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Ovarian granulosa cell lines

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Abstract

The ovary is a complex endocrine gland responsible for production of sex steroids and is the source of fertilizable ova for reproduction. It also produces various growth factors, transcription factors and cytokines that assist in the complex signaling pathways of folliculogenesis. The ovary possesses two primary steroidogenic cell types. The theca cells (and to a lesser extent, the stroma) are responsible for androgen synthesis, and the granulosa cells are responsible for conversion of androgens to estrogens, as well as progesterone synthesis. These cells undergo a transformation in the luteal phase of the menstrual cycle, converting them from estrogen producing, to predominantly progesterone producing cells. Understanding the molecular mechanisms regulating these cells is essential in understanding the regulation of steroidogenesis and reproduction. Creation of appropriate in vitro cell model systems can provide important tools for the study of ovarian function. This has led to the development of ovarian steroidogenic cell lines in several laboratories. Developing theca cell lines has met with limited success. Conversely, numerous human and animal granulosa cell lines have been developed. This review will discuss the existing granulosa cell lines and their characteristics.

Keywords: Granulosa cell; Folliculogenesis; Estrogen

1. Background

The ovaries are complex endocrine organs developed from the bipotential gonad (Gillman, 1948). In adult life and under the stimulatory actions of the gonadotropins, they are responsible for the production of the sex steroids and are the source of fertilizable ova. The sex steroids act in an autocrine/paracrine fashion in the human ovary to produce the complex interactions necessary for folliculogenesis and act in an endocrine fashion to regulate the orderly hormonal cycle required for menses. The sex steroids are also responsible for the secondary sexual characteristics that appear with puberty. Ovulatory dysfunction is common at the extremes of reproductive age. Reproductive disorders such as polycystic ovarian syndrome (PCOS), manifested by chronic anovulation and hyperandrogenism, are common in reproductive-aged women and result in hirsutism, infertility and menstrual disturbances. Greater understanding of the mechanisms that regulate and control ovarian function and the perturbations in ovarian steroidogenesis in these disorders may lead to novel therapies for a variety of reproductive disorders.

The ovary is composed of three distinct regions: an outer cortex containing the germinal epithelium and the follicles, a central medulla consisting of stroma, and a hilum around the area of attachment of the ovary to the mesovarium (Carr, 1998). The steroidogenic cells of the ovary are the granulosa cells, which are the avascular cellular compartment surrounding the oocyte, and the theca cells, which reside in the ovarian stroma. These two cellular compartments are separated by the basal lamina (Weakly, 1966). The ovary secretes a number of steroids, including pregnenolone, progesterone, 17α-hydroxyprogesterone, 17α-hydroxypregnenolone, dehydroepiandrosterone, androstenedione, testosterone, estrone, and 17β-estradiol (Baird et al., 1974; Baird and Fraser, 1969). Under the control of various hormones, second-messenger signalling such as cyclic AMP (cAMP), cytokines and transcription factors, ovarian steroid produc-

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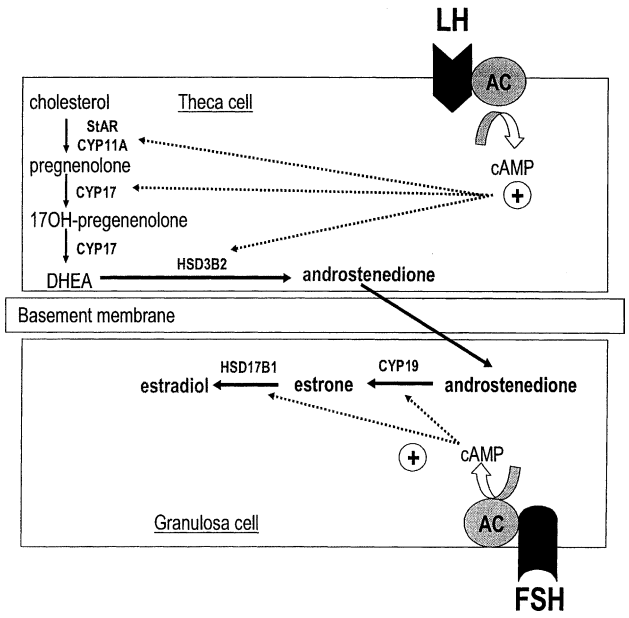

Fig. 1. Follicular phase steroid biosynthesis in the ovary with the illustration of the two-cell/two-gonadotropin theory. It should be noted that there is some species variation in steroidogenic pathways within the ovary and the pathway provided in this figure is characteristic of human. AC, adenylate cyclase; FSH, follicle stimulating hormone; LH, luteinizing hormone. Nomenclature for the enzymes: StAR, steroidogenic acute regulatory protein; CYP11A, cholesterol side-chain cleavage; CYP17, 17α-hydroxylase, 17,20-lyase; HSD3B2, 3β-hydroxysteroid dehydrogenase type II; HSD17B1, 17β-hydroxysteroid dehydrogenase type I; CYP19, aromatase.

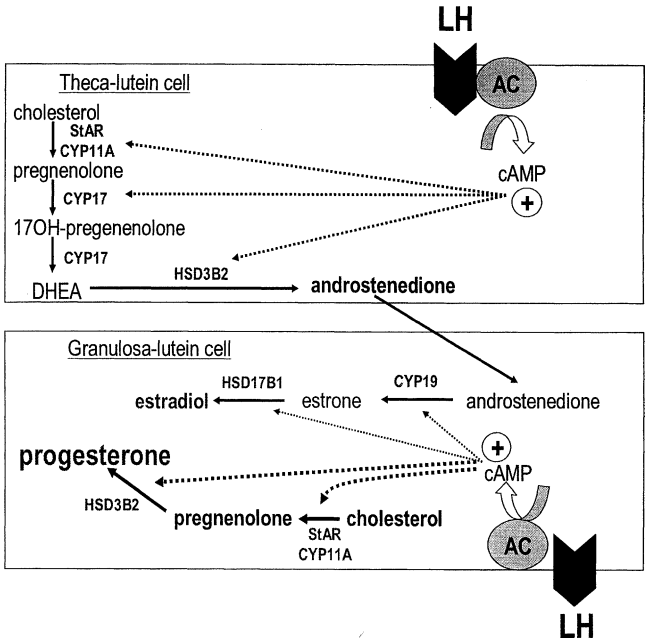

Fig. 2. Steroidogenic pathways for luteinized theca and granulosa cells. AC, adenylate cyclase; LH, luteinizing hormone. It should be noted that there is some species variation in steroidogenic pathways within the ovary and the pathway provided in this figure is characteristic of human. Some species lose CYP17 expression in theca-lutein cells, resulting in progesterone synthesis. AC, adenylate cyclase; FSH, follicle stimulating hormone; LH, luteinizing hormone. Nomenclature for the enzymes: StAR, steroidogenic acute regulatory protein; CYP11A, cholesterol side-chain cleavage; CYP17, 17α-hydroxylase, 17,20-lyase; HSD3B2, 30-hydroxysteroid dehydrogenase type II; HSD17B1, 17β-hydroxysteroid dehydrogenase type I; CYP19, aromatase.

tion qualitatively and quantitatively changes throughout the ovulatory cycle, with increased steroidogenic activity in the luteal phase, accompanied by a shift from a predominant estrogen producing to a progesterone producing organ.

In humans, ovarian steroidogenesis occurs according to the two-cell/two-gonadotropin theory (Ryan and Petro, 1966). For estrogen biosynthesis to occur, synthesis of C_{19} androgens from cholesterol occurs in the luteinizing hormone (LH) stimulated theca cell compartment, as CYP17 activity is predominantly limited to the theca cells (Sasano et al., 1989) (Fig. 1). These androgens then diffuse into the avascular, CYP17 deficient granulosa cell compartment. Under follicle stimulating hormone (FSH) stimulation, these androgens either undergo aromatization to estrogens via aromatase (CYP19) activity (Bjersing, 1968), or are preferentially 5α-reduced when present in an androgen-rich environment (McNatty et al., 1979). In the luteal phase, the luteinized granulosa cell compartment becomes vascularized, allowing delivery of cholesterol to the previously avascular granulosa cell compartment. Increased expression of steroidogenic enzymes also occurs, resulting in increased production of progesterone as well as estrogen derived from theca cell androgens (Fig. 2). While this model also holds for primates and some animals, variations exist, such as in the porcine ovary, that demonstrate cellular differences in steroidogenic enzyme expression, and as a result, steroidogenic capability (Conley et al., 1995). In addition, many animal species lose the ability to produce estrogen in the luteal phase. Thus, ovarian steroid production has evolved a number of species-specific differences in cell function.

Understanding the mechanisms which regulate ovarian steroidogenesis and folliculogenesis at the molecular and cellular level requires readily available cells for in vitro studies. For humans, the major source of granulosa cells for in vitro studies has been patients undergoing in vitro fertilization (IVF). These cells are limited in number, and under stimulation with supraphysiologic doses of FSH and human chorionic gonadotropin (hCG) in vivo, they have a limited life span in vitro (Breckwoldt et al., 1996). In addition, due to hyperstimulation with gonadotropins, these cells are normally fullly luteinized at the time of isolation. Because of this, utilizing primary cultures of human granulosa cells for research has been difficult. Furthermore, theca cells are even more difficult to obtain. The use of theca cell models through long-term theca-cell cultures or tumor cell models has allowed for greater understanding of these androgen producing cells, but development of thecal cell lines has met with limited success (Rainey et al., 1996a,b; McAllister et al., 1989; Magoffin and Erickson, 1988).

Due to these limitations, the use of granulosa cell lines for in vitro model systems has become an attractive alternative. As a result, granulosa cell lines have been developed from ro-

Table 1
The steroidogenic properties of rodent ovarian granulosa cell lines

Cell line	Progesterone synthesis	cAMP responsive	Aromatase activity	Inhibin production (α/β_A)	Steroidogenic enzyme expression	FSH responsive	LH/CG responsive
GC48C	+	+	ND	ND	ND	–	ND
Rao-gcl-29	+	+	+	ND	+	–	–
Grs	+	+	ND	ND	ND	–	ND
P0-GRS1	+	+	ND	ND	+	–	–
PA-GRS1	+	+	ND	ND	ND	ND	ND
$G\beta_2$AR13	+	+	ND	ND	+	–	–
KsOG	+	+	ND	ND	ND	–	ND
DC3	+	+	+	ND	ND	–	–
RGA-41S	–	–	–	ND	ND	–	–
RGA-1	ND	+	ND	+/+	+	ND	ND
GLHR-15	+	+	ND	ND	+	–	+
GFSHR-17	+	+	ND	ND	+	+	–
GRM01	+	+	+	+/+	ND	+	+
GRM02	+	+	+	+/+	ND	+	+
KK-1	+	+	+	+/ND	+	+	+
OV3121	–	ND	+	ND	ND	ND	ND
ROG	+	+	–	ND	ND	+	–
SIGC	+	ND	+	ND	+	ND	ND

(+) Positive; (–) negative; ND: not documented.

Table 2
The steroidogenic properties of porcine and bovine granulosa cell lines

Cell line	Progesterone synthesis	cAMP responsive	Aromatase activity	Inhibin production (α/β_A)	Steroidogenic enzyme expression	FSH responsive	LH/CG responsive
Porcine							
MDG2.1	ND	ND	ND	ND	+	–	ND
PGC-2	+	+	+	ND	ND	–	–
jc-410	+	+	+	ND	+	–	–
Bovine							
BGC-1	–	–	–	ND	ND	ND	ND

(+) Positive; (–) negative; ND: not documented.

dent (Zeleznik et al., 1979) (Table 1), bovine (Bernath et al., 1990) porcine (Leighton et al., 1993) (Table 2), and primate (Husen et al., 2002; Ishiwata et al., 1984) (Table 3) granulosa cells. These cell lines have been developed through various methods including spontaneous immortalization, oncogenic transformation, radiation induced tumorigenesis, chemical mutagenesis, explants of human tumors, and tumorigenesis in transgenic animal models. To various extents, each cell line has been characterized with respect to steroidogenic activity, gonadotropin responsiveness, growth factor regulation, and cellular differentiation. As culture conditions often vary between cell lines, direct comparison of cell lines is somewhat difficult. In addition, characteristics of the cell lines may change with time in culture or passages, making comparisons to primary cultures, or even subclones from the same cell line, somewhat complex. While recognizing the limitations of cell

Table 3
The steroidogenic properties of non-human primate and human ovarian granulosa cell lines

Cell line	Progesterone synthesis	cAMP responsive	Aromatase activity	Inhibin production (α/β_A)	Steroidogenic enzyme expression	FSH responsive	LH/CG responsive
Nonhuman							
G1SV1	+	+	ND	ND	+	ND	ND
Human							
HTOG	+	ND	+	ND	ND	ND	ND
COV434	+	+	+	ND	–	+	–
KGN	+	+	+	ND	–	+	–
HGL5	+	+	+	ND	+	–	–
HO-23	+	+	ND	ND	+	ND	–
GC1a	–	ND	–	ND	–	–	–
HGP53	+	+	ND	ND	+	+	ND

(+) Positive; (–) negative; ND: not documented.

lines as in vitro model systems, a thorough understanding of the available granulosa cell lines will allow investigators the ability to determine the most appropriate model for evaluating ovarian function.

2. Rodent granulosa cell lines

2.1. Cell lines developed through oncogenic transformation

2.1.1. SV40 transformed cell lines

2.1.1.1. Origins and steroid production. The first granulosa cell line developed that retained some of the differentiated functions of granulosa cells was the GC48C cell line (Zeleznik et al. 1979) (Table 1). Using freshly isolated rat granulosa cells that continued to produce progesterone, a hybrid was created with an SV40-transformed rat ovarian granulosa cell line that lost the capacity to produce progesterone. Three cell lines were developed, each producing progesterone, with a concentration dependent increase in response to cAMP and dibutyryl cAMP stimulation. Of these three cell lines, the GC48C cell line was studied in detail, due to its greater progesterone stimulation in response to the protein kinase A pathway agonist, dibutyryl cAMP. In comparison to primary rat granulosa cells, these cells produced a similar amount of progesterone in the same time-dependent fashion. These cells, however, were not responsive to FSH. After 11 months of continuous culture, these cells lost their properties of steroid synthesis. As such, this cell line did not retain any useful properties for in vitro studies.

The Rao-gcl-29 cell line was an SV40 transformed cell line developed through culture of diethylstilbestrol (DES) treated day 25 immature rat ovarian granulosa cells in G418 fortified culture following infection by ψ_2-SV40-6 cells (Rao et al., 1993) (Table 1). These cells release SV40 T antigen and retain the neo gene that confers resistance to the antibiotic G418. This cell line was found to produce progesterone when stimulated by cAMP or cAMP analogs, but was not responsive to FSH or hCG. Very low levels of estradiol production were detected when stimulated with cAMP analogs, similar to controls (22 pmol/10^6 cells/48 h). Upregulation of cholesterol side-chain cleavage cytochrome P450 (CYP11A) mRNA was demonstrated by 48 hours.

The inability of rat granulosa cells that have undergone SV40-transformation alone to retain robust steroidogenic capacity was later confirmed by others, who then modified these cells through cotransfection with the Ha-RAS oncogene to develop the Grs cell line (Amsterdam et al., 1988)(Table 1). The morphology of the Grs cell line was distinguishable from the primary granulosa cells and the SV40-transformed cell line. The Grs cell line formed a network of spindle-shaped, refractile cells that rapidly converted into densely packed, small, rounded cells. This cell line did not respond to FSH stimulation, but responded to cAMP analogs and forskolin, by increasing progesterone. Progesterone production kinetics of this cell line in response to 8-Br-cAMP were compared to primary granulosa cells. Whereas significant progesterone production was detected by 3 hours in primary granulosa cells, similar levels were not detected in the Grs-21 cell line until 12 hours. It was postulated that this may be due to de novo synthesis of the steroidogenic enzymes responsible for progesterone synthesis. Similar to primary granulosa cells, the Grs cell lines produced substantial amounts of the progesterone metabolite 20α-dihydroprogesterone in response to 8-Br-cAMP. The population doubling time of this cell line was 18 hours, and it retained its steroidogenic capacity through 20 passages and two freeze-thaw cycles. During 48 hours of stimulation of 1 mM 8-Br-cAMP, the range of progesterone production was 10.8–24.0 ng/10^6 cells. It was thought that the restoration of steroidogenesis to the SV40-transformed granulosa cells by Ha-RAS cotransfection may be due to a reduction of the dedifferentiating influence of large T antigen that had previously been demonstrated (Garcia et al., 1986).

This group developed other similar cell lines designated PO-GRS1 (Hanukoglu et al., 1990) and PA-GRS1 (Suh et al., 1992a) to designate preovulatory or preantral transformed granulosa cells, respectively (Table 1). The PO-GRS1 cell line demonstrated CYP11A expression and progesterone production 24 hours after 8-Br-cAMP stimulation and not at basal stimulation. The PA-GRS1 cell line demonstrated progesterone production, but further analysis was limited to the mechanisms of cAMP-mediated tumorigenesis in nude mice.

Regulation of steroidogenesis in granulosa cells by adrenergic agents was characterized by a cell line obtained by triple transfection of granulosa cells obtained from PMSG primed immature rats with SV40, Ha-RAS oncogene, and an expression vector containing human β_2-adrenergic receptors (Selvaraj et al., 2000). The Gβ_2AR13 cell line demonstrated progesterone synthesis and a concentration dependent increase in response to the adenylyl cyclase stimulant, forskolin (Table 1). This cell line also demonstrated a concentration dependent increase in cAMP and progesterone production when stimulated by isoproterenol, a β_2-adrenergic receptor agonist. This cell line was not responsive to gonadotropins, but responded to adrenalin in a concentration dependent fashion, by increasing cAMP and progesterone production. Incubation with propranolol, a β_2 blocker, inhibited the actions of isoproterenol. StAR expression was induced by forskolin at 1 hour and maximal at 24 hours incubation, whereas progesterone production was evident at 4 hours and increased through 24 hours.

The KsOG cell line was another cell line developed by cotransfection of pregnant mare serum gonadotropin (PMSG) primed day 27 rat ovarian granulosa (ROG) cells with SV40 and Kirsten murine sarcoma virus, a v-Ki-ras oncogene (Pan et al., 1995) (Table 1). Consistent with other similarly developed cell lines, low level constitutive steroidogenesis, cAMP responsiveness, and nonresponsiveness to gonadotropins was demonstrated. The ROG cell line was also shown to express keratin, a characteristic of fetal ovaries

and not adult rat ovarian granulosa cells (Fridmacher et al., 1992; Pan et al., 1992; Frojdman et al., 1993), suggesting that ras oncogenic transformation induces a combination of characteristics that resemble a fetal, rather than adult, ROG phenotype.

The DC3 cell line was similarly derived from SV40-transformed granulosa cells (Fitz et al., 1989) (Table 1). Because this cell line secreted significant amounts of estradiol, it was described as the first model that retained the properties of immature, non-luteinized granulosa cells. This cell line was a monoclonal derivative of the originally SV40 transformed cell line previously described (Hillier et al., 1978). Basal steroid production revealed detectable levels of estrone, 17β-estradiol, and progesterone, but not pregnenolone, 17α-hydroxyprogesterone, or Δ4-androstenedione. FSH receptor binding was demonstrated, but FSH incubation did not result in stimulation of progesterone synthesis. LH receptor binding could not be detected. Progesterone production was increased in a concentration dependent manner in response to incubation with 25-hydroxycholesterol, but estradiol production increased slightly in response to the highest concentration of 25-hydroxycholesterol (20 μg/ml). In response to 48 hours of incubation in the presence of increasing concentrations of androstenedione, a concentration dependent increase in estradiol production was observed. cAMP production was dramatically increased by forskolin in a dose dependent manner. Forskolin, but not FSH, increased production of both estradiol and progesterone in a dose dependent manner in the presence of 25-hydroxycholesterol. The increase in estradiol was not as great as seen for progesterone, in response to forskolin.

2.1.1.2. Temperature sensitive variants. Establishment of a temperature sensitive granulosa cell line occurred by oncogenic transformation of DES simulated granulosa cells from 27-day-old immature rats with the SV40 tsA255 mutant (Zilberstein et al., 1989). This mutant has a temperature sensitive mutation in the gene required for maintenance of transformation (Chou, 1985). The resulting RGA-41S cell line demonstrated unrestrained growth and a transformed phenotype at the permissive temperature (33 °C), while demonstrating a differentiated, growth arrested, nontransformed phenotype at the nonpermissive temperature (40 °C) (Table 1). This cell line was developed to analyze the mitogenic and differentiating role of IGF-1 in granulosa cells. This cell line was found to lack gonadotropin receptors and did not demonstrate steroidogenic capacity. The cells grown at the nonpermissive temperature demonstrated limited replication to monolayer formation, with flat, tetrahedral shaped cells with a decreased nuclear to cytoplasm ratio. In contrast, the cells grown at the permissive temperature (33 °C) demonstrated multilayer growth, with smaller, cobblestone appearing cells with a larger nuclear to cytoplasmic ratio. As granulosa cells can secrete IGF-1 and interact in a paracrine fashion (Baranao and Hammond, 1984) and IGF-1 binds to IGF-1 receptors in rat granulosa cells (Davoren et al., 1986), these products were studied in the RGA-41S cell line. While significant IGF-1 levels of 1.7 ng/ml were detected in the cells grown at 33 °C, IGF-1 levels were barely detectable in the cells grown at 40 °C, indicating the production of IGF-1 correlates with the mitotic activity of the cells. IGF-1 receptors were detected in cells grown at either temperature.

A similar temperature sensitive cell line, RGA-1, was developed (Thompson et al., 2001) (Table 1). As this cell line was obtained in the same manner as the RGA-41S cell line, it might be assumed that it would also be unresponsive to gonadotropin stimulation and lack steroidogenic capacity, although this was not directly determined. These cells expressed inhibin α and β_A when grown at the nontransformed, differentiated temperature (39 °C), but not at the transformed temperature (33 °C). Steroidogenic acute regulatory (StAR) protein expression was demonstrated in RGA-1 cells grown at both temperatures, but were 3-fold greater at 39 °C. The initial reason for development of this cell line was to determine the role of prohibitin (a protein that is associated with cellular differentiation, atresia and luteolysis in the rat ovary) in cell differentiation and apoptosis in granulosa cells.

2.1.1.3. Gonadotropin responsive variants. As primary granulosa cells cease to divide after prolonged gonadotropin stimulation (Hsueh et al., 1984), immortalizing gonadotropin sensitive granulosa cells provide an essential in vitro model. Granulosa cell lines are not responsive to FSH in culture, possibly due to loss of the native FSH receptor upon transformation (Amsterdam and Selvaraj, 1997). Amsterdam and coworkers modified their SV40 and Ha-RAS cotransfected cell line to develop an LH responsive cell line (Suh et al., 1992b). This was done by additional transfection with the LH/CG receptor expression plasmid (McFarland et al., 1989). Development of the GLHR-15 cell line demonstrated expression of LH/CG receptors and gonadotropin stimulated progesterone production (50–60 ng/48 h/mg cell protein versus 1–2 ng/48 h/mg cell protein at basal) (Table 1), but the cell line was not responsive to FSH stimulation. Progesterone and 20α-dihydroprogesterone increased in response to hCG in a concentration dependent manner. Progesterone production after hCG stimulation was detected at 6 hours and progressively increased during the 96 hours studied, and cAMP elevated within 15 minutes and returned to basal levels by 12 hours. Stimulation with hCG demonstrated an increase in CYP11A expression, with a 5-fold increase in response to hCG.

Amsterdam and coworkers subsequently developed an FSH responsive granulosa cell line in a similar fashion to the GLHR-15 cell line, using an FSHR expression plasmid (Keren-Tal et al., 1993). The resulting GFSHR-17 cell line increased progesterone production 15-fold in response to 1.6 nM FSH in serum free medium, although basal progesterone levels were barely detectable (Table 1). GFSHR-17 cells demonstrated a similar FSH stimulated pro-

gesterone increase as primary granulosa cells. The stimulated cAMP levels increased 20-fold within 30 minutes and returned to basal levels after 3 hours. Progesterone production was detected after 12 hours of FSH stimulation. CYP11A expression was also increased in response to FSH stimulation. In contrast, primary rat granulosa cells responded to LH and hCG, whereas the GFSHR-17 cells did not.

2.1.2. v-myc transformed cell lines

2.1.2.1. Origins. In 1992, three granulosa cell lines were developed by v-myc transfection of granulosa cells from PMSG primed day 22 mice (Briers et al., 1993). The resulting cell lines GRM01, GRM02, and GRM01L, were obtained (Table 1). The GRM01 cell line was obtained by using calcium phosphate for the SVv-myc plasmid precipitation, with the GRM01L cell line developing from a subculture as a rapidly growing cell line (36 hours versus 10 hours). The GRM02 cell line was developed using lipofection for transfection. The GRM01 cell line had the appearance of primary granulosa cells, which were small cuboidal and larger pleomorphic cells. The smaller cells were multilayered, while the larger cells formed monolayers. The GRM01L tended to form monolayers, and only became multilayered when very confluent. The GRM02 cell line had a similar doubling time to GRM01, and similar morphology, but formed multilayered plaques. Myc expression was evident, although cross-reactivity of the antibodies to c-myc could not be ruled out. Only GRM01L cell line demonstrated tumorigenicity in nude mice.

2.1.2.2. Steroid and growth factor production. GRM01L cell line did not secrete steroids, while GRM01 and GRM02 retained steroidogenic activity (Vanderstichele et al., 1994). 3β-hydroxysteroid dehydrogenase type II (HSD3B2) and 17β-hydroxysteroid dehydrogenase (17β-HSD) were expressed by GRM01 and GRM02 cell lines. Estradiol and progesterone were secreted by GRM01 cells (34.1 and 520 ng/ml/10^6 cells/24 h, respectively) while GRM02 secreted only progesterone (163.2 ng/ml/10^6 cells/24 h), which is an order of magnitude lower than primary granulosa cell cultures (Kreeger et al., 2003). Addition of androstenedione or testosterone to cultured media resulted in an accumulation of estradiol, demonstrating an active aromatase (CYP19) system in both GRM01 and GRM02 cells (57.3 and 119.5 ng/10^6 cells/24 h, respectively). This conversion was inhibited by aromatase inhibitors. Both cell lines responded to LH/hCG, FSH, and forskolin, and cAMP analogs. Time dependent induction of steroidogenesis by LH was demonstrated, with a 2.3-fold increase in progesterone production at 8 hours after LH incubation, to 20-fold by 48 hours. Growth factors TGFa and EGF were also found to increase progesterone production on the GRM02 cell line. Immunoreactive inhibin was detected in the GRM01 cell line (0.24 U/ml) and GRM02 cell line (14.7 U/ml).

2.2. Cell lines developed from tumors in transgenic mice

2.2.1. KK-1 cell line

An immortalized murine ovarian granulosa tumor cell line has been developed using a transgenic mouse model (Kananen et al., 1995). This was done using the SV40 T-antigen as the transgene and fragments of the inhibin α-subunit as the appropriate tissue specific promoter. Development of the KK-1 cell line resulted from culturing of granulosa cell tumor explants from founder transgenic mice that were confirmed to express the transgene in the tumor (Table 1). The cells were fibroblast-like in appearance. These cells displayed a dose-dependent increase in cAMP production in response to hCG (10-fold) and forskolin (40-fold), but minimally to FSH (2.6-fold). The cells demonstrated CYP19 activity by converting androstenedione to estradiol. LH and FSH receptor responsiveness was maintained out to 10 and 20 passages, respectively. A constant supply of gonadotropin responsive cells is maintained from frozen batches of cells from earlier passages. CYP19 gene expression and inhibin α-subunit gene expression was documented.

2.3. Cell lines from radiation induced tumorigenesis

2.3.1. OV3121 cell line

A nonmetastatic tumor cell line, OV3121, was developed from an ovarian granulosa cell tumor in B6C3F1 mice irradiated with ^{60}Co-gamma rays (Yanagihara et al., 1995) (Table 1). This was a tumorigenic, nonmetastatic cell line following injection into syngenic mice. Estradiol production was demonstrated, but estriol, pregnenolone, and progesterone production could not be demonstrated, and cAMP and gonadotropin responsiveness were not determined.

2.4. Cell lines developed through chemical mutagenesis

2.4.1. ROG Cell Line

The ROG cell line was developed through 9,10-dimethyl-1,2-benzanthracene (DMBA) treatment in serum free culture in the presence of undifferentiated ROG cells isolated from day 14 immature rats (Li et al., 1997). The cell line is grown in the presence of recombinant human activin A, which is a known granulosa cell mitogen and induces FSH receptors (Li et al., 1995; Xiao et al., 1992; Nakamura et al., 1995). In the absence of activin, these cells failed to grow and subsequently died. The cell line maintained FSH binding and FSH responsiveness and in the presence of FSH, the cells underwent a growth spurt, but after 72 hours, these cells became quiescent and failed to grow. FSH withdrawal then induced apoptosis within 24 hours in FSH treated cells, which could be rescued by forskolin, but not FSH. Progesterone production was not detected until FSH stimulation, when progesterone levels of 500 pg/ml could be induced after 72 hours of 1 ng/ml FSH stimulation. This cell line maintained the features of predifferentiated granulosa cells, as it did not demonstrate responsiveness to LH. These cells then undergo rapid differentiation and steroidogenic capability in the presence of FSH.

2.5. Cell lines developed through spontaneous immortalization

2.5.1. SIGC cell line

This spontaneously immortalized cell line was developed from 45-day-old Berlin Duckery rats (Stein et al., 1991) (Table 1). These cells do not appear to luteinize in culture and remain relatively undifferentiated. These cells express CYP11A and synthesize limited amounts of estrogen and progesterone. FSH, forskolin, and cAMP responsiveness were not determined. These cells undergo apoptosis when deprived of serum or bFGF (Lynch et al., 2000).

3. Porcine granulosa cell lines

3.1. MDG2.1 cell line

This cell line was established through pSV3neo plasmid transfection of freshly cultured granulosa cells from porcine follicles 4–6 mm in diameter (Leighton et al., 1993) (Table 2). This plasmid contained the SV40 T antigen and conferred resistance to the G418 antibiotic. Culture conditions were similar to those required to produce the rodent Rao-gcl-29 cell line (Rao et al., 1993) The resulting MDG2.1 cell line was found to have a 24–36-hour doubling time, which was 2–3-fold faster than primary granulosa cells. This cell line was found to express CYP11A. Steroidogenic capacity of this cell line was not determined, as it was primarily developed to determine insulin-like growth factor (IGF) and insulin-like growth factor binding protein (IGFBP) expression in porcine granulosa cells. Gonadotropin dependent steroidogenesis was not determined, although FSH did not alter IGFBP expression, unlike in primary porcine granulosa cell cultures (Leighton et al., 1994).

3.2. PGC-2 cell line

The PGC-2 cell line arose spontaneously from the continuous culturing of equine CG treated primary granulosa cells from follicles 2–3 mm from prepubertal gilts (Kwan et al., 1996) (Table 2). This cell line has been maintained in culture for >100 passages, maintains contact inhibition, and has a doubling time of 20 hours. Forskolin stimulation resulted in a five-fold increase in cAMP production over 60 minutes, but its effect on progesterone production was not determined. Basal progesterone synthesis was 5.6 ng/3 $\times$ 10^5 cells/48 h. Progesterone secretion was not enhanced by FSH or LH, but it was significantly increased with the addition of androstenedione or pregnenolone, and was significantly decreased in the presence of estradiol. Estradiol secretion was demonstrated with the addition of androstenedione, confirming the presence of a functional CYP19 system. FSH incubation did not increase CYP19 activity. FSH receptor mRNA was not detected.

3.3. jc-410 cell line

The jc-410 cell line is another spontaneously arising porcine cell line (Chedrese et al., 1998; Gillio-Meina et al., 2000) (Table 2). This cell line was developed from granulosa cells obtained from 4 to 6 mm follicles obtained from prepubertal gilts, and characteristics were compared to primary granulosa cells from small (1–3 mm) and medium (4–6 mm) sized follicles. Progesterone synthesis was 10 and 1% of the amount produced by granulosa cells from small and medium follicles, respectively. Estradiol synthesis was only detected in the presence of androstenedione. The cell line did not respond to gonadotropins, but responded to forskolin and cAMP in a concentration dependent manner. Expression of HSD3B2, CYP19 and CYP11A was comparable to primary granulosa cells, and cAMP analogs increased steroidogenic enzyme expression (Rodway et al., 1999).

4. Bovine granulosa cell lines

4.1. BGC-1 cell line

The BGC-1 cell line is a cell line obtained by spontaneous immortalization of a primary bovine granulosa cell culture (Bernath et al., 1990; Lerner et al., 1995) (Table 2). This cell line was obtained after 75 passages. Granulosa cells were obtained from small size follicles (2–5 mm) from the ovaries of beef cows and heifers. These cells did not produce any detectable progesterone in serum free medium, and this was not altered in the presence of cAMP or 25-hydroxycholesterol. In the presence of pregnenolone, primary granulosa cell cultures almost completely converted it to progesterone, whereas the BGC-1 cell line converted a small fraction to progesterone, indicating a much lower HSD3B2 activity in the cell line. 5α-reducing activity was greater in the cell line, as measured by 5α-reduced [^{3}H] progesterone metabolites. Primary cell cultures could synthesize 17β-hydroxyprogesterone from either pregnenolone or progesterone as substrate, whereas the cell line could not synthesize 17α-hydroxyprogesterone from pregnenolone. Neither primary cell cultures nor BGC-1 cells showed significant conversion of [^{3}H]testosterone to estradiol, indicating loss of aromatase activity. In the BGC-01 cells, most of the testosterone was converted to dihydrotestosterone, and 5α-androstane-3α,17β-diol, whereas primary cultures demonstrated minimal testosterone metabolism. Steroidogenesis was not increased by cAMP, and gonadotropin sensitivity was not determined.

5. Primate ovarian granulosa cell lines

5.1. G1SV1 cell line

The G1SV1 cell line is the only known non-human primate ovarian granulosa cell line (Husen et al., 2002) (Table 3).

Granulosa cells were obtained from large, antral, preovulatory follicles (>2 mm) from the marmoset monkey, *Callithrix jacchus*, and transfected with a plasmid containing the SV40 genome. The cell line had undergone up to 40 passages before characterization. Unlike SV40 transformed ovarian granulosa cell lines in other animal models, SV40 large T-antigen is not detected by nested PCR in the G1SV1 cell line. Basal progesterone production was barely detectable, but increased greater than 50-fold in the presence of 8-Br-cAMP after 48 hours (55 ng/mg protein). Estrogen receptor-α (ER-α) mRNA is expressed in G1SV1 cell lines, and is downregulated in the presence of 8-Br-cAMP, similar to in vivo ER-α expression in primates (Einspanier et al., 1997; Saunders et al., 2000). Progesterone receptor mRNA was not demonstrated with or without 8-Br-cAMP stimulation. Immunofluorescence staining revealed the presence of HSD3B2 enzyme. The status of gonadotropin responsiveness was not described.

6. Human ovarian granulosa cell lines

As many studies are directed towards defining normal and abnormal physiology of the human situation, a model of human origin could be of importance. Furthermore, due to species-specific differences in spatial and temporal regulation of ovarian steroidogenesis, understanding mechanisms of human ovarian steroidogenesis becomes relevant.

6.1. Cell lines developed from ovarian tumors

6.1.1. HTOG cell line

6.1.1.1. Origins. In 1981, a 74-year-old gravida 9, para 7, Japanese female presented with abdominal swelling and postmenopausal bleeding. She subsequently underwent a hysterectomy and bilateral salpingo-oophorectomy, revealing a 12 cm × 15 cm × 10 cm right ovarian tumor that was solid, partially necrotic, and yellow/brown in appearance (Ishiwata et al., 1984). The tumor was examined histologically and found to be a granulosa-theca cell tumor. The granulosa cells were isolated, the tumor was cultured and underwent over 100 passages in 25 months. Chromosomal number varied considerably and showed hyperploidy. Doubling time accelerated with subcultures, and was 23.8 hours at passage 40. The cultured cells grew in multilayers and did not demonstrate contact inhibition.

6.1.1.2. Steroid production. The HTOG cell line was steroidogenically active, producing large quantities of estrone (1250 pg/5 × 10^5 cells/96 h at passage 7) and estradiol (2470 pg/5 × 10^5 cells/96 h at passage 7), but minimal progesterone (132 pg/5 × 10^5 cells/96 h at passage 7) and 17-hydroxyprogesterone, and no testosterone (Table 3). By passage 41, the estrogen production was less than 1% of its original production (estrone: 14,272 pg/5 × 10^5 cells/96 h passage 1 versus 84 pg/5 × 10^5 cells/96 h passage 41; estradiol: 7750 pg/5 × 10^5 cells/96 h passage 1 versus 76 pg/5 × 10^5 cells/96 h passage 41). Gonadotropin and cAMP responsiveness was not determined. It was unclear where the steroid precursors for estradiol synthesis originated.

6.1.2. COV434 cell line

6.1.2.1. Origins. In 1984, a metastatic granulosa cell tumor was obtained from a 27-year-old female (Van den Berg-Bakker et al., 1993; Zhang et al., 2000). The cells were isolated and cultured, and characterized on passage 24. The cells retained the morphological appearance of luteinized granulosa cells.

6.1.2.2. Steroid production. In the presence of androstenedione and 100 ng/ml of FSH, the cells started to secrete estradiol after 8 hours, and increased to 175 pM in the culture medium at 18 hours (Table 3). LH and hCG did not significantly alter estradiol concentration, and LH receptor mRNA was not detected. Progesterone production was not altered by gonadotropins, although cAMP production was enhanced by FSH and forskolin. This was the first gonadotropin responsive human ovarian granulosa cell line.

6.1.3. KGN cell line

6.1.3.1. Origins. The KGN cell line originated from a Stage III granulosa cell carcinoma removed from a 63-year-old Japanese women in 1984 (Nishi et al., 2001) (Table 3). The tumor recurred and was surgically removed in January 1994. A portion of the tumor recurrence was removed for establishment of the cell line. The cells grew as a monolayer, with a doubling time of 46.4 hours. The cells underwent over 100 passages over 5 years.

6.1.3.2. Steroid production. Immunohistochemical staining of the cells for CYP19 revealed the presence of this steroidogenic enzyme. KGN cells demonstrated production of pregnenolone and progesterone, with a dose dependent increase in the presence of dibutyryl-cAMP. 17α-hydroxypregnenolone and 17α-hydroxyprogesterone were barely detectable and not affected by dibutyryl cAMP. DHEA, androstenedione, testosterone, and estradiol were undetectable. CYP19 activity (0.84 pM/mg protein/12 h), measured by the conversion of [1β-^{3}H] androstenedione to estrone, was 50 times higher than in cultured skin fibroblasts, but was 50 times lower than primary human granulosa cells. This activity was increased in the presence of dibutyryl cAMP. CYP19 activity was stimulated by FSH, with a minimal effective dose of 50 mIU/ml hMG, whereas it was not affected by hCG. In the presence of 10 μM of androstenedione for 72 hours, 171 pM/10^6 cells of estradiol was produced, which increased 23.8 fold in the presence of 1 mM dibutyryl cAMP. As no estradiol or androgens were detected in this cell line, it provided a good model for the two-cell, two- gonadotropin model for ovarian steroidogenesis. The small levels of 17-hydroxylated steroids detected were likely due to cross-reactivities in the radioim-

munoassay used. As the previous granulosa tumor cell line produced estrogens, and other granulosa cell tumors have been shown to synthesize estrogens and androgens, the question of this inconsistency can be raised. This may be due to differences in culture conditions or the stage of follicular development (Yong et al., 1992; Schipper et al., 1993). In the KGN cell line, RT-PCR for CYP17 transcripts was undetectable. Presence of FSH receptors was documented through [^{125}I] FSH binding assay. Documentation of FSH receptors and gonadotropin responsiveness makes this cell line attractive for studying ovarian G-protein coupled receptor signal transduction (King et al., 2003).

6.2. Cell lines developed through oncogenic transformation

6.2.1. HGL5 cell line

6.2.1.1. Origins. Using the E6 and E7 open reading frames of the high risk strain of the human papilloma virus strain 16 (HPV16) that had been previously shown to transform human cells (Hudson et al., 1990; Sedman et al., 1991; Shay et al., 1993), transformation of luteinized granulosa cells was performed to establish the HGL5 cell line (Rainey et al., 1994) (Table 3). Transfection was performed by inserting the HPV16 E6/E7 DNA into the retroviral vector pLXSN under the transcriptional regulation of the Moloney murine leukemia virus promoter–enhancer sequences. This vector also contained the neomycin resistance gene under control of the SV40 promoter. These cells had undergone at least 75 passages and had been maintained in culture for over 1 year. Cell doubling time was 96 hours at passage 75.

6.2.1.2. Steroid production. Progesterone production was demonstrated, and then stimulated progesterone production was measured after 72 hours. LH and FSH stimulation was without effect. At maximal concentrations, forskolin and dibutyryl cAMP increase progesterone release 30 and 20-fold, respectively. In medium containing androstenedione (1 μM), cells treated for 72 hours synthesized estradiol, which was increased 60- and 50-fold above basal values when treated with forskolin and dibutyryl cAMP, respectively. Gonadotropin treatment had no effect. When compared to primary granulosa cell cultures, CYP19 expression levels were semiquantitatively lower when measured by Northern blotting. Subsequent studies have revealed that this cell line does not produce inhibin α-subunit (Rainey et al., 1996).

6.2.2. HO-23 cell line

6.2.2.1. Origins. The HO-23 cell line was developed by triple transfection of primary granulosa cells with SV40 DNA, Ha-ras oncogene, and a temperature sensitive variant of tumor suppressor gene p53 (p53val135) that allows wild-type activity at 32 °C but not 37 °C (Hosokawa et al., 1998a) (Table 3). The cell line was steroidogenically stable after 20 passages and several freeze-thaw cycles.

6.2.2.2. Steroid production. Progesterone production was 100 ng/10^6 cells/48 h, which was 40-fold higher than the POGRS-1 rat granulosa cell line. Stimulation of cells with forskolin revealed at least a 33-fold elevation of pregnenolone progesterone, and 20α-dihydroprogesterone compared to basal levels. The cells increased progesterone production in response to 8-Br-cAMP, but did not respond to hCG. In the presence of forskolin, progesterone increase was not detected for 3–6 hours, due to de novo synthesis of CYP11A (Hosokawa et al., 1998b). The orphan nuclear hormone receptor steroidogenic factor-1 (SF-1), was expressed in nonstimulated cells and was augmented 1.9-fold in the presence of forskolin. StAR expression was increased 5.4-fold in the presence of forskolin, as measured by Western blot analysis. CYP11A expression, measured indirectly as the electron carrier adrenodoxin, demonstrated a 14-fold elevation in the presence of forskolin. This was the first cell line where levels of SF-1 were examined, and the first human line determining the inducibility of progesterone synthesizing enzymes.

6.2.3. GC1a cell line

6.2.3.1. Origins. The GC1a cell line was developed by transfection of human granulosa cells using the SV40 large T antigen (Nitta et al., 2001; Okamura et al., 2003) (Table 3). Granulosa cells were obtained from 6 mm follicles from specimens obtained in women undergoing hysterectomy and oophorectomy for gynecological disorders in Japanese women aged 45–64. The cells underwent at least 100 passages. The cells arranged into cuboidal epithelial-like monolayers and expressed inhibin-α. These cells were subsequently cotransfected with mouse SF-1 cDNA subcloned into plasmid expression vector pIND, where downstream gene expression is inducible by ecdysone (GC1a+SF-1). The cells should provide an interesting model to study SF-1 regulated gene expression.

6.2.3.2. Steroid production. These cells were unable to synthesize steroids, and this was not inducible by gonadotropin stimulation. This was thought to be due to the cell's inability to express SF-1 (Amsterdam and Selvaraj, 1997). After cotransfection with SF-1 and induction of expression with ecdysone, gene expression for StAR, CYP11A, and CYP19 were analyzed and were evident within 48 hours, as measured by RT-PCR. The cells do not produce progesterone but produce estradiol. Interestingly, estradiol production is not altered by the addition of androstenedione, but is increased by estrone addition. 17β-hydroxysteroid dehydrogenase type I (17βHSD type 1) mRNA is detected. From these experiments, it appears that the cells do not possess significant aromatase activity, and the predominant synthesis of estradiol is through 17βHSD type 1 conversion of estrone. However, it is unclear what steroid precursors were used in the culture media. Forskolin, cAMP analog, and gonadotropin stimulation were not determined.

6.2.4. HGP53 cell line

6.2.4.1. Origins. In an effort to develop a gonadotropin responsive granulosa cell line, the investigators responsible for the HO-23 cell line developed the HGP53 cell line (Table 3). Essentially, this was done by co-transfection of primary granulosa cells with the Ha-ras oncogene and the p53val135 tumor suppressor gene, in the absence of SV40 T antigen transfection (Tajima et al., 2002).

6.2.4.2. Steroid production. FSH stimulated (1 lU/ml) progesterone secretion was 3–10 ng/10^6 cells/24 hours, comparable to the rFSHR-17 rat granulosa cell line (Keren-Tal et al., 1993) and 60-fold higher than basal levels. A dose-dependent increase in progesterone production was evident for forskolin and 8-Br-cAMP, and a similar, but attenuated dose–response curve is seen for FSH, likely due to desensitization phenomenon also seen in primary granulosa cells (Zor et al., 1976). FSH stimulated gene expression of StAR and CYP11A were demonstrated by Western blot analysis, and resulted in a 64- and 3.1-fold increase, respectively.

7. Summary

The development of granulosa cell lines has provided a readily accessible, reproducible and renewable model for studying the molecular events regulating granulosa cell steroidogenesis and folliculogenesis. In future, this may allow for a greater understanding in the mechanisms of ovarian steroidogenesis and the intraovarian factors involved in follicular differentiation and growth. With the more recent development of gonadotropin responsive granulosa cell lines, the mechanisms of ovarian G-protein coupled receptor signal transduction and gonadotropin regulation of steroidogenesis may be determined. The role of putative growth factors and transcription factors in the human ovary may be determined through the use of various granulosa cell lines at various stages of cellular differentiation. These cell model systems will allow for better elucidation of the mitogenic, luteotropic and apoptotic mechanisms responsible for selection and support of the dominant ovarian follicle. It must be recognized that each cell line has different properties with respect to its steroidogenic, mitogenic, and differentiating capabilities. As a result, choosing the appropriate cell line to study will be essential. Finally, further development of new and novel granulosa cell lines with additional differentiated functions will allow for additional studies that further our understanding of ovarian biology at the molecular level.

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