyrosine kinase other than the four characterized) (211). Because the concentration of imatinib is increased, a range of other tyrosine kinases are known to be inhibited, including FLT3 (FMS-related tyrosine kinase 3; STK1), CSF1R (colony-stimulating factor 1 receptor), Src, and the EGFR (212). Given that overexpression of colony-stimulating factor (*CSF*) with its receptor, *CSF1R*, in normal granulosa cells resulted in proliferation and tumorigenesis (213), Chu *et al.* (211) also examined the two GCT-derived cell lines for expression of *CSF1R* and *FLT3*. They found neither gene to be expressed in both cell lines, and where expressed, the levels were very low, indicating that CSF1R and FLT3 were unlikely to be mediating the response to imatinib (211). Although these findings suggest that the tyrosine kinases targeted by therapeutic concentrations of imatinib do not have a significant pathogenic role in GCT, they do suggest that a TKI of appropriate specificity may represent a therapeutic option (211).

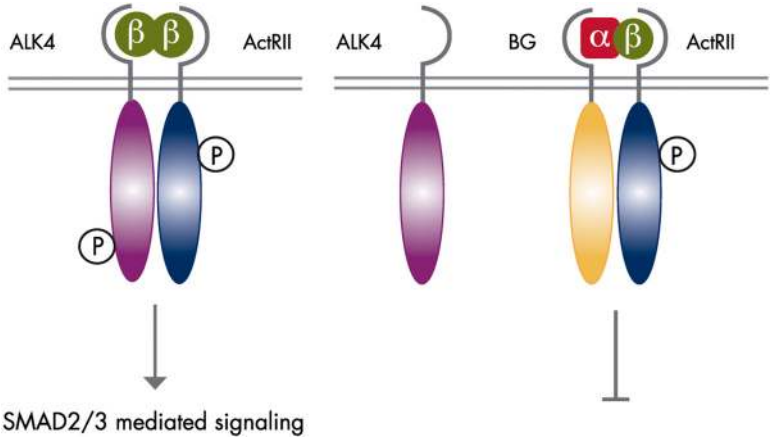
### B. TGFβ superfamily members

The important role members of the TGFβ superfamily play in ovarian follicle development is well established (reviewed in Refs. 214–216).

#### *1. Inhibin subunits*

As previously described, the inhibins are peptide hormones comprising heterodimers of an α-subunit linked via a single disulfide bond to either a βA subunit or a βB subunit, forming inhibin A and inhibin B, respectively. The β-subunits are also able to homo- or heterodimerize (βAβA, βAβB, βBβB) to form a closely related subgroup, the activins. Subunit expression and secretion of α-subunit monomers or αβ-subunit dimers are independently regulated in the normal ovary (126). Inhibin B is predominantly produced by small primary follicles, whereas inhibin A is produced in all follicle stages including the dominant follicle and corpus luteum. At the onset of menopause, the accompanying depletion of ovarian follicles results in total serum inhibin dropping to undetectable levels (110, 111). In contrast, serum inhibin levels are markedly raised in women with GCT, and inhibin may be used as a reliable tumor marker for GCT recurrence (100,

**Figure 5.**




Schematic representation of activin and inhibin mechanisms of action. Activin binding to ActRII promotes the recruitment and phosphorylation of its type I receptor (Alk4), which in turn activates specific downstream receptor-regulated SMAD transcription factor proteins. Inhibin is also capable of binding to ActRII where it recruits the ancillary binding protein betaglycan (BG; TGF$\beta$ receptor type III, TGFBR3). This high-affinity interaction blocks the recruitment of Alk4 to ActRII, thereby antagonizing the action of activin.

114, 172, 217). In addition, the high serum inhibin levels are associated with suppressed plasma FSH levels (217), indicating that the tumor-derived inhibin is biologically active and that tumor growth proceeds independently of FSH (1).

In contrast to the human disease, mice null for the inhibin $\alpha$-subunit gene (*Inha*$^{-/-}$), and therefore completely inhibin deficient, develop invasive sex cord-stromal tumors with 100% penetrance (218) (discussed in *Section IV.A*). Matzuk and colleagues propose that inhibins are secreted tumor suppressors with gonadal specificity, and they suggest that the apparent contradiction with respect to human GCT may be explained by resistance to inhibin in human GCT (218, 219). No evidence for loss of heterozygosity at the inhibin $\alpha$-subunit gene (*INHA*) was found in a study of 17 human GCT (220). As discussed in *Section II.H.2*, in a study of inhibin $\alpha$-subunit expression using immunohistochemistry in 30 GCT, three were $\alpha$-subunit immunonegative, whereas one exhibited slight staining for the $\alpha$-subunit (129). These studies raise the question of whether loss of inhibin $\alpha$-subunit expression plays a role in the pathogenesis of human GCT or whether it is simply a bystander effect in advanced-stage disease.

#### *2. Inhibin/activin receptors*

Despite extensive investigation, the receptor complex and molecular mechanisms by which inhibins act on their target cells remain poorly understood, in comparison with the situation for the structurally related family of activins. Activins and other members of the TGF$\beta$ superfamily signal via pairs of specific type I and type II serine/threonine kinase receptor complexes (reviewed in Ref. 221). Activin binds to its type II receptor (ActRII or ActRIIB), which recruits and phosphorylates a type I receptor (Alk4 or ActRIB), which in turn phosphorylates specific intracellular receptor-regulated Sma and Mad-related protein (SMAD) transcription factors (Fig. 5). Inhibins also bind to ActRII via their $\beta$-subunit, albeit with an approximate 10-fold lower affinity than that of activins (222). This binding does not, however, induce recruitment or phosphorylation of the type I receptor, enabling inhibin to competitively bind ActRII and antagonize the action of activin and other TGF$\beta$ superfamily members that signal via ActRII (223, 224). The type III TGF$\beta$ receptor betaglycan (TGFBR3), also functions as an inhibin receptor (225, 226). Betaglycan enhances the binding of inhibin to the ActRII receptor, thereby blocking activin from binding to ActRII and antagonizing activin signaling (227) (Fig. 5).

Expression of the genes encoding the ActR subunits and betaglycan is widespread in ovarian tumors, including GCT (173). Bilandzic *et al.* (228) have shown that nine of 17 (53%) GCT exhibit reduced betaglycan expression compared with normal premenopausal ovary, suggesting that absence and/or reduced expression of betaglycan on the cell surface may provide a mechanism via which human GCT become inhibin resistant. Furthermore, exogenous betaglycan expression in the KGN and COV434 cell lines promoted cellular behaviors consistent with that of aggressive or metastatic disease, such as adhesion, migration, and invasion (228). In addition, exogenous expression of a mutant form of betaglycan defective in inhibin binding, or endogenous *INHA* gene silencing, abrogated these behaviors (228).

#### *3. SMAD proteins*

The SMAD family of transcription factors, comprising SMAD1–8, are essential intracellular mediators of TGF$\beta$ signaling. More specifically, TGF$\beta$, activin, and nodal signal through the receptor-regulated SMAD2 or -3 whereas bone morphogenetic protein (BMP) and MIS signal through SMAD1, -5, or -8. Once phosphorylated, the receptor-regulated SMAD associate with the co-SMAD, SMAD4, and enter the nucleus to regulate gene transcription (229). The role of SMAD in normal ovarian function is well documented (214, 230, 231), and although the generation of various SMAD knockout mouse models have facilitated investigations into the action of SMAD in murine granulosa cells and the development of GCT (dis-

cussed in *Section IV.D*), little is known about their role in human granulosa cell tumorigenesis. Given that loss of heterozygosity or inactivation mutations in SMAD4 have been associated with 70–80% of pancreatic cancers, and to a lesser extent other malignancies, including biliary tract, cervical, non-small cell lung carcinoma, breast, and bladder (232), an involvement of SMAD in the pathogenesis of GCT is not improbable.

### C. Nuclear receptors (NR)

NR are critical for endocrine signaling and have long been implicated in several hormone-dependent malignancies including those of the breast, prostate and endometrium. Although several NR known to be involved in normal granulosa cell biology have previously been examined in GCT, including ERβ (233), ERα, the progesterone receptor, (234) and steroidogenic factor-1 (SF-1) (135), the role of other NR and their pattern of expression in GCT had not been investigated. The expression levels of all 48 NR were systematically evaluated in a panel of 14 human adult GCT and the KGN and COV434 cell lines using low-density gene profiling arrays (235). Results revealed that chicken ovalbumin upstream promoter-transcription factor 2 (*COUP-TF2*) was the most abundantly expressed NR, with peroxisome proliferator-activated receptor γ (*PPARγ*), *SF-1*, and thyroid hormone receptor-α also exhibiting prominent expression (235). Perhaps not surprisingly, ERβ was the most abundantly expressed steroid receptor, with expression of the androgen receptor, ERα, and the progesterone receptor also of note. The concordance of expression between individual tumor samples was extremely high for the vast majority of NR. In addition, expression levels, but for a few NR, were parallel in the COV434 and KGN cell lines (235).

#### *Estrogen and the ER*

Estrogens signal via two NR subtypes, ERα and ERβ (236–239), with ERβ being the predominant form expressed in the ovary (240, 241). Ligand-bound receptors mediate both autocrine and endocrine actions of estrogen by binding to estrogen response elements present on estrogen-inducible genes (242) and may also act as coregulators of other transcription factors (243, 244).

Several knockout mouse models have highlighted the importance of intraovarian estrogen action. Mice that carry a null mutation in the genes encoding ERα [ERα knockout (αERKO)] or ERβ (βERKO) lack functional receptor activity and therefore cannot respond to estrogen (245–249). The aromatase knockout (ArKO) mouse, generated by targeted disruption of the *cyp19* gene, is able to respond to but does not synthesize estrogen (250). Female αERKO mice are completely infertile (251), whereas βERKO females are subfertile and have fewer and smaller litters than wild-type mice due to a reduced ovarian efficiency (247). In ArKO females, folliculogenesis is disrupted, corpora lutea are completely absent, and mice are rendered infertile due to an inability to ovulate, demonstrating the crucial role estrogen plays in the development of follicles beyond the antral stage (250, 252, 253). The ovarian phenotype of the various ERKO and ArKO models has been extensively reviewed (248, 254–264).

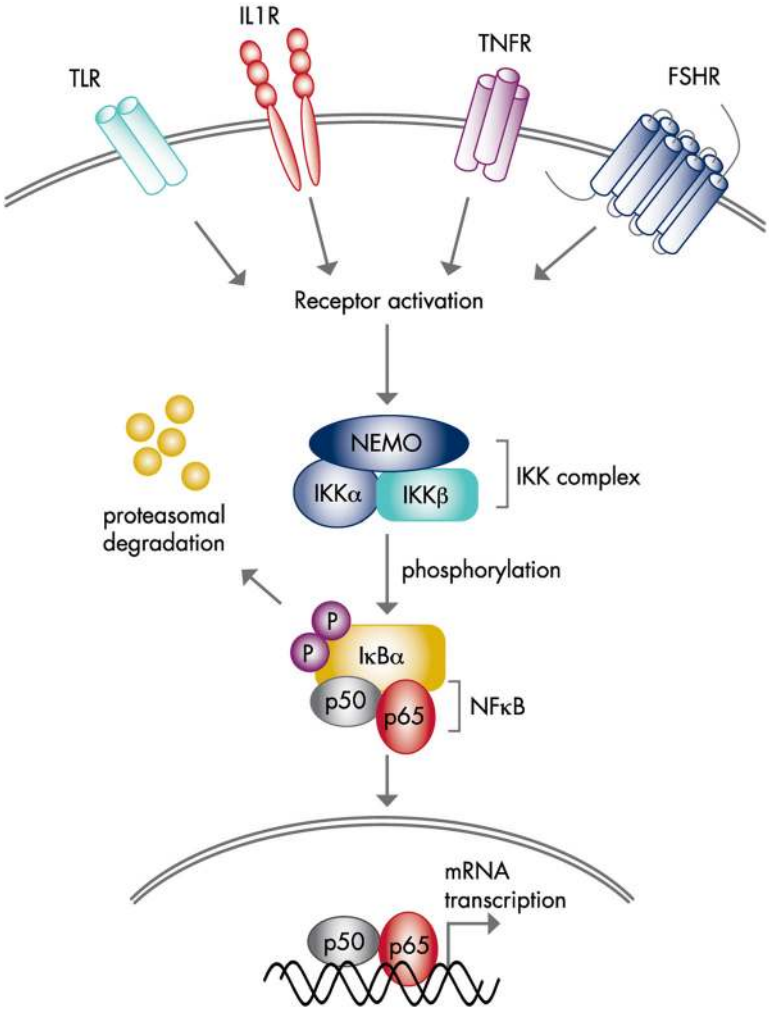
Despite their absolute requirement for the maintenance of the ovarian phenotype, follicle development and optimum female fertility, the role of estrogen and the ER in granulosa cell biology remains incompletely understood. In the nonpregnant premenopausal female, the developing preovulatory follicle is the primary site of estrogen synthesis, evident by the FSH-induced expression of aromatase in granulosa cells. Because granulosa cells also express both ERα and ERβ, it has been suggested that they may mediate an autocrine estrogen action within the follicle (265). ERβ is predominantly and abundantly expressed in GCT, in contrast to ERα, which shows moderate expression in GCT (233). The important role ERβ plays in normal ovarian function has recently been reviewed (264).

Although a limited number of studies have demonstrated a response to hormonal treatment in GCT (*Section II.I.3*), the mechanism of its action remains to be addressed. Indeed, it can be argued that a direct role for estrogen in granulosa cell tumorigenesis is unlikely. Transrepression of ERβ signaling by the constitutive and inducible activation of the NFκB signaling pathway in the COV434 and KGN cell lines suggests the role of ERβ in GCT is likely to be antiproliferative (203). Furthermore, ERβ acts as an antiproliferative factor in other cancer types, including breast, prostate, and colon (266). Thus, if estrogen action is relevant to tumorigenesis, it is likely to involve ERα, which, in contrast to ERβ, is expressed at low levels in GCT (233). Therefore, we have speculated that estrogen is more likely to be acting on the tumor environment, such as stroma or on angiogenesis, rather than the tumor itself, if indeed it does play a role in granulosa cell tumorigenesis (267).

### D. Nuclear factor κB

To gain insight into the function of ERβ in granulosa cells and GCT, Chu *et al.* (203) used COV434 and KGN cell lines as an *in vitro* model. Like human GCT (233), the cell lines were shown to predominantly express both ERβ mRNA and protein, with no ERα protein observed (203). Interestingly, however, despite ERβ expression and the ability to functionally bind estradiol, when cells were transfected with estrogen-responsive reporter constructs and treated with estradiol, no response was observed

**Figure 6.**




The canonical pathway of NFκB activation. The canonical NFκB pathway is mediated by the IκB kinase (IKK) complex [consisting of the IKKα and IKKβ catalytic subunits bound to the IKKγ/NFκB essential modulator (NEMO) scaffold protein]. Activation of the IKK complex can be initiated by a wide range of stimuli leading to the phosphorylation of IκBα. The phosphorylated IκBα is targeted for polyubiquitination and 26S proteasomal degradation. Free NFκB dimers can then enter the nucleus to activate transcription of target genes.

(203). To investigate whether this transcriptional repression was restricted to ERβ, a range of other reporter constructs were employed, containing the glucocorticoid receptor (GR) response element, as well as reporter constructs containing enhancer elements for second messenger pathways, including CREB, heat shock, and the MAPK reporters AP-1 and NFκB. The results revealed two interesting findings; first, GR-mediated transactivation was also repressed, demonstrating that transrepression was not limited to the ER, and second, although the CREB, heat shock, and MAPK reporters could all be induced by the appropriate stimulus, both the AP-1 and NFκB reporters exhibited constitutive activation under serum-free conditions (203). Furthermore, although inhibition of AP-1 using MAPK inhibitors had no effect on ER transcriptional repression, inhibition of the NFκB pathway using the inhibitor of κBα (IκBα)-specific inhibitor, BAY11-7082, restored both ER- and GR-mediated transactivation (203). These data demonstrate that the functional consequence of both constitutive and ligand-dependent NFκB activity is the transrepression of ERβ-mediated transcription in the COV434 and KGN cell lines (203).

Furthermore, we have also shown that although inhibition of NFκB signaling by blocking phosphorylation of IκBα down-regulated the constitutive activity (Fig. 6), it also dose-dependently decreased cell proliferation and cell viability while dose-dependently increasing cellular apoptosis in both cell lines (Jamieson, S., and P.J. Fuller, unpublished data). These findings indicate that unopposed NFκB signaling mediates the properties of oncogenic transformation in granulosa cells, that is, enhanced growth activity and protection from apoptotic cell death, and that inhibition of this pathway attenuates these cellular functions.

To date, little is known about the function of NFκB in normal granulosa cells. Wang *et al.* (268) reported that the NFκB pathway mediates the FSH-induced up-regulation of X-linked inhibitor of apoptosis expression in rodent granulosa cells via the PI3K/AKT pathway, thereby contributing to follicular growth. The activation of NFκB may therefore provide GCT with a survival advantage not only through its antiapoptotic effects but also through transrepression of ERβ signaling (203). The expression of cell surface signaling complexes involved in the inflammatory response, including toll-like receptor 4 (TLR4), has previously been reported in murine (269), bovine (270), and human (271) granulosa cells. Woods *et al.* (272) recently reported that TLR4 activation by bacterial lipopolysaccharide activates the NFκB pathway in the KGN and COV434 cell lines; however, the significance of TLR4 expression in GCT remains to be explored.

In view of the constitutive activation of NFκB the effect of the proteasome inhibitor bortezomib (MG-341, PS-341, Velcade) was examined in KGN and COV434 cells (273). Bortezomib, rationally designed to target NFκB activity, was approved by the FDA in 2003 for the treatment of relapsed and refractory multiple myeloma and, when combined with chemotherapy, may achieve a complete response (274). Chu *et al.* (273) showed that bortezomib

dose-dependently inhibits cell proliferation and viability in COV434 and KGN cells while promoting apoptosis. NFκB constitutive activity was not decreased, however, suggesting that although advanced-stage GCT may respond to bortezomib treatment in a clinical setting, its effect appears to be independent of NFκB signaling (273). In addition, this study suggests that alteration of proteasome function is not contributing to the constitutive NFκB activity observed in KGN and COV434 cells (273).

### E. Oncogenes and tumor suppressors

Known oncogenes have been investigated as possible candidates in the pathogenesis of GCT, including c-myc, p21-ras, and c-erbB2 (53); K-, N-, and H-ras and B-raf (275); and WT1 (276), but no evidence of mutation or overexpression was revealed. Likewise, mutation or loss of heterozygosity in the tumor suppressor p53 is not a feature of GCT (53, 277, 278).

### F. Other signaling factors

#### *1. Wnt/β-catenin*

The *Wnt* ligands comprise a large family of highly conserved, extracellular signaling glycoproteins that act locally to regulate a diverse range of developmental and homeostatic processes (279). To date, 19 WNT members have been identified in humans, and two subclasses of Wnt proteins have been loosely classified based on functional assays: the canonical Wnt, which use a common β-catenin-dependent signaling cascade, and the noncanonical Wnt, which are less well understood but appear to signal in a β-catenin-independent manner (279). Canonical Wnt transduce their signals by binding to the N-terminal extracellular cysteine-rich domain of the Frizzled family of seven-transmembrane G protein-coupled receptors (280), 10 of which are encoded in the human genome (281). In the absence of Wnt signals, β-catenin (CTNNB1) accumulates in the cytoplasm where it is constitutively engaged to a large destruction complex in which axin and adenomatous polyposis coli function as the scaffold proteins to which glycogen synthase kinase 3β (GSK3β) and casein kinase I (CKI) bind and rapidly phosphorylate β-catenin. Phosphorylated β-catenin becomes ubiquitinated and is targeted for proteasomal degradation, resulting in little or no free β-catenin in the cytoplasmic pool in the resting state. Upon Wnt binding to their respective cognate Frizzled receptors, the signal is transduced via a cytosolic phosphoprotein, Dishevelled (DSH), which recruits the destruction complex to the plasma membrane, thereby blocking β-catenin phosphorylation. Unphosphorylated β-catenin then accumulates in the cytoplasm and is translocated to the nucleus where it forms a complex with the T cell-specific transcription factor/lymphoid enhancer-binding factor 1 (TCF/LEF) family of transcription factors to regulate the transcription of WNT target genes (279).

The essential role WNT signaling plays in mammalian sex determination and ovarian development is well established (reviewed in Refs. 282 and 283). In granulosa cells of the adult ovary, the expression of WNT2 (284) and WNT4 (285, 286) has been observed. WNT2 binds the frizzled-9 receptor (FZD9; cluster of differentiation 349, CD349) to signal via the canonical pathway in cultured human cumulus cells (287). Furthermore, knockdown and overexpression studies of Wnt2 in cultured mouse granulosa cells have revealed that WNT2/β-catenin signaling regulates granulosa cell proliferation, suggesting that it may also play an important role in human folliculogenesis (288).

Aside from being essential for the formation of the female reproductive system during embryogenesis (289), WNT4 expression persists in the adult ovary and has been detected in granulosa cells at all stages of folliculogenesis from the small growing follicle through to the corpora lutea (283, 286). Conditional knockout studies ablating Wnt4 gene expression in granulosa cells resulted in mice that, although born with a normal ovarian reserve, underwent premature follicle depletion and had only approximately 25% of healthy antral follicles when compared with controls or, in some cases, complete loss of antral follicles or corpora lutea (290). This finding suggests that WNT4 mediates normal follicular development and is required for granulosa cell survival during the later phases of folliculogenesis (290).

Dysregulation of WNT signaling is associated with many forms of human cancer (291, 292). In particular, mutations in the genes encoding axin, adenomatous polyposis coli, and β-catenin have been found to cause aberrant activation of WNT/β-catenin signaling. Boerboom *et al.* (293) examined archival human and equine GCT samples for β-catenin expression by immunohistochemistry and found that a large proportion of equine GCT (14 of 18) displayed β-catenin expression localized to the nucleus, indicative of hyperactivation of the WNT/β-catenin pathway. In contrast, only one of six human GCT samples exhibited β-catenin nuclear localization (293), a finding that was supported by Ohishi *et al.* (294) who observed β-catenin nuclear localization to be absent in all of the 32 human GCT samples examined. Interestingly, mice expressing a dominant-stable mutant form of β-catenin in their granulosa cells developed late-onset GCT with high penetrance (293) (discussed further in *Section IV.E*).

Although the Wnt4 conditional knockout (290) and β-catenin-overexpressing (293) animal models further im-

plicate a role for the WNT/β-catenin pathway in granulosa cell proliferation and follicular development, its possible involvement in the etiology of human GCT remains to be established.

#### 2. IGF system

The autocrine/paracrine action of the IGF system is hormonally regulated in the ovary where it contributes to granulosa cell growth and function (295). The response of granulosa cells to gonadotropins is regulated by locally produced IGF, which play a key role in sensitizing granulosa cells to the actions of FSH during the terminal stages of folliculogenesis (296).

The IGF system is composed of two ligands, IGF-I and IGF-II; two receptors, the type I receptor, which mediates most of the somatomedin-like actions of both IGF-I and IGF-II, and the type II receptor, which binds IGF-II alone and appears to be involved in degradation of IGF-II; and six IGF-binding proteins (IGFBP), which bind IGF-I and IGF-II with high affinity and increase IGF's half-life, thereby maintaining a stable pool of IGF in all biological fluids of the organism. Furthermore, IGFBP can be subdivided into two groups that either inhibit or enhance IGF action on target cells (reviewed in Refs. 296–298).

The somatic cells of the ovarian follicle express genes encoding IGF-I, IGF-II, and the IGFBP in a spatiotemporally regulated manner (265). In addition, they also exhibit distinct species-specific patterns of expression. For example, in rodents and pigs, the expression of mRNA encoding IGF-I is confined to granulosa cells (299). In contrast, in humans, mRNA encoding IGF-II but not IGF-I is localized to granulosa cells (299), whereas *in situ* hybridization experiments have shown that IGF-I is expressed at low levels in the theca cells of antral follicles (297). Based on this pattern of expression, it is suggested that IGF-I and IGF-II drive follicle antrum formation (300). Indeed, although IGF-I has no effect on primordial follicle development, both IGF-I and IGF-II promote granulosa cell proliferation in secondary follicles (298). Furthermore, Kamada *et al.* (301) suggest that IGF-II may be a general stimulator in the proliferation and differentiation of granulosa cells and that cAMP may be a second messenger for the effects of IGF-II in granulosa cells. IGFBP produced in the ovary may also contribute to the local modulation of gonadotropin action via interaction with components of the cAMP system (265) (reviewed in Refs. 296–299 and 302).

The IGF system and the signal transduction networks it regulates play important roles in cancer development (reviewed in Ref. 303), including epithelial ovarian cancer (304). In the human follicle, the predominant IGF is IGF-II, and its actions are modulated by IGFBP-4 and the IGFBP-4 protease, pregnancy-associated plasma protein-A (PAPP-A) (305, 306). Alexiadis *et al.* (307) characterized the expression of IGF-I, IGF-II, IGFBP-4, and PAPP-A in a panel of GCT samples and compared levels with those observed in normal ovary and in epithelial ovarian tumor samples. Although both IGF-I and IGF-II were expressed in GCT, the levels were lower than in the normal ovary and in epithelial ovarian tumors. IGFBP-4 expression was also low in the GCT, whereas PAPP-A gene expression was highest in the GCT (307). Given the prominent role that the IGF signaling system plays in normal granulosa cells, these observations suggest that the IGF system does not play a role in the pathogenesis of GCT, with PAPP-A likely to be subserving a function other than IGFBP-4 proteolysis (307).

#### 3. GATA-4

GATA-4 is one of six members of the GATA family of zinc finger transcription factors that regulate the expression of genes in which the promoter or enhancer contains the GATA sequence motif (A/T)GATA(A/G) (308). The sexually dimorphic pattern of GATA-4 expression during mouse embryogenesis suggests it may be a regulator of genes involved in gonadal development and sex differentiation in mammals (309). Indeed, GATA-4 has been shown to regulate the sex-determining genes *SRY* (sex-determining region Y chromosome), *SOX9* (SRY-box containing gene 9), and *MIS* as well as key steroidogenic factors in the ovary, including steroidogenic acute regulatory protein (StAR), P450 aromatase (CYP19A1), the inhibin α-subunit (INHA), and 17β-hydroxysteroid dehydrogenase type 1 (HSD17B1) (310).

GATA-4 is also implicated in postnatal gonadal development in both males and females, with granulosa cells being the major site of GATA-4 mRNA expression in the adult human and murine ovary (311). GATA-4 is spatiotemporally expressed in granulosa cells, with GATA-4 mRNA expression negligible in primordial follicles, high from the primary through to antral stages of folliculogenesis, followed by rapidly diminishing levels during ovulation and corpora lutea formation (171, 311–314). Furthermore, within the proliferating follicle, GATA-4 mRNA levels are higher in cumulus granulosa cells than mural granulosa cells (311).

This pattern of expression is consistent with mediation of granulosa cell proliferation, and given the evidence for FSH-mediated up-regulation of GATA-4 expression in the mouse ovary and testis (311), these data suggest GATA-4 activity is likely induced by the classical FSH/cAMP/PKA pathway in granulosa cells and plays a role in normal granulosa cell proliferation (310, 315, 316). Furthermore, GATA-4 has also been shown to participate in TGFβ-mediated activation of the inhibin α-subunit via interaction with SMAD3 in cultured mouse ovarian tumor cells (316).

Consistent with its localization to preovulatory granulosa cells, GATA-4 and a GATA family transcriptional cofactor, Friend of GATA 2 (FOG-2), are also expressed in GCT (135, 171). Using immunohistochemical analyses of primary GCT, Anttonen *et al.* (135) reported that 35 of 80 (44%) exhibited high GATA-4 expression; that is, expression was equivalent to that seen in normal granulosa cells. Expression correlated positively with clinical stage (stage Ic or higher) and risk of recurrence, suggesting GATA-4 immunostaining may serve as a prognostic tool in predicting tumor aggressiveness (135).

Following on from these studies, the same group also reported that GATA-4 expression correlated with Bcl-2 (BCL2; B-cell lymphoma 2) and cyclin D2 (CCND2) expression in both human and murine GCT and in particular that GATA-4 participates in the regulation of Bcl-2 expression in human GCT (314). Given that the antiapoptotic gene Bcl-2 is under GATA-4 control in other cell types (317, 318), one may postulate that increased GATA-4 expression promotes a prosurvival mechanism in granulosa cells by up-regulating the activation of Bcl-2, thereby protecting cells from apoptosis and contributing to granulosa cell tumorigenesis (314).

### G. Apoptosis

To date, much of the research into the pathogenesis of GCT has focused on the aberrant activation of pathways and oncogenes known to exert a pro-proliferative or prosurvival effect on granulosa cells. Follicular growth and differentiation from the primordial stage through to a fully competent corpus luteum is, of course, a highly complex process that is realized by less than 0.1% of follicles (319). The maintenance of optimum fertility in females relies on a delicate balance between signals for cell survival in follicles that are recruited for maturation during each ovulatory cycle and signals for cell death in those that, at some point, must be eliminated by atresia. Therefore, it is reasonable to postulate that impaired apoptosis is likely to be a contributing mechanism in granulosa cell tumorigenesis (174).

Several studies have examined the potential involvement of the naturally occurring cytokine, TNF-related apoptosis-inducing ligand (TRAIL; cluster of differentiation 253, CD253) and its death domain-containing transmembrane receptors, TRAIL receptor 1 (TRAIL-R1)/death receptor 4 and TRAIL-R2/DR5, in GCT. A member of the TNF superfamily, TRAIL has been under intense focus due to its ability to preferentially induce programmed cell death in a number of human malignancies while exhibiting little or no toxicity in normal cells (320). This discovery prompted a rapid surge in studies evaluating the efficacy of cancer therapeutic agents that can activate the TRAIL apoptotic pathway, and several phase II clinical trials are underway. These therapeutic agents include recombinant human TRAIL (rhTRAIL) and DR4-/DR5-specific agonistic monoclonal antibodies (321). Resistance to TRAIL therapy is frequently encountered, requiring the resensitization of malignant cells to TRAIL by combinatorial treatment with chemotherapy or radiation (321).

TRAIL mRNA expression has been reported in avian and porcine granulosa cells (322, 323) as well as human nonneoplastic granulosa cells (derived from *in vitro* fertilization) and the KGN and COV434 cell lines (324). *In situ* hybridization and immunohistochemical analysis has also shown TRAIL and its receptors to be expressed in adult human granulosa cells at multiple stages of folliculogenesis (325). Immunohistochemical analysis of a tissue microarray containing 80 primary and 12 recurrent GCT samples showed DR4 expression to be strong or intermediate in 18 and 73% of samples, respectively, with only 9% exhibiting low DR4 expression levels (326). Similar results were seen for DR5 with expression regarded as strong, intermediate, or low in 17, 75, and 8% of samples, respectively (326).

*In vitro* studies using the two GCT-derived cell lines showed that although rhTRAIL induced a slight decrease in viability for both cell lines, treatment in combination with a proteasome inhibitor (Z-LLF-CHO) synergistically enhanced the TRAIL-induced loss of viability (327). This occurred independently of p53 activity and was, at least in part, due to the up-regulation of DR5 and the proapoptotic protein Bax (327). Moreover, in comparison with treatment with TRAIL alone, the reduction in cell viability observed in combination with the proteasome inhibitor occurred in a caspase-8-independent manner (327). Another study by the same group also showed that the TRAIL-induced loss of viability in the COV434 and KGN cell lines could be enhanced through combinatorial treatment with the conventional chemotherapeutic cisplatin (324). Unlike proteasomal inhibition, however, the cisplatin-induced cell death and enhanced TRAIL sensitivity occurred in a partially p53-dependent manner, suggesting multiple mechanisms and sites of action are involved in synergistic activity (324).

As discussed in *Section II.F.3*, increased GATA-4 expression correlates with increased expression of the antiapoptotic protein Bcl-2 in human GCT (314). Targeted overexpression of Bcl-2 in ovarian somatic cells has been shown to result in decreased apoptosis and enhanced folliculogenesis in mice (328). Kyrönlahti *et al.* (326) showed that rhTRAIL dose-dependently activated caspase-3 and induced apoptosis in isolated primary human GCT cells and the KGN cell line (326). The ability of GATA-4 to modify TRAIL-induced apoptosis in human GCT was examined, and although the treatment of KGN cells with

TRAIL had no effect on endogenous GATA-4 levels, inhibition of GATA-4 expression by either dominant-negative adenovirus or short hairpin RNA-producing constructs sensitized KGN cells to TRAIL-induced apoptosis (326). However, this effect was observed even in the absence of exogenous TRAIL, suggesting that GATA-4 alone may function as an antiapoptotic factor in GCT and that the effect of GATA-4 knockdown is not TRAIL specific (326).

#### H. *FOXL2*

As described in *Section II.F.1*, adult GCT have recently been characterized by a single somatic missense mutation in the *FOXL2* gene (c.402C→G; p.C134W) (4, 6–11). The *FOXL2* gene comprises a single exon that encodes a member of the forkhead domain/winged-helix family of transcription factors. Like the other members of the forkhead family, FOXL2 contains a characteristic winged helix DNA-binding domain of approximately 100 amino acids that binds DNA at a 7-bp core recognition motif (5′-G/A-T/C-C/A-A-A-C/T-A-3′) (329). During mammalian embryogenesis, FOXL2 is the earliest identified sexually dimorphic marker of ovarian differentiation (330–333). FOXL2 is specifically expressed in eyelids and in fetal and adult ovarian follicular cells (331); its expression persists in the ovary through reproductive life in the granulosa cells of developing follicles and the cumulus oophorus of the preovulatory follicle (334). Animal knockout studies have highlighted the important role FOXL2 plays in multiple stages of follicle development. Germline deletion of *Foxl2* in the mouse results in a high level of perinatal mortality and premature ovarian failure in those females that survive (332, 335). FOXL2 is required for optimal formation of primary follicles. In *Foxl2*-null ovaries, granulosa cells fail to complete the squamous to cuboidal transition, resulting in the arrest of folliculogenesis at the primordial follicle stage, which ultimately leads to premature follicular depletion (332, 335). Conditional deletion of *Foxl2* in adult female mice induces the transdifferentiation of granulosa cells into functional Sertoli cells, which then produce androgens rather than estrogens (336). This finding reveals an essential role for FOXL2 in the maintenance of the ovarian phenotype and normal granulosa cell function in adults. Furthermore, heterozygous *FOXL2* loss-of-function mutations result in the autosomal dominant disease blepharophimosis-ptosis-epicanthus inversus, which is associated with eyelid malformation and premature ovarian failure (330).

The expression levels of *FOXL2* in the adult GCT differ little from those in normal ovary with only occasional (two of 56), somewhat anomalous tumors exhibiting increased expression (6). Given the crucial role FOXL2 plays in normal granulosa cell biology, the molecular consequences of the C134W mutation in adult GCT remain to be elucidated. The mutation lies in the wing 2 domain of the forkhead-DNA binding domain of the FOXL2 protein (4), a highly conserved residue across species. Homology modeling indicates the mutation appears unlikely to compromise DNA binding but may influence the interaction with other transcription factors (4).

A number of targets of FOXL2 have been identified in both pituitary (337) and gonadal cells (334). In the ovary, these include a number of genes fundamental to granulosa cell function and follicle development, including growth differentiation factor 9 (GDF9), inhibin βA (INHBA), MIS (338), aromatase (339), and follistatin (340). FOXL2 has also been shown to repress promoter activity of the *StAR* gene (341). The regulation of gene expression by FOXL2 may also involve coregulatory interactions with other transcription factors including AP-1 (342, 343), the nuclear receptor SF-1 (344, 345), BMP2 (340), and the SMAD family of transcription factors (342, 346–348), all of which play fundamental roles in ovarian biology.

Fleming *et al.* (13) investigated whether the C134W mutation had any effect on the ability of FOXL2 to regulate the expression of its steroidogenic targets in GCT. Using the KGN and COV434 cell lines, it was shown that the mutation altered the regulation of the aromatase promoter but not the StAR promoter (13). Moreover, there was no evidence to suggest that the mutant protein directly alters DNA binding; instead, an alteration in an associated protein-protein interaction is likely (13). Fleming *et al.* (13) suggest that the unknown candidate protein may be FOXL2 itself; with the mutation inhibiting FOXL2's ability to form a homodimer, for instance. This interaction may provide the mechanism via which hyperestrogenism is manifested, as seen in 70% of patients with GCT.

Given their previous finding that FOXL2 overexpression induces apoptosis in rat granulosa cells (349), Kim *et al.* (7) examined the effect of the C134W mutation on granulosa cell apoptosis. Overexpression of wild-type FOXL2 in the KGN cell line significantly increased cellular apoptosis in comparison with cells in which C134W FOXL2 was overexpressed (14). Moreover, it was shown that FOXL2-induced apoptosis was dependent on caspase-8-mediated B-cell lymphoma-2 homology 3 interacting domain death agonist and Bcl-2 homologous antagonist signaling and that wild-type FOXL2 significantly up-regulated the death receptors, TNFR1 and Fas, in comparison with mutant FOXL2 (14). These data suggest that FOXL2 acts as a tumor suppressor in normal granulosa cells and that the C134W mutation impairs FOXL2's ability to mediate death ligand-induced apoptosis (14). Fur-

ther investigation is required to determine the underlying mechanism via which this occurs.

The C134W mutant might be postulated to be either an activating mutation or a gain-of-function mutation; however, Benayoun *et al.* (12) found limited evidence to support activation in an *in vitro* assay. Given the high homology with other members of the FOX family, several of which also play fundamental roles in ovarian function, such as FOXO3a (350), a gain-of-function mutation is a distinct possibility. Based on their findings, Benayoun *et al.* (351–353) have argued that FOXL2 is a tumor suppressor gene. Although decreased FOXL2 expression is a feature of advanced disease in juvenile GCT (92), this may be a bystander rather than a driver effect (354). Of 233 adult GCT reported to be C134W mutation positive, with the exception of six cases, no evidence of loss of the wild-type *FOXL2* allele was reported (4, 6–8, 10, 11), as would be expected for a tumor suppressor gene. Although it remains formally possible that the mutation represents very targeted inactivation of a specific tumor suppressor activity that is dosage dependent, the presence of a single, specific, heterozygous mutation would seem rather more analogous to the situation seen for other oncogenic or pro-proliferative mutations, as is observed with the *RET* (M918T) protooncogene in multiple endocrine neoplasia (MEN) type 2 (355), the *RAS* and *BRAF* (V600E) mutations in ovarian, thyroid, and other tumors (356, 357), and the *JAK2* (V617F) mutation in myeloproliferative disease (358). Conversely, inactivation of a tumor suppressor gene such as the *MEN1* gene in MEN type 1 (359) or the *BRCA1/2* genes in breast cancer (360, 361) is associated with a myriad of mutations.

## IV. Transgenic Mouse Models

A number of transgenic mouse models that develop GCT have been identified; however, whether any truly recapitulate the human situation is not clear.

### A. Inhibin α-subunit knockout

To investigate the role of inhibin in mammalian reproduction and development, Matzuk *et al.* (218) employed homologous recombination in mouse embryonic stem cells to delete the α-inhibin gene, creating a transgenic model that is completely inhibin deficient. Mice homozygous for the deletion were susceptible to the development of bilateral, mixed, or incompletely differentiated sex cord-stromal tumors that developed with 100% penetrance in both sexes and, in some cases, appeared as early as 4 wk of age (218). In females homozygous for the deletion, tumors were typically multifocal, hemorrhagic, and of mixed granulosa/Sertoli cell appearance, whereas the male littermates developed intratubular testicular lesions of Sertoli cell origin that often resembled juvenile GCT (218, 219). In all homozygous null animals, tumor development was rapidly accompanied by a severe cancer cachexia-like wasting syndrome that was caused by a >10-fold increase in circulating activin and ultimately resulted in death (362, 363). Inhibin-deficient mice gonadectomized at an early age were rescued from the cachexia syndrome but went on to develop adrenal sex cord steroidogenic tumors with nearly 100% penetrance and ultimately succumbed to a wasting syndrome similar to that seen in the intact α-inhibin-null animals (362). Therefore, it is suggested that inhibin is a tumor suppressor with specificity to the gonads and adrenal cortex (218, 219, 362).

Using the α-inhibin-null animal as a model system for gonadal sex cord-stromal tumorigenesis, Matzuk and colleagues have performed comprehensive cross-breeding programs with other conditional knockout models to investigate the role of potential modifiers in disease progression, including the gonadotropins, sex steroid hormones and receptors, cell cycle regulators, and activin signaling (reviewed in Ref. 364). In general, deletion of the gonadotropins and activin signaling modulators resulted in complete loss or delayed onset of tumors accompanied by absence of the cachexia-like syndrome when compared with $Inha^{-/-}$ mice (364). The cachexia-like syndrome was shown to be directly caused by increased activin signaling through the ActRII receptor, with symptoms minimized in double-homozygous mutant mice null for both inhibin and ActRII (363). In contrast, loss of the sex steroid hormones and receptors resulted in accelerated tumor development and an earlier onset of the wasting syndrome (364).

Additional studies employing a transgenic model in which inhibin-deficient animals carry the mouse metallothionein I-follistatin transgene ($inha^{m1}/inha^{m1}$, MT-FS$^{+}$) revealed that although histologically similar, gonadal tumors still developed, mice exhibited a less severe wasting syndrome, lower serum activin levels, and a statistically significant prolonged survival in a number of cases compared with inhibin-null mice alone (365). These data suggest that follistatin can act as a physiological modifier to block the activin-mediated cachexia-like syndrome and/or slow the progression of gonadal tumors in these mice (365).

### B. Targeted overexpression of luteinizing hormone

To investigate the direct role of hypersecretion of LH on reproductive abnormalities, Risma *et al.* (366) generated a transgenic mouse model in which elevated serum LH levels were chronically maintained. This was achieved by introducing a transgene containing a bovine α-subunit promoter to drive the expression of a chimeric LHβ sub-

unit containing the carboxyl-terminal peptide (CTP) of the human chorionic gonadotropin β-subunit (hCGβ) linked to the carboxy terminus of the bovine LHβ subunit (366). This bLHβ-CTP insert, expressed exclusively in the gonadotropes of the anterior pituitary, resulted in elevated levels of serum LH by 1) increasing the secretion of hormones from the pituitary and 2) extending the half-life of LH heterodimers containing the chimeric β-subunit by slowing their elimination rate from serum (366). The resultant transgenic animals had serum LH levels that were elevated 5- to 10-fold above that of nontransgenic controls (366). By 4–9 months of age, a subset of the bLHβ-CTP females developed GCT and theca-interstitial cell tumors, suggesting that altered gonadotropin levels are tumorigenic (366). It was subsequently shown that LH induction of GCT in bLHβ-CTP transgenics is strain specific (367). When the transgene was present in a CF-1 background, all females developed GCT and pituitary hyperplasia by 5 months of age (n = 8), whereas in hybrid mice, generated by crossing CF-1 males with C57BL/6 (n = 6), SJL (n = 4), or CD-1 (n = 13) females, their ovaries developed a luteoma rather than GCT, and the pituitary developed pituitary hyperplasia and subsequent adenoma (367, 368). Moreover, 5-month-old transgenic females had serum LH levels that were elevated only 2-fold over that of their nontransgenic littermates (366, 367, 369). Compared with the 5- to 10-fold increase observed in younger mice of the same strain, a situation analogous to the elevated serum inhibin levels observed in the human disease state, this observation suggests GCT-derived inhibin negatively impacts transgene expression (367, 368). Together these results suggest that although chronically elevated serum LH levels can result in the development of GCT, the effect is also linked to an underlying genetic predisposition. Indeed, the strain dependency of tumor development provides a platform via which the mechanism of LH-induced tumorigenesis may be elucidated (368).

As discussed previously, no definitive association between gonadotropin hyperstimulation, in the context of infertility treatment for example, and the development of ovarian tumors has been observed in humans. The results of these animal studies may therefore add weight to the argument that abnormal gonadotropin stimulation is tumorigenic (366). This finding is also supported by studies in which the suppression of FSH and LH levels in inhibin-deficient animals ($Inha^{-/-}$ $Gnrh1^{hpg/hpg}$) resulted in loss of both tumor development and suppression of the cachexia-like syndrome observed in the inhibin-α-knockout mouse (370).

### C. Simian virus 40 T-antigen driven by inhibin α-subunit promoter

With the aim of establishing *in vivo* gonadal tumor models and immortalized gonadal somatic cell lines, Kananen *et al.* (371) generated transgenic mice in which the simian virus 40 T-antigen (SV40 TAg) was driven by either 6- or 2.1-kb fragments of the mouse inhibin α-subunit promoter (inhα/TAg). Female animals carrying the 6-kb fragment were infertile and developed GCT with 100% penetrance by 5–7 months of age (n = 36) (371–373). Further investigation of this model revealed a physiological state congruous with that of human GCT patients, including continued folliculogenesis, depressed serum gonadotropins, elevated serum inhibin levels, and similar histopathological features (374). Suppression of circulating gonadotropins by administration of a GnRH antagonist or crossbreeding onto a gonadotropin-deficient hypogonadal mutant (*hpg*) background prevented tumor development in gonad-intact mice (374, 375). Prepubertal gonadectomy resulted in adrenocortical tumors that were also lost after induced hypogonadotropic hypogonadism, suggesting tumor development was related to elevated gonadotropin secretion (372, 375).

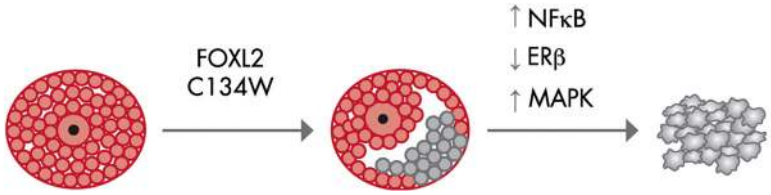
When the inhα/TAg animals were crossed with those producing constitutively elevated levels of LH (bLHβ-CTP), the resultant double transgenics (bLHβ-CTP/inhα/TAg) displayed earlier tumor formation and more rapid disease progression than inhα/TAg animals (376). This occurred in conjunction with suppressed FSH secretion, suggesting that either high-level exposure to LH or altered gonadotropin ratios have a tumor promoter effect (376).

### D. SMAD knockouts

Given the role of elevated serum activin levels in gonadal tumorigenesis and subsequent fatal, cachexia-like syndrome in inhibin-α-deficient mice, various Smad knockout models have been developed to further investigate the contribution of downstream components of TGFβ receptor complex signal transduction.

Activins signal via the activin/TGFβ-specific receptor-regulated SMAD, SMAD2 and SMAD3. $Inha^{-/-}$ $Smad3^{-/-}$ double-knockout mice exhibited delayed tumor development and increased survival due to the uncoupling of activin signaling from the cell cycle machinery, thus attenuating ovarian tumor progression and delaying the onset of the cachexia-like syndrome (377, 378). Ovarian tumors developed by 26 wk of age in the majority of double-knockout females, compared with 4 wk in $Inha^{-/-}$ animals, suggesting that multiple genes contribute to inhibin-deficient gonadal tumorigenesis in females (377). In addition, delayed tumor development was significantly more pronounced in the male littermates with tumors either absent or unilaterally

**Figure 7.**




Adult GCT are defined by a single somatic missense mutation in the *FOXL2* gene (c. 402C→G; p.C134W). Although the high frequency with which the mutation occurs suggests it is etiological for adult GCT, it does not explain differences in tumor stage, disease recurrence, or aggressiveness. The molecular changes that drive disease progression are likely to involve the subversion of signaling pathways essential for normal granulosa cell biology. Evidence suggests these may include NFκB, MAPK/ERK, and ERβ.

slow growing, indicating that although SMAD3 may be the principal transducer of gonadal tumorigenesis in males, it potentially overlaps with SMAD2 function in granulosa cells (377).

To examine the effect of loss of the BMP-specific receptor-regulated SMAD, SMAD1, SMAD5, and SMAD8, which modulate BMP and MIS signaling, conditional gonadal somatic cell Smad1/5 and Smad5/8 double knockouts and Smad1/5/8 triple knockouts were generated (379). Smad5/8 double knockouts were viable and fertile (379). In contrast, both Smad1/5 double knockouts (n = 15) and Smad1/5/8 triple knockouts (n = 15) developed poorly differentiated, unilateral or bilateral GCT with 100% penetrance by 3 months of age (379). Approximately 80% of aged mice developed peritoneal and lymphatic tumor metastases (379). Further characterization of the Smad1/5 double-knockout model revealed close physiological and histological similarities to that of human juvenile GCT (380). Together these models imply roles for Smad1 and Smad5 as tumor suppressors with redundant functions (379).

#### E. Constitutively activated Wnt/β-catenin

Boerboom *et al.* (293) showed that targeted constitutive activation of β-catenin (CTNNB1) in granulosa cells, via generation of mice that express a dominant stable β-catenin mutant (*Catnb*$^{flox(ex3)/+}$;*Amhr2*$^{Cre/+}$), resulted in the development of multiple ovarian lesions resembling disorganized follicles and cystic structures, which often evolved into GCT in older mice (293). These tumors, which exhibited many histopathological similarities to the human disease, were not detected before 19 wk of age (n = 0 of 28), appeared in 44% of animals by 6 months of age (n = 4 of 9), and reached a maximum penetrance of 57% by 7.5 months of age (n = 8 of 14) (293). Although these data suggest a causal link between misregulated Wnt/β-catenin signaling and GCT development, the late onset of the tumors may indicate that this pathway alone is insufficient to cause GCT. Building upon this study, the same *Amhr2*$^{Cre/+}$ line was crossed with *Pten*$^{flox/flox}$ animals to conditionally target the PI3K antagonist gene *Pten* in granulosa cells (*Pten*$^{flox/flox}$;*Amhr2*$^{Cre/+}$) (172). In the resultant model, in which the PI3K/AKT pathway was constitutively activated, five of 70 mice (~7%) developed aggressive and metastatic GCT (172). Interestingly, when crossed with the *Catnb*$^{flox(ex3)/+}$;*Amhr2*$^{Cre/+}$ model (*Pten*$^{flox/flox}$;*Ctnnb1*$^{flox(ex3)/+}$;*Amhr2*$^{cre/+}$), mice developed perinatal-onset, bilateral GCT with 100% penetrance, suggesting a synergistic effect between the Wnt/β-catenin and PI3K/AKT pathways (172).

#### F. Two-yr-old βERKO

As mentioned previously, ERα and ERβ are differentially expressed and regulated in specific tissues. It has recently been reported that one of the lines of female mice null for ERβ (βERKO) (247) develop sex cord tumors (less differentiated) and GCT (differentiated and estrogen secreting) with 100% penetrance by 2 yr of age (n = 23) (217). Spontaneous tumor development was not observed in αERKO or αβERKO female animals suggesting ERα is required for tumor development (217). Furthermore, it was shown that phospho-SMAD2/3 was highly expressed in the nuclei of tumor cells, as was LH receptor expression (217). Given the similarities with *Inha*$^{-/-}$ females, Fan *et al.* (217) suggest that in the absence of ERβ, proliferative actions via the FSH/SMAD3 pathway are able to signal unopposed. In addition, the increased expression of ERα further contributes to tumor progression by increasing estrogen-stimulated granulosa cell proliferation (217).

## V. Future Directions

The evidence is now compelling that the somatic C134W mutation in the *FOXL2* gene is etiological in the development of adult GCT. Because the mutation is absent from juvenile GCT it confirms the suggestion, previously based on tumor morphology, that juvenile GCT are a distinct disease (Table 2). Although the mutation is clearly necessary in the adult subtype, it remains to be determined whether it is sufficient for tumorigenesis. It obviously does not explain differences in tumor stage, disease recurrence, aggressiveness, *etc.* Therefore, it follows that other, presumably mutational, events must be contributing to the pathogenesis of adult GCT (Fig. 7). The elucidation of these changes remains an important goal, particularly because they are likely to have prognostic and therapeutic

implications. The existing mouse models, although offering biological insights, have not contributed greatly to addressing the clinical problem; a mouse model in which expression of the *FOXL2* C124W mutation is induced in granulosa cells may be informative.

It is surprising that substantive transcription and/or gene expression studies have not been reported for adult GCT (45). The study of Shah *et al.* (4) includes a full transcription analysis, but this is restricted to four GCT only, without regard to tumor stage, *etc.* They do, however, note the relative genomic stability of these tumors; this suggests that a transcriptome analysis of a larger cohort, perhaps filtered by stage or behavior, might be both rewarding and revealing.

Although it is to be hoped that a more detailed understanding of the molecular pathogenesis of these tumors will identify key therapeutic targets, the existing evidence for upregulation of specific signaling pathways together with an emerging array of new therapeutic agents provides exciting applications for novel therapeutic approaches. Although the *in vitro* studies discussed suggest several possible approaches, these can only be validated through formal clinical trials. In view of the relative rarity of GCT, particularly beyond stage I tumors where surgical cure is achieved, international collaboration will be required to mount successful trials. Such trials might, for instance, focus on the use of TKI of the MAPK/ERK pathway and/or of NF$\kappa$B signaling. Given the relatively vascular nature of large GCT, it may be that they are susceptible to inhibition of angiogenesis using VEGF inhibitors, a strategy that may not target the tumor cells specifically. In reality it is unlikely that any one agent alone will be fully effective, but strategic coupling of agents targeting several pathways may allow lower doses, less toxicity, and greater efficacy (324).

## VI. Summary

The past few years have seen significant progress in the field of GCT research. In particular, the finding that the somatic *FOXL2* C134W mutation is characteristic of adult-type GCT is an exciting advance in clinical molecular oncology, yet many questions remain to be resolved. At a molecular level, the consequence of the mutation and its contribution to the mechanisms of GCT pathogenesis remains to be determined. In addition, because the majority of patients are diagnosed with stage I disease, the major clinical challenge in the management of adult GCT remains the identification of prognostic factors and/or tumor markers that are able to predict disease recurrence.

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