

Ovulatory Surges of Human CG Prevent Hormone-Induced Granulosa Cell Tumor Formation Leading to the Identification of Tumor-Associated Changes in the Transcriptome

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Granulosa cell tumors comprise approximately 10% of ovarian tumors and, although rare, are clinically important due to their potential for malignancy and recurrence. Although their morphological features have been carefully described, the global changes in gene expression associated with their formation remain undetermined. To initiate this characterization, we used a transgenic mouse model in which granulosa cell tumors occur with 100% penetrance in CF-1 mice that harbor a novel transgene encoding a chimeric LH β subunit. When this transgene is expressed in other strains of mice, including (C57BL/6 \times CF-1, Tg) F₁ hybrids, luteomas develop even though levels of LH remain high. This dichotomous response permits a longitudinal comparison of global changes in transcriptomes uniquely associated with either granulosa cell tumors or luteomas. Herein we report numerous changes in the transcriptome, including a decrease in LH receptor mRNA and increases in several mRNAs that encode secreted proteins pre-

viously associated with granulosa cell tumors. Furthermore, we identified a constellation of mRNAs that encode proteins that may serve as new markers for this tumor phenotype. Additional experiments indicated that periodic treatment with human CG prevented formation of granulosa cell tumors in mice genetically predisposed to tumor development and, instead, led to the appearance of luteomas. More importantly, ovarian transcriptomes from the luteomas induced by ovulatory doses of human CG permitted refined confirmation of gene expression changes that were uniquely associated with either granulosa cell tumors in the permissive CF-1 genetic background or in luteomas in the F₁ hybrids. Together, these dynamic changes in the ovarian transcriptome indict various signaling pathways potentially involved in mediating the actions of LH over time and, depending on genetic background, the formation of either a luteoma or a granulosa cell tumor. (*Molecular Endocrinology* 16: 1230–1242, 2002)

MOLECULAR EVENTS underlying the formation of granulosa cell tumors in women remain poorly understood despite their importance (1, 2). Although these tumors are rare and comprise only 10% of all ovarian tumors (3, 4), they exhibit the potential for malignancy and recurrence (3–6), making them clinically significant. Thus, efforts to identify the molecular mechanisms leading to development of these tumors are important as they may reveal new therapeutic targets or agents.

Alterations in expression of several genes have been associated with granulosa cell tumors including Müllerian inhibiting substance (7), inhibin (8–11), and p53 (12). Likewise, altered expression of protooncogenes such as *c-erbB2* and *c-myc* has also been implicated (13). Furthermore, germ-line mutations such as in the chromosomal locus *LKB1*, associated with Peutz-Jeghers syndrome (14), have been suspected as playing a role in the formation of some granulosa cell

tumors. Despite these investigations, however, the complete network of genes and/or mutations necessary and sufficient for tumorigenesis has yet to be delineated.

In addition to alterations in gene expression, dysregulation of hormonally controlled signal transduction pathways has also been associated with granulosa cell tumors. For example, studies in rodents initially identified FSH as a potential tumorigenic contributor, specifically through its regulation of cyclin D2 expression (15). D-type cyclins are important for cell proliferation and the transition between G₁ and S phase (16). Cyclin D2 is the only D-type cyclin expressed in granulosa cells (17), regulating entry into M phase through interaction with Cdk4 or Cdk6 (16, 18). Furthermore, cyclin D2 mRNA is elevated in a subset of granulosa cell tumors in women (15). In rodents, FSH positively regulates, whereas LH negatively regulates, cyclin D2 expression in granulosa cells (17). Thus, it has been postulated that activating mutations in FSH receptors (FSH-R) may induce granulosa cell tumors, perhaps through its effect on cyclin D2. However, to date no such mutations have been discovered in gran-

Abbreviations: EST, Expressed sequence tag; FSH-R, FSH receptor; LH-R, LH receptor; PRL-R, PRL receptor; SOM, self-organizing map; StAR, steroidogenic acute regulatory protein.

ulosa cell tumors from women (1). In addition, mice lacking the FSH-R develop granulosa cell tumors, suggesting that this pathology can occur in the absence of any FSH signaling (19).

Clinical studies have also suggested that elevated gonadotropin levels can lead to tumorigenesis. The strongest correlative evidence for this comes from postmenopausal women who represent the largest cohort of patients with granulosa cell tumors (2). These women are anovulatory and have high levels of LH and FSH due to the lack of negative feedback from estrogen (20). Infertility treatment with gonadotropins has also been suspected of increasing the risk for granulosa cell tumor development; however, these data remain controversial (21–24).

The use of murine animal models has provided additional clues regarding how genetic contributions may underlie formation of at least some granulosa cell tumors. For example, the SWR inbred strain of mice spontaneously develops granulosa cell tumors early in life, resembling juvenile granulosa cell tumors in girls (25). Tumor incidence is increased after dehydroepiandrosterone or testosterone treatment, indicting androgens as possible tumor promoters (26). Furthermore, Beamer and colleagues (27) have identified four chromosomal loci responsible for tumor susceptibility in SWR mice, illustrating that a complex genetic trait underlies tumor development in juvenile females.

Other rodent models of granulosa cell tumorigenesis have been created using ectopic ovarian transplants (28, 29), chemical carcinogens (30–34), irradiation (35), neonatal thymectomy (36–38), and pharmacological treatment with steroids (31). In most of these models, gonadotropins are either elevated or become elevated with additional treatments, supporting the notion that gonadotropins are important tumor promoters. Studies using transgenic mice also support this view. Mice deficient in inhibin α develop granulosa cell tumors at an early age (39). The lack of inhibin α leads to an increase in LH, FSH, and estrogens (39, 40). When these animals are bred with mice deficient in GnRH (hpg/hpg), and hence gonadotropins, tumors fail to form, further underscoring the importance of LH and FSH (41). In contrast, mice deficient in FSH and inhibin continue to develop tumors, although with increased latency and decreased penetrance (40). These data suggest that LH alone can induce tumor formation in this model, and that FSH may accelerate this process.

In addition to these models, mice that hypersecrete LH (LH β CTP) also develop granulosa cell tumors (42). Female transgenic mice have elevated serum levels of estrogens, androgens, progesterone, corticosteroids, and PRL (43–46). These altered hormone levels lead to anovulation (42) and midpregnancy failure (46), culminating in infertility. LH hypersecretion also causes an early depletion of primordial follicles (47) and induces advanced folliculogenesis leading to the formation of hemorrhagic ovarian cysts (43) and the eventual formation of granulosa cell tumors in CF-1 mice (42, 48). The development of these tumors is strain dependent,

with at least three loci contributing to tumor development (44). This suggests that a complex genetic trait governs granulosa cell tumor susceptibility of CF-1 mice that chronically hypersecrete LH and might partially explain the rarity of granulosa cell tumors in postmenopausal women, who also have chronically elevated levels of LH (20). In addition to these loci, however, we suspect that there must be other genomic or transcriptomic events involved in granulosa cell transformation that are induced by altered hormones and interact with the underlying genetic factors.

Herein, we report an analysis of global gene expression patterns related to granulosa cell tumorigenesis in LH-hypersecreting mice. We also describe a surprising finding, that ovulatory doses of human CG (hCG) prevent the formation of granulosa cell tumors in CF-1 transgenic mice that are predisposed to tumor formation and instead induce the formation of a differentiated luteoma. These hCG-induced luteomas were used to facilitate refinement of tumor-associated changes in the transcriptome. Using this approach, a cohort of candidate genes that potentially cooperate with the genetic loci that underlie tumor formation were identified and should serve as targets for future studies on granulosa cell tumor formation.

RESULTS AND DISCUSSION

Expression Profiling Strategy

To begin elucidating the molecular mechanisms associated with granulosa cell tumorigenesis in LH β CTP mice, global expression profiling analyses were performed. We predicted that overlapping expression profiles from multiple, closely related samples spanning different ages would identify a specific set of genes whose changes in expression were uniquely associated with tumorigenesis. This was accomplished by taking advantage of the strain and age dependency observed in the formation of granulosa cell tumors. Specifically, when LH β CTP transgenic mice are bred with numerous other strains of mice, first generation transgenic progeny fail to form granulosa cell tumors. In contrast, other hormonal phenotypes including elevated levels of LH, estrogens, and androgens are maintained (44). Transgenic F₁ hybrid (C57BL/6 \times CF 1 δ , Tg) females are also infertile and develop multicystic ovaries. However, instead of forming a granulosa cell tumor, these mice develop completely luteinized ovaries (44), reminiscent of a luteoma of pregnancy observed in women (49). Thus, because many, if not all, early morphological and endocrinological phenotypes except granulosa cell tumorigenesis are common between CF-1 and F₁ hybrid transgenic females, inclusion of the hybrids in the expression profiling experiment should eliminate expression changes induced by the transgene that are

unrelated to tumor formation. This subtractive method is displayed diagrammatically in Fig. 1.

C57BL/6-derived F₁ hybrids (C57BL/6♀ × CF-1♂, Tg) were used in this study and also complemented ongoing genetic studies aimed at identifying the genetic modifiers permissive for formation of granulosa cell tumors (44). Eight experimental groups were used for the initial accumulation of gene expression profiles.

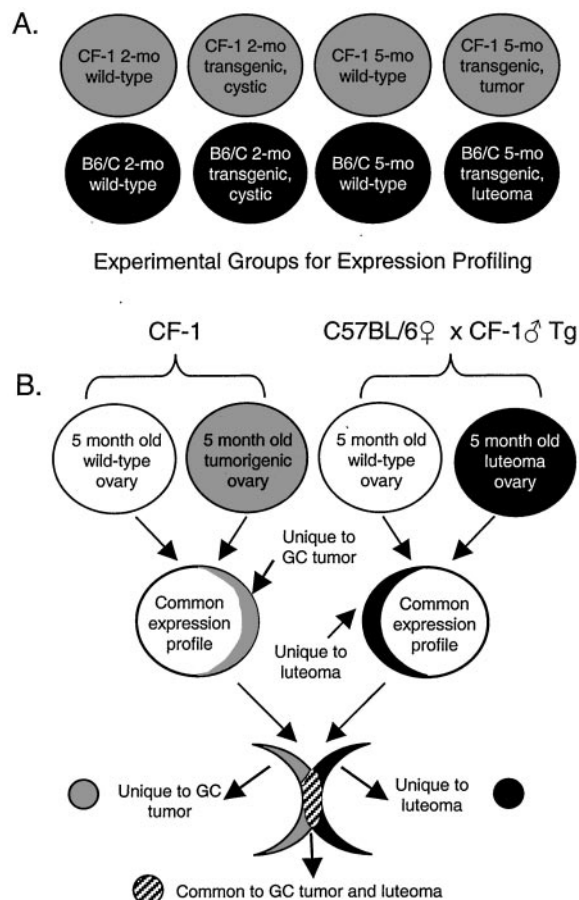


Fig. 1. Differences in Age and Mouse Strain Enabled Identification of Tumor-Associated Expression Changes

A, Each circle represents the expression profile of one experimental group. There were eight experimental groups in the initial experiment. These included 2- and 5-month-old wild-type and transgenic CF-1 and (C57BL/6♀ × CF-1♂, Tg) F₁ hybrid mice (B6/C). B, The expression profiling technique is diagrammed with four of the eight experimental groups for simplicity. The schematic illustrates how a majority of the genes between wild-type and transgenic CF-1 animals will not change, with only a small percentage differing between the two. These changes may be associated with the formation of a granulosa cell tumor because CF-1 transgenic animals at this age have tumors. Furthermore, when the same comparison between B6/C F₁ hybrid animals is performed, a similar phenomenon occurs; however, the gene changes found in 5-month-old B6/C animals will not be associated with granulosa cell tumors, but rather with luteoma formation. When the small percentage of genes from each comparison is then overlapped, genes associated only with the formation of a granulosa cell tumor are identified.

These groups consisted of 2- and 5-month-old transgenic and wild-type mice from both CF-1 and (C57BL/6♀ × CF-1♂, Tg) F₁ hybrid strains (Fig. 1). Total RNA was collected from ovaries, and biotinylated cRNA was produced. Equal amounts of total RNA were pooled from a minimum of four mice for each experimental group to minimize changes due to inter-individual variation. This pooling strategy identifies only uniform changes in an experimental group instead of changes that may be associated with a single animal. Global changes in gene expression profiles were assessed with Affymetrix Mu11K oligonucleotide microarrays containing approximately 11,000 genes and expressed sequence tags (ESTs). Once hybridization data were obtained, all samples were normalized for chip intensity and GAPDH levels. Raw data were then analyzed using self-organizing maps (SOMs) to reveal informative patterns of gene expression (50). This mathematical analysis clusters genes with similar expression patterns throughout all eight samples. Once informative clusters were identified, the data were filtered using Affymetrix parameters to reveal significant expression changes that were at least 3-fold because changes of this magnitude are highly reproducible in replicate samples (51).

Global Analyses Revealed Down- and Up-Regulated Genes Associated with Granulosa Cell Tumors

This initial gene expression profiling experiment revealed many informative expression patterns. A cluster of genes whose expression is dramatically decreased upon development of granulosa cell tumors was identified (Fig. 2A). Expression of these genes does not decrease in younger transgenic animals or in F₁ hybrids with luteomas compared with age-matched nontransgenic controls. In addition, another cluster of genes whose expression increased significantly in granulosa cell tumors was revealed (Fig. 2B). These changes reflect characterized cDNAs or annotated ESTs. In all, 75 expression increases and 47 expression decreases were revealed from this initial combinatorial analysis.

Although all 122 tumor-associated gene expression changes potentially contribute significantly to tumorigenesis, we decided to identify those genes whose expression consistently changes to generate initial targets for future analyses. We reasoned that changes that are not common to most tumors might be indicative of slight variations in tumor phenotype or progression. To identify consistent patterns in the tumor-associated transcriptome, we repeated the analysis of RNA from tumors and luteomas because they represented the two ovarian endpoints of chronically elevated LH. Specifically, another pooled granulosa cell tumor sample (three tumors) and a pooled F₁ hybrid luteoma sample (three luteomas) were analyzed. These samples were distinct from the samples used in the initial analysis. When compared with the initial

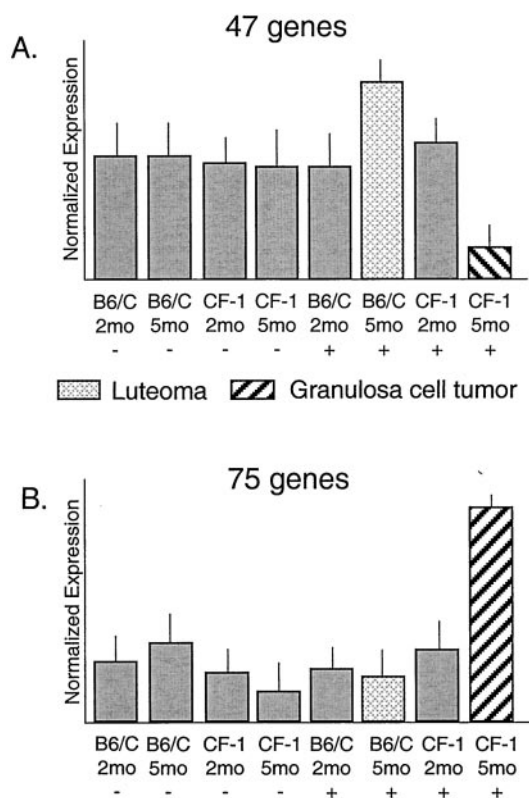


Fig. 2. Genes Down- and Up-Regulated in Granulosa Cell Tumors

SOMs were generated to cluster genes sharing the same expression pattern across the eight samples. Twelve clusters were generated by GENECLUSTER; raw data have been published as supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org> and have been deposited into the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>). The *bar graph* is a recapitulation of a cluster generated by GENECLUSTER. The x-axis represents the individual experimental groups, and the y-axis represents normalized expression where the expression levels of each gene was normalized to have the mean = 0 and variance = 1 across all time points. The *error bars* represent sd of average expression as calculated by GENECLUSTER. The *graph* displays both wild-type (-) and transgenic (+) CF-1 animals and F₁ hybrid animals (B6/C) at 2 and 5 months of age. A, This cluster displays genes that decrease in expression in granulosa cell tumors and are increased in B6/C transgenic animals. Only genes changing more than 3-fold were considered to be significant. B, A reciprocal cluster was also identified that included 75 significant gene expression increases specific to granulosa cell tumors.

analysis, a cohort of gene expression changes consistent in two independent tumor and luteoma samples was identified (Fig. 3A). Specifically, there were 26 genes with reduced expression and 46 genes with increased expression in this group. These expression changes, listed in Tables 1 and 2, may represent common expression alterations that contribute to granulosa cell tumor formation in LH β CTP mice.

In evaluating the cohort of consistent gene changes, it was not surprising that protooncogenes such as *c-fos* (52, 53), *N-myc* (54), and platelet-derived growth

factor A (55) demonstrated expression increases (Table 2). These genes have been associated with other ovarian tumors and may also play tumorigenic roles in this model. Genes encoding many extracellular matrix proteins [such as fibronectin (56–58), FISP-12 (59), fibulin (60), and laminin B1 (61, 62)] were also included in this cluster. Interestingly, several of these are increased in other tumors and/or are hormonally regulated in ovaries. Inhibin β A and β B subunit mRNA, which are produced by granulosa cells (63), were also represented in this group. This was expected because excess inhibin production is a hallmark of human granulosa cell tumors (8) and tumors from SWR mice (64). Thus, many genes previously associated with ovarian tumors were identified in this cohort of expression changes, implicating them as contributors to the tumor phenotype.

As discussed above, it has been suggested that elevated cyclin D2 mRNA contributes to granulosa cell tumorigenesis (15). However, a significant elevation of cyclin D2 was not identified as a consistent change using this rigorous, combinatorial analysis. These results do not exclude cyclin D2 as a potential contributor to tumor formation, but because the alterations are not consistently observed, it may not be a common phenomenon in tumorigenesis in this model. Indeed, Sicinski and colleagues (15) observed elevations in cyclin D2 mRNA in many, but not all, human granulosa cell tumors, illustrating again that cyclin D2 elevation in granulosa cell tumors may not be a consistent phenomenon.

Gene Expression Changes Potentially Associated with LH-R Signaling

Interestingly, LH receptor (LH-R) mRNA expression was significantly decreased in granulosa cell tumors but elevated in luteomas. This pattern was surprising because tumor development is dependent on high levels of LH, and continued expression of LH-R was predicted. However, there are precedents for this observation from human samples and other mouse models of granulosa cell tumors (65, 66). There are at least two explanations that may underlie the significant reduction of LH-R mRNA in the granulosa cell tumors. LH β CTP mice are infertile, in part due to anovulation, resulting from a failure to mount an ovulatory surge of LH (42, 46). Thus, chronic exposure to elevated levels of LH results in continued anovulation, potentially causing a subsequent down-regulation of LH-R mRNA and tumorigenesis. Alternatively, chronic exposure to elevated LH may indirectly induce uncontrolled proliferation and eventual tumorigenesis of immature granulosa cells that never express LH-R; hence, receptor expression is decreased, through an indirect mechanism, compared with more differentiated luteomas.

Steroidogenic acute regulatory protein (StAR), which mediates cholesterol transport into the mitochondrial matrix during steroid biosynthesis (67), also displayed decreased expression in granulosa cell tu-

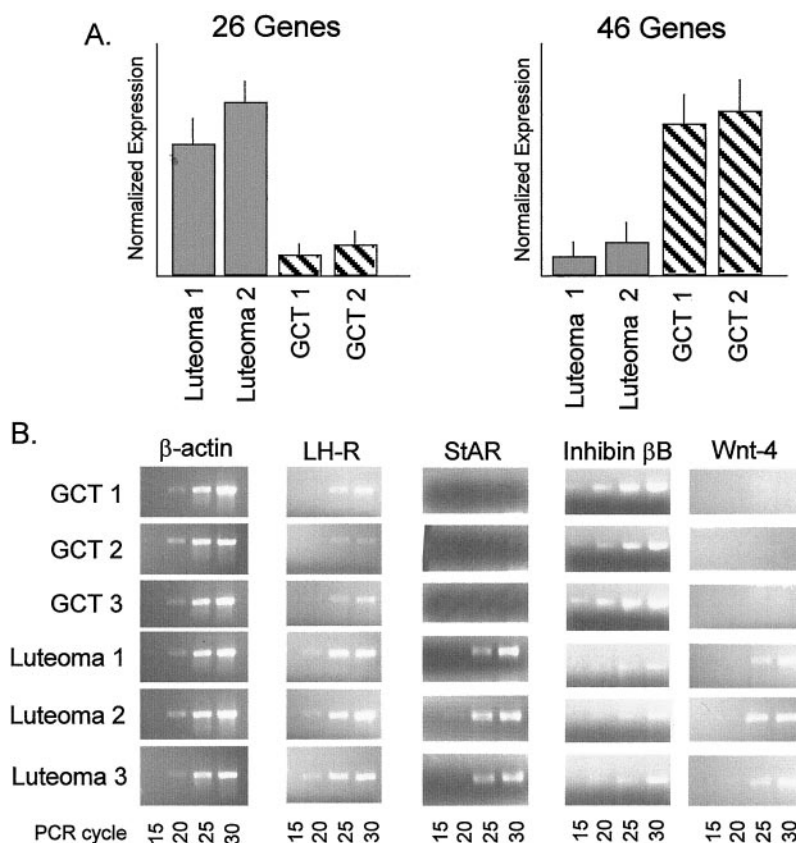


Fig. 3. Consistent Expression Patterns Were Identified with Duplicate Samples and Confirmed by RT-PCR

A, Duplicate, pooled samples encompassing distinct granulosa cell tumors (GCT) and luteomas from F₁ hybrids were used to identify consistent, tumor-specific gene expression changes. SOMs were used to normalize expression and illustrate the pattern of expression as described in Fig. 2. A cohort of genes was identified whose expression changed consistently between two luteoma and two tumor samples. Specifically, 26 genes demonstrated consistent expression decreases, whereas 46 genes displayed consistent expression increases. These genes have now been distinguished as strong candidates for further study. These consistent gene expression changes are illustrated in Tables 1 and 2. B, Semiquantitative RT-PCR was performed to confirm specific expression changes in individual animals with either a tumor or luteoma phenotype. All samples have approximately the same level of expression of β -actin at cycle 25, allowing for comparison of the specific mRNAs at this cycle number. Both LH-R and StAR expression are decreased in all granulosa cell tumors (GCT 1–3) as compared with the luteoma samples. Conversely, inhibin β -B is increased in all granulosa cell tumors compared with luteomas. These data recapitulate the microarray analyses and illustrate that the expression changes for these genes are consistent between animals of a particular phenotype.

mors (Table 1). This dramatic decrease in StAR mRNA was surprising because animals harboring tumors retain elevated serum androgens (44). This may suggest potential adrenal involvement or a StAR-independent mechanism of steroid production in tumor cells. Of note, LH β CTP mice have elevated levels of LH-R in their adrenal glands, again implicating the adrenals in the pathology (45). On the other hand, high expression of StAR in luteomas correlates with previous studies reporting elevated expression of StAR in corpora lutea and luteomas of pregnancy in women (68). Furthermore, the decreased expression of StAR correlates with the decrease in LH-R mRNA. This phenomenon is also observed in LH-R knockout mice (69), in which StAR expression is also decreased (data not shown). Thus, because LH and the LH surge regulate StAR expression (70, 71), there may be linkage between these two observations, functionally connecting their changes in gene expression. In addition, PRL receptor

(PRL-R) expression parallels StAR and LH-R. PRL-R mRNA is elevated in differentiated granulosa cells found in mouse corpora lutea (72, 73), consistent with its higher expression in luteomas compared with tumors in LH β CTP mice. Hence, decreased expression of PRL-R, in combination with decreased StAR and LH-R expression, suggests that granulosa cell tumors are less differentiated compared with wild-type ovaries and luteomas and that some of these changes may be functionally interrelated.

Other signaling pathways were also identified in the refined cohort of expression changes. For instance, alterations in members of the Wnt signaling pathway were revealed. The Wnt signaling pathway is involved in development, cellular proliferation and differentiation, and tumorigenesis (74–77). Specifically, Wnt-4 and secreted frizzled related protein 4 (sFRP-4) displayed dramatically decreased expression in granulosa cell tumors, while remaining high in luteomas.

Table 1. Gene Expression Decreases Associated with Granulosa Cell Tumors

Accession No.	Gene Name	Average Fold Decrease (Tumor vs. Luteoma)	Reproduced in hCG-Induced Luteoma
AA087277	Secreted frizzled related protein 4	400	Yes
Z27088	Relaxin precursor	300	Yes
AA122502	NG-CAM related adhesion molecule precursor	150	Yes
M14757	Multidrug resistant protein	100	No
M89797	Wnt-4	100	Yes
W15994	Laminin B receptor	49.0	Yes
D17433	PGF receptor	43.0	Yes
L36062	StAR	33.0	Yes
D14636	PEBP2a1 protein	32.0	Yes
D32137	MOPG	31.0	Yes
W41963	Acetyl-CoA synthetase	16.0	No
M62766	HMG-CoA reductase	14.0	Yes
Z22532	Syndecan-1	13.0	No
I13593	PRL-R receptor	11.5	Yes
D42048	Squalene epoxidase	9.5	No
X61940	Growth factor-inducible immediate early gene	9.0	Yes
AA066425	Tumor-associated antigen	8.5	No
M81310	LH-R	8.0	Yes
X56304	Tenascin	7.5	Yes
U49507	Lisch 7	7.0	Yes
M28698	Cytokeratin 19	7.0	Yes
AA016727	Farnesyl pyrophosphate synthetase	7.0	No
I05781	Cytosolic epoxide hydrolase	6.0	Yes
M37761	Calycyclin	6.0	Yes
M31419	204 interferon activatable protein	5.5	Yes
AA000961	Hemoglobinase precursor	3.0	Yes

Interestingly, mice deficient in Wnt-4 have significant ovarian pathology (78), suggesting that this pathway is essential for normal ovarian physiology and, from these data, potentially involved in tumorigenesis. Furthermore, Wnt-4 mRNA expression mirrors LH-R mRNA levels, suggesting that these signaling pathways may also interact and that Wnt-4 may be important for granulosa cell differentiation. In addition, there are emerging data (79) that Wnt-4 is expressed in granulosa cells, regulated by LH, and highly expressed in corpora lutea, supporting the data from this study. Frizzled-10 mRNA, a receptor for Wnts (80), was also dramatically increased in granulosa cell tumors from LH-hypersecreting mice. This may be in response to the decreased expression of Wnt-4 ligand observed in granulosa cell tumors (Table 1), further suggesting that the Wnt signaling pathway may play an important role in ovarian tumorigenesis. Thus, from the refined cohort of consistent expression changes, uncharacterized signaling pathways representing potentially important contributors to granulosa cell tumorigenesis and ovarian physiology have been identified and targeted for future studies.

The consistent changes in gene expression, observed through processing of two independent tumor and luteoma samples, suggests that the cohort of expression changes presented in Tables 1 and 2 may represent common phenomena in granulosa cell tumors in LH β CTP mice. To provide another means of verification, we analyzed a smaller subset of these genes in granulosa cell tumors and luteomas obtained

from individual animals using semiquantitative RT-PCR. This was distinct from the pooling technique used in the microarray analysis. As shown in Fig. 3B, the expression differences in LH-R, StAR, inhibin β -B, and Wnt-4 observed between luteomas and granulosa cell tumors through microarray analyses were recapitulated. Thus, from these additional analyses, this subset of tumor-associated expression changes has been shown to be consistent throughout individuals, distinguishing them and their related signaling pathways as potentially important contributors in both normal ovarian physiology and in ovarian tumorigenesis.

Ovulatory Doses of hCG Prevent Tumorigenesis in LH β CTP Mice

Having defined the partial transcriptomes that are associated with LH-induced luteomas or granulosa cell tumors, we wondered whether providing even further elevated, but surge like, levels of LH in genetically predisposed animals (CF-1) would shift the pathogenic outcome from tumor to luteoma through initiation of a differentiation cascade. The initial expression profiling data suggested that granulosa cell tumors in this model are composed of undifferentiated granulosa cells with decreased expression of LH-R, StAR, and PRL-R as compared with the more differentiated luteomas. Furthermore, although LH β CTP mice have chronically elevated levels of LH, they fail to mount a preovulatory surge of LH (data not shown) that is necessary for granulosa cell differentiation. Thus, we hy-

Table 2. Gene Expression Increases Associated with Granulosa Cell Tumors

Accession No.	Gene Name	Average Fold Increase (Tumor vs. Luteoma)	Reproduced in hCG-Induced Luteoma
X13586	2,3-Bisphosphoglycerate mutase	130	Yes
U81603	Eya2 homolog	40.0	Yes
W35058	Frizzled 10	39.0	Yes
U85610	Indian hedgehog protein	27.0	Yes
AB004048	Neuronatin	26.0	Yes
J04946	Angiotensin-converting enzyme	22.0	Yes
AA111277	Visinin-like protein	22.0	Yes
U37459	Glia-derived neurotrophic growth factor (GDNF)	21.0	Yes
M69069	MHC class I mRNA	21.0	No
X68837	Secretogranin II	17.0	Yes
M29464	Platelet-derived growth factor A-chain	14.0	Yes
X69620	Inhibin β -B subunit	12.0	Yes
AA105452	Glia-derived nexin precursor	10.0	Yes
M15525	Laminin B1	10.0	Yes
X03919	N-myc	9.5	Yes
X69619	Inhibin β -A subunit	9.0	Yes
I056439	IGFBP-2	9.0	Yes
M18194	Fibronectin	9.0	Yes
AA097626	Pol polyprotein	9.0	Yes
X94322	Melanoma-inhibitory-activity protein	8.5	Yes
V00727	c-fos oncogene	8.0	Yes
X13945	L-myc	8.0	Yes
M70642	FiSP-12	8.0	Yes
U17961	p62 mRNA	7.0	No
AA035915	Ras-like protein TC21	6.5	Yes
U79766	Ajuba	6.0	No
AF004326	Angiopoietin-2	6.0	Yes
W48402	SIR2	5.5	Yes
X89627	17- β -Hydroxysteroid dehydrogenase	5.5	Yes
AA064226	RAB-11B	5.5	Yes
M31131	N-cadherin	5.0	Yes
X70853	Fibulin C	5.0	Yes
W82053	EF-2	5.0	Yes
W41733	PKC substrate	5.0	Yes
X75285	Fibulin 2	5.0	Yes
X70854	Fibulin D	4.0	Yes
Z28532	Follistatin	4.0	Yes
AA063914	Tubulin α -chain	3.5	Yes
L04538	Amyloid precursor-like protein	3.5	Yes
AF119416	GM3 synthase	3.5	Yes
U79748	DPC4	3.5	No
X89749	mTGIF protein	3.0	Yes
W13162	CDK4	3.0	Yes
W43968	Myosin heavy chain 1B	3.0	Yes
W81863	Extensin precursor	3.0	Yes
Z22784	Troponin 1	3.0	Yes

MHC, Major histocompatibility complex.

pothesized that the lack of ovulatory surges may be an important determinant of granulosa cell tumor formation in CF-1 mice. Without ovulatory doses of LH, granulosa cells that express LH-R will fail to differentiate, causing an imbalance between cells that proliferate and those that differentiate. We found previously that ovulatory doses of hCG can induce ovulation in LH β CTP mice. This provides evidence that the ovulation machinery, including LH-R expression on granulosa cells, is intact in young transgenic animals (46). Thus, activating this machinery by providing ovulation-inducing doses of hCG may stimulate differentiation

and antagonize the protumorigenic influence of chronically high LH and possibly other hormones and growth factors.

To address these possibilities, LH β CTP mice were treated with ovulatory doses of hCG every fourth day for 5 months to determine whether an alteration in the tumorigenic outcome would occur. Treatment began at 2 wk of age and was halted once the animals reached 5 months of age. Transgenic female littermates received injections of saline as controls. All transgenic animals ($n = 4$) receiving hCG injections failed to develop granulosa cell tumors, whereas, as

expected, all littermate controls ($n = 3$) receiving saline injections developed tumors at 5 months of age. Representative sections from one of the four hCG-treated and one of the three saline-treated mice is shown in Fig. 4. Interestingly, ovaries from hCG-treated females displayed a histological phenotype indistinguishable from the F_1 hybrid mice, *i.e.* a luteoma in which most cells in the ovary are luteinized and differentiated. Thus, providing CF-1 transgenic females with ovulatory doses of hCG prevents tumor formation and induces an ovarian phenotype histologically identical with that observed in other strains of mice where LH is chronically elevated. This result indicates that ovulatory surges of hCG are capable of antagonizing the genetic predisposition for granulosa cell tumor formation in CF-1 transgenic mice.

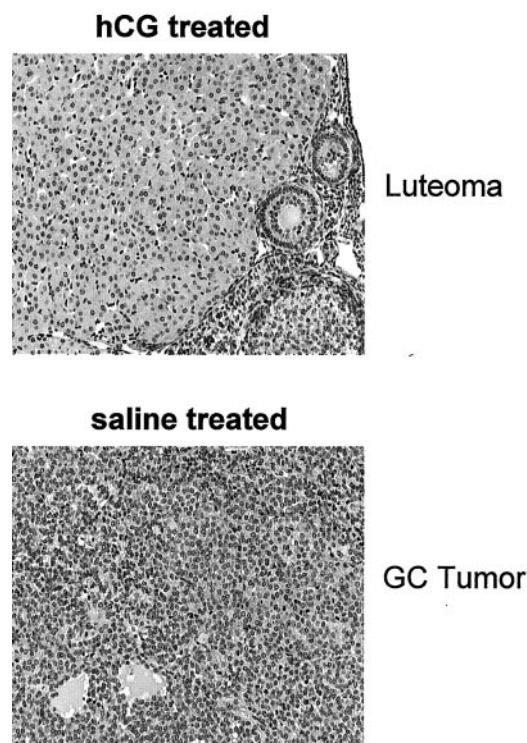


Fig. 4. Surges of hCG Prevent Tumorigenesis in LH β CTP Mice

Injections of hCG (10 IU) every 4 d beginning at 2 wk of age in CF-1 transgenic animals prevented the formation of granulosa cell tumors. Representative hematoxylin and eosin-stained sections from one of four hCG-treated, and one of three saline-treated, controls are illustrated. All samples from each experimental group had the same histological phenotypes. The granulosa cell tumors from saline controls displayed several hallmarks of granulosa cell tumors including increased nuclear-cytoplasm ratio, increased mitoses, and Call-Exner bodies. Conversely, in hCG-treated animals there is a decrease in the nuclear-cytoplasm ratio and extensive luteinization of the ovary.

Surges of hCG Induce a Transcriptional Profile Similar to a Luteoma as Opposed to a Granulosa Cell Tumor

Because hCG treatment induced formation of a luteoma in CF-1 mice that was morphologically and histologically identical with those in F_1 hybrid mice, we examined whether the ovarian transcriptome of CF-1 mice treated with ovulatory doses of hCG resembled that from F_1 hybrid mice. Specifically, from the initial analyses, 46 genes were found to increase in expression in tumors when compared with wild-type and F_1 hybrid luteomas (Fig. 2). In contrast, ovaries from genetically predisposed CF-1 mice receiving hCG treatment failed to show increases in expression in these genes and, in fact, had levels more similar to those found in luteomas from F_1 hybrids (Fig. 5A and Table 2). For example, expression of the *c-fos*, *L-myc*, and *N-myc* genes was increased in granulosa cell tumors; however, the expression of these genes was quite low in hCG-induced luteomas and similar to levels found in F_1 hybrid luteomas and wild-type animals. Increases in inhibin β A-B mRNAs, and most of the extracellular matrix protein encoding mRNAs seen in granulosa cell tumors, were also not observed with surges of hCG (Table 2).

Similarly, most genes with reduced expression in granulosa cell tumors were not significantly altered in ovaries from hCG surge-treated mice (Fig. 5B and Table 1). PRL-R, StAR, Wnt-4, and sFRP-4 were all expressed at levels similar to those in F_1 hybrid luteomas compared with granulosa cell tumors. Of note is the retention of LH-R mRNA in hCG-treated animals compared with tumor-bearing animals, in which its expression was decreased. Surges of LH normally induce luteinization of granulosa cells and expression of LH-R to support pregnancy (81). Thus, because hCG-treated transgenic ovaries and ovaries from F_1 hybrids are highly luteinized, it is not surprising that LH-R gene expression in these samples is high. These data again suggest that appropriate LH-R expression and sufficient LH levels may be critical for proper control of granulosa cell proliferation, differentiation, and tumorigenesis when accompanied by a genetic predisposition toward tumor formation.

Refining the Transcriptome Alterations Associated with Granulosa Cell Tumors

The diagram in Fig. 6 illustrates that chronically elevated LH, as well as potential alterations in androgens and other growth factors, initiates a series of events that, depending on the genetic predisposition of transgenic mice that chronically hypersecrete LH, leads inevitably to one of two tumorigenic outcomes: granulosa tumors or luteomas. In the preceding sections, we have shown that specific transcriptomes are linked to each tumorigenic outcome. Several of the changes in gene expression involve important components of major signal transduction cascades associated with

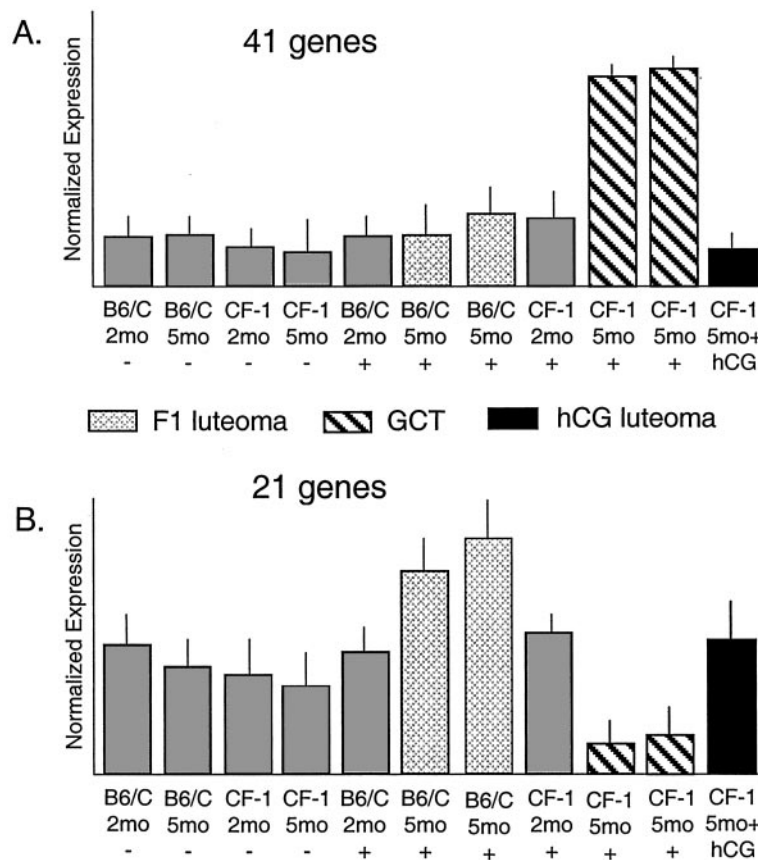


Fig. 5. Ovaries from hCG-Treated Mice More Closely Resemble F₁ Hybrid Luteomas when Analyzing Tumor-Associated Expression Changes

New clusters were created with the addition of RNA from hCG-treated animals (CF-1 5 mo + hCG). Remarkably, hCG-induced luteomas had a transcriptome that more closely resembled F₁ hybrid luteomas as compared with granulosa cell tumors. Specifically, hCG luteomas mimicked F₁ hybrid luteomas in 86% of the gene expression changes previously identified as associated with granulosa cell tumor development. SOMs of tumor-associated expression increases (A) and decreases (B) are illustrated with the incorporation of hCG-induced luteomas. The specific gene expression changes are displayed in Tables 1 and 2, respectively.

LH action. We have also discovered that administration of surges of hCG that mimic ovulatory levels of LH block formation of granulosa tumors in genetically predisposed mice and trigger, instead, formation of luteomas. The transcriptome associated with luteomas induced by this pathway bears striking resemblance to luteomas that form in transgenic mice that are not genetically predisposed to granulosa cell tumors.

Although this type of comparative gene expression profiling points to a number of interesting candidates worthy of additional functional characterization, we suspect that only a few of these will consistently associate with every granulosa cell tumor or luteoma. Therefore, the next challenge is to identify a smaller subset of transcriptomes that fulfill this criterion. We predict this can be achieved by continued expression profiling of ovarian tumors that arise through different initiating events. For example, we have recently performed comparisons between granulosa cell tumors from LH β CTP mice and tumors from inhibin α knock-out mice (data not shown). The inclusion of these tumors has further reduced the size of the transcrip-

tome linked with granulosa tumors to approximately 30 genes including LH-R and members of the Wnt signaling pathway. By further extending this approach to ovaries from LH β CTP mice deficient in other hormones, such as FSH, androgens, or estrogens, we predict that a stable transcriptome will emerge that uniquely associates with most granulosa cell tumors.

Once transcriptome alterations have been refined, the tumorigenic sufficiency of the identified genes and their encoded proteins, such as *c-fos*, N-myc, and members of the Wnt signaling pathway, can be examined indirectly through assessment of cell-specific localization and quantification and directly via additional transgenic approaches. Additionally, coupling these studies with proteomic approaches that include assessment of protein modification and activity should provide an ultimate test of their functional significance. Although proteomic approaches can be pursued independently, gene expression profiling can enhance proteomic analysis by identifying potential signaling pathways that include components that do not show parallel changes between a specific mRNA and its

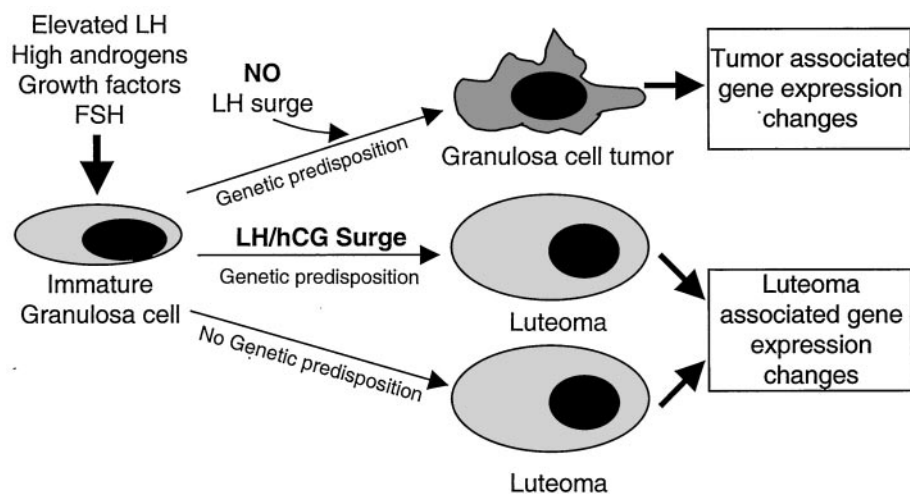


Fig. 6. Identifying Unique Transcriptomes that Couple with Genetic Factors to Induce Granulosa Cell Tumors or Luteomas

This model illustrates that chronically elevated LH, as well as potential alterations in androgens and other growth factors, initiates one of two basic tumorigenic pathways. The pathway taken depends on genetic predisposition and can be modified by hormonal intervention. Although the different tumors share overlapping transcriptomes, each has a unique pattern of gene expression.

encoded protein. For example, our preliminary data indicate that the mRNA for β -catenin, an important member of the Wnt signaling pathway, does not change during the course of tumorigenesis, whereas processing of its encoded protein changes dramatically (data not shown). Thus, our initial transcriptome data lay the groundwork for continued definition of genetic networks and signaling pathways that consistently associate with formation of ovarian tumors. How these networks and pathways interact with genetic factors governing tumor susceptibility may ultimately lead to a better understanding of human granulosa cell tumors, particularly those from postmenopausal women.

MATERIALS AND METHODS

Expression Profiling

Total RNA was collected from ovaries from individual mice using TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD). Equal amounts of total RNA were pooled from at least four animals in each experimental group. The average amount of pooled total RNA was approximately 20 μ g. Biotin-labeled cRNA was created from pooled total RNA, following protocols provided by Affymetrix (Affymetrix, Santa Clara, CA). Briefly, total RNA was used in a reverse transcription reaction (Life Technologies, Inc.) with an oligo-dT primer containing a T7 polymerase promoter. Double-stranded cDNA was generated and used in an *in vitro* transcription reaction containing biotinylated UTP and CTP (Enzo Diagnostics, Farmingdale, NY). Equal amounts of biotinylated cRNA from each sample were used to probe Affymetrix Mu11K expression array GeneChips. These arrays contain 11,000 genes and ESTs distributed between two chips (A and B). Once all samples were hybridized and scanned, expression data between chips were normalized to GAPDH expression levels using GeneChip software.

Generation of SOMs and Data Analysis

SOMs were generated using data collected from Affymetrix expression arrays. Values for "average difference intensity," a quantitative level of hybridization and hence relative expression, were used to generate SOMs. GENECLUSTER was used to create the SOMs (50). Data were filtered and normalized (thresholds: minimum (min), 20; maximum (max), 30,000; row variation: max/min = 3, max - min = 100; row normalization: mean 0, variance 1) across samples so as to eliminate genes that exhibited no change throughout the samples. Once informative SOMs and clusters were generated, the data were filtered further using parameters generated by Affymetrix Microarray Suite that are not considered in the GENECLUSTER program. For example, genes are identified as "absent" or "present" by Affymetrix software. Therefore, genes identified as absent throughout all the samples were removed from the analysis. In addition, only genes identified as "increased" or "decreased" between informative samples by Affymetrix software were recorded as significant changes. Fold change was also considered. Gene changes 3-fold or greater were considered significant because changes greater than 3-fold were previously found to be highly repeatable and reliable between duplicated samples (51). Once genes were removed using these criteria, the data was reclustered in GENECLUSTER to generate the true expression pattern, utilizing only the row normalization parameter in GENECLUSTER. In the end, only genes changing more than 3-fold and those that were identified as "present," "increased," and/or "decreased" in particular samples were accepted in each cluster. Expression profiling data can be viewed at <http://mend.endojournals.org>, and have been deposited in the Gene Expression Omnibus database (<http://www.ncbi.nlm.nih.gov/geo>).

RT-PCR

Total RNA was purified from the ovaries of both CF-1 and F₁ hybrid (C57BL/6 \times CF-1, Tg) 5-month transgenic mice harboring either granulosa cell tumors or luteomas, respectively. The RNA was deoxyribonuclease 1 treated and used in a reverse transcription reaction to generate single-stranded cDNA using SuperScript reverse transcriptase (Life Technologies, Inc.). Equal amounts of RNA were used for each re-

action. Specific cDNA species were generated through the use of gene-specific 3'-primers for β -actin (5'-GGATTCCAT-ACCCAAGAAGGAAGG-3'), LH-R (5'-TCTGTTACCCAAGACACTC-3'), StAR (5'-CTGAAGATGGACAGACTT-3'), Wnt-4 (5'-AACTGTGCATTCCGAGGCAC-3'), and inhibin β -B (5'-GTCGTACGAGTACAGTTTCG-3'). Single-stranded cDNAs were used as templates for PCR for each of the gene transcripts. Additional gene-specific 5'-primers were used for these reactions [β -actin (5'-GAACATGGCATTGTTACC-AACTGG-3'), LH-R (5'-TCTGTTACCCAAGACACTC-3'), StAR (5'-GTCAAGGAGATCAAGGTC-3'), Wnt-4 (5'-GCTTC-CAGTGGTCAGGATGC-3'), inhibin β -B (5'-AACAACTGACAGGTCAGTGG-3')]. Fifteen-microliter aliquots were removed from each 100- μ l reaction every five cycles beginning at cycle 15 and ending at cycle 35. Aliquots were visualized on agarose gels. Comparison of gene expression levels between samples was normalized to β -actin expression.

hCG Injections and Histological Analyses

All mice for these experiments were on a 12-h day/night cycle and had food and water *ad libitum*. The Case Western Reserve University Institutional Animal Care of Use Committee approved all animal studies. Four transgenic and four wild-type CF-1 mice were given hCG in saline (Wyeth-Ayerst Laboratories, Inc., Philadelphia, PA) (10 IU) injections beginning at 14 d of age. This dose has been shown to induce ovulation in wild-type and young LH β CTP mice (46). Injections were given every 4 d, mimicking the normal cycle of a mouse (82). At 5 months of age, the mice were killed and ovaries harvested for histological analysis and RNA collection. Tissue was fixed in Kalhe's solution, paraffin embedded, sectioned, and hemotoxylin and eosin stained. Sections were then evaluated for the presence of granulosa cell tumors.

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REFERENCES

- Fuller PJ, Verity K, Shen Y, Mamers P, Jobling T, Burger HG 1998 No evidence of a role for mutations or polymorphisms of the follicle-stimulating hormone receptor in ovarian granulosa cell tumors. *J Clin Endocrinol Metab* 83:274–279
- Amsterdam A, Selvaraj N 1997 Control of differentiation, transformation, and apoptosis in granulosa cells by oncogenes, oncoviruses, and tumor suppressor genes. *Endocr Rev* 18:435–461
- Cooke I, O'Brien M, Charnock FM, Groome N, Ganesan TS 1995 Inhibin as a marker for ovarian cancer. *Br J Cancer* 71:1046–1050
- Wynder EL, Dodo H, Barber HR 1969 Epidemiology of cancer of the ovary. *Cancer* 23:352–370
- Lee WL, Yuan CC, Lai CR, Wang PH 1999 Hemoperitoneum is an initial presentation of recurrent granulosa cell tumors of the ovary. *Jpn J Clin Oncol* 29:509–512
- Fontanelli R, Stefanon B, Raspagliesi F, Kenda R, Tomasic G, Spatti G, Riboldi G, Di Donato P, Pilotti S, De Palo G 1998 Adult granulosa cell tumor of the ovary: a clinico-pathologic study of 35 cases. *Tumori* 84:60–64
- Lane AH, Lee MM, Fuller Jr AF, Kehas DJ, Donahoe PK, MacLaughlin DT 1999 Diagnostic utility of Mullerian inhibiting substance determination in patients with primary and recurrent granulosa cell tumors. *Gynecol Oncol* 73:51–55
- Lappohn RE, Burger HG, Bouma J, Bangah M, Krans M, de Bruijn HW 1989 Inhibin as a marker for granulosa-cell tumors. *N Engl J Med* 321:790–793
- Burger HG, Baillie A, Drummond AE, Healy DL, Jobling T, Mamers P, Robertson DM, Susil B, Cahir N, Shen Y, Verity K, Fuller PJ, Groome NP, Findlay JK 1998 Inhibin and ovarian cancer. *J Reprod Immunol* 39:77–87
- Robertson DM, Cahir N, Burger HG, Mamers P, Groome N 1999 Inhibin forms in serum from postmenopausal women with ovarian cancers. *Clin Endocrinol (Oxf)* 50:381–386
- Robertson DM, Cahir N, Burger HG, Mamers P, McCloud PI, Pettersson K, McGuckin M 1999 Combined inhibin and CA125 assays in the detection of ovarian cancer. *Clin Chem* 45:651–658
- Gebhart JB, Roche PC, Keeney GL, Lesnick TG, Podratz KC 2000 Assessment of inhibin and p53 in granulosa cell tumors of the ovary. *Gynecol Oncol* 77:232–236
- King LA, Okagaki T, Gallup DG, Twiggs LB, Messing MJ, Carson LF 1996 Mitotic count, nuclear atypia, and immunohistochemical determination of Ki-67, c-myc, p21-ras, c-erbB2, and p53 expression in granulosa cell tumors of the ovary: mitotic count and Ki-67 are indicators of poor prognosis. *Gynecol Oncol* 61:227–232
- Wang ZJ, Churchman M, Campbell IG, Xu WH, Yan ZY, McCluggage WG, Foulkes WD, Tomlinson IP 1999 Allele loss and mutation screen at the Peutz-Jeghers (LKB1) locus (19p13.3) in sporadic ovarian tumours. *Br J Cancer* 80:70–72
- Sicinski P, Donaher JL, Geng Y, Parker SB, Gardner H, Park MY, Robker RL, Richards JS, McGinnis LK, Biggers JD, Eppig JJ, Bronson RT, Elledge SJ, Weinberg RA 1996 Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. *Nature* 384:470–474
- Lukas J, Bartkova J, Welcker M, Petersen OW, Peters G, Strauss M, Bartek J 1995 Cyclin D2 is a moderately oscillating nucleoprotein required for G₁ phase progression in specific cell types. *Oncogene* 10:2125–2134
- Robker RL, Richards JS 1998 Hormone-induced proliferation and differentiation of granulosa cells: a coordinated balance of the cell cycle regulators cyclin D2 and p27Kip1. *Mol Endocrinol* 12:924–940
- Hunter T, Karin M 1992 The regulation of transcription by phosphorylation. *Cell* 70:375–387
- Danilovich N, Roy I, Sairam MR 2001 Ovarian pathology and high incidence of sex cord tumors in follitropin receptor knockout (FORKO) mice. *Endocrinology* 142:3673–3684
- Wise PM, Krajnak KM, Kashon ML 1996 Menopause: the aging of multiple pacemakers. *Science* 273:67–70
- Willemsen W, Kruitwagen R, Bastiaans B, Hanselaar T, Rolland R 1993 Ovarian stimulation and granulosa-cell tumour. *Lancet* 341:986–988
- Rossing MA, Daling JR, Weiss NS, Moore DE, Self SG 1994 Ovarian tumors in a cohort of infertile women. *N Engl J Med* 331:771–776
- Bristow RE, Karlan BY 1996 Ovulation induction, infertility, and ovarian cancer risk. *Fertil Steril* 66:499–507

24. Mosgaard BJ, Lidegaard O, Kjaer SK, Schou G, Andersen AN 1997 Infertility, fertility drugs, and invasive ovarian cancer: a case-control study. *Fertil Steril* 67:1005–1012
25. Beamer WG, Shultz KL, Tennent BJ, Azumi N, Sundberg JP 1998 Mouse model for malignant juvenile ovarian granulosa cell tumors. *Toxicol Pathol* 26:704–710
26. Beamer WG, Shultz KL, Tennent BJ 1988 Induction of ovarian granulosa cell tumors in SWXJ-9 mice with dehydroepiandrosterone. *Cancer Res* 48:2788–2792
27. Beamer WG, Shultz KL, Tennent BJ, Nadeau JH, Churchill GA, Eicher EM 1998 Multigenic and imprinting control of ovarian granulosa cell tumorigenesis in mice. *Cancer Res* 58:3694–3699
28. Guthrie MJ 1957 Tumorigenesis in intrasplenic ovaries in mice. *Cancer* 10:190–203
29. Beamer WG, Shultz KL, Tennent BJ, Shultz LD 1993 Granulosa cell tumorigenesis in genetically hypogonadal-immunodeficient mice grafted with ovaries from tumor-susceptible donors. *Cancer Res* 53:3741–3746
30. Armuth V, Berenblum I 1979 Mechanism of ovarian carcinogenesis: effect of 7,12-dimethylbenz[a]anthracene administration on intrasplenic ovarian grafts in unilaterally ovariectomized C3HeB/Fe mice. *J Natl Cancer Inst* 63:1047–1050
31. Capen CC, Beamer WG, Tennent BJ, Stitzel KA 1995 Mechanisms of hormone-mediated carcinogenesis of the ovary in mice. *Mutat Res* 333:143–151
32. Hilfrich J 1975 Comparative morphological studies on the carcinogenic effect of 7,12-dimethylbenz(a)anthracene (DMBA) in normal or intrasplenic ovarian tissue of C3H mice. *Br J Cancer* 32:588–595
33. Rao AR 1981 Effects of carcinogen and/or mutagen on normal and gonadotropin-primed ovaries of mice. *Int J Cancer* 28:105–110
34. Taguchi O, Michael SD, Nishizuka Y 1988 Rapid induction of ovarian granulosa cell tumors by 7,12-dimethylbenz(a)anthracene in neonatally estrogenized mice. *Cancer Res* 48:425–429
35. Tennent BJ, Beamer WG 1986 Ovarian tumors not induced by irradiation and gonadotropins in hypogonadal (hpg) mice. *Biol Reprod* 34:751–760
36. Nishizuka Y, Sakakura T, Taguchi O 1979 Mechanism of ovarian tumorigenesis in mice after neonatal thymectomy. *Natl Cancer Inst Monogr* 51:89–96
37. Michael SD, Taguchi O, Nishizuka Y 1981 Changes in hypophyseal hormones associated with accelerated aging and tumorigenesis of the ovaries in neonatally thymectomized mice. *Endocrinology* 108:2375–2380
38. Michael SD, Taguchi O, Nishizuka Y 1980 Effect of neonatal thymectomy on ovarian development and plasma LH, FSH, GH and PRL in the mouse. *Biol Reprod* 22:343–350
39. Matzuk MM, Finegold MJ, Su JG, Hsueh AJ, Bradley A 1992 α -Inhibin is a tumour-suppressor gene with gonadal specificity in mice. *Nature* 360:313–319
40. Kumar TR, Palapattu G, Wang P, Woodruff TK, Boime I, Byrne MC, Matzuk MM 1999 Transgenic models to study gonadotropin function: the role of follicle-stimulating hormone in gonadal growth and tumorigenesis. *Mol Endocrinol* 13:851–865
41. Kumar TR, Wang Y, Matzuk MM 1996 Gonadotropins are essential modifier factors for gonadal tumor development in inhibin-deficient mice. *Endocrinology* 137:4210–4216
42. Risma KA, Clay CM, Nett TM, Wagner T, Yun J, Nilson JH 1995 Targeted overexpression of luteinizing hormone in transgenic mice leads to infertility, polycystic ovaries, and ovarian tumors. *Proc Natl Acad Sci USA* 92:1322–1326
43. Risma KA, Hirshfield AN, Nilson JH 1997 Elevated luteinizing hormone in prepubertal transgenic mice causes hyperandrogenemia, precocious puberty, and substantial ovarian pathology. *Endocrinology* 138:3540–3547
44. Keri RA, Lozada KL, Abdul-Karim FW, Nadeau JH, Nilson JH 2000 Luteinizing hormone induction of ovarian tumors: oligogenic differences between mouse strains dictates tumor disposition. *Proc Natl Acad Sci USA* 97:383–387
45. Kero J, Poutanen M, Zhang FP, Rahman N, McNicol AM, Nilson JH, Keri RA, Huhtaniemi IT 2000 Elevated luteinizing hormone induces expression of its receptor and promotes steroidogenesis in the adrenal cortex. *J Clin Invest* 105:633–641
46. Mann RJ, Keri RA, Nilson JH 1999 Transgenic mice with chronically elevated luteinizing hormone are infertile due to anovulation, defects in uterine receptivity, and midgestation pregnancy failure. *Endocrinology* 140:2592–2601
47. Flaws JA, Abbud R, Mann RJ, Nilson JH, Hirshfield AN 1997 Chronically elevated luteinizing hormone depletes primordial follicles in the mouse ovary. *Biol Reprod* 57:1233–1237
48. Owens GE, Keri RA, Nilson JH 2001 LH hypersecreting mice: a model for ovarian granulosa cell tumors. In: Castro MG, ed. *Transgenic models in endocrinology*. Boston: Kluwer Academic Publishers; 59–78
49. Piana S, Nogales FF, Corrado S, Cardinale L, Gusolfino D, Rivasi F 1999 Pregnancy luteoma with granulosa cell proliferation: an unusual hyperplastic lesion arising in pregnancy and mimicking an ovarian neoplasia. *Pathol Res Pract* 195:859–863
50. Tamayo P, Slonim D, Mesirov J, Zhu Q, Kitareewan S, Dmitrovsky E, Lander ES, Golub TR 1999 Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. *Proc Natl Acad Sci USA* 96:2907–2912
51. Cho RJ, Campbell MJ, Wenzler EA, Steinmetz L, Conway A, Wodicka L, Wolfsberg TG, Gabrielian AE, Landsman D, Lockhart DJ, Davis RW 1998 A genome-wide transcriptional analysis of the mitotic cell cycle. *Mol Cell* 2:65–73
52. Gercel-Taylor C, Taylor DD 1996 Effect of patient-derived lipids on *in vitro* expression of oncogenes by ovarian tumor cells. *Gynecol Obstet Invest* 42:42–48
53. Luthra K, Chapekar TN 1998 Oncogene expression as detected by immunocytochemical staining in hormonally induced ovarian cell lines. *Indian J Exp Biol* 36:447–455
54. Tyson FL, Boyer CM, Kaufman R, O'Brian K, Cram G, Crews JR, Soper JT, Daly L, Fowler Jr WC, Haskill JS 1991 Expression and amplification of the HER-2/neu (c-erbB-2) protooncogene in epithelial ovarian tumors and cell lines. *Am J Obstet Gynecol* 165:640–646
55. Versnel MA, Haarbrink M, Langerak AW, de Laat PA, Hagemeyer A, van der Kwast TH, Berg-Bakker LA, Schrier PI 1994 Human ovarian tumors of epithelial origin express PDGF *in vitro* and *in vivo*. *Cancer Genet Cytogenet* 73:60–64
56. Gentry PA, Zareie M, Liptrap RM 1996 Fibronectin concentrations correlate with ovarian follicular size and estradiol values in equine follicular fluid. *Anim Reprod Sci* 45:91–102
57. Morley P, Armstrong DT, Gore-Langton RE 1987 Fibronectin stimulates growth but not follicle-stimulating hormone-dependent differentiation of rat granulosa cells *in vitro*. *J Cell Physiol* 132:226–236
58. Morley P, Armstrong DT, Gore-Langton RE 1987 Adhesion and differentiation of cultured rat granulosa cells: role of fibronectin. *Am J Physiol* 253:C625–C632
59. Bork P 1993 The modular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Lett* 327:125–130
60. Roger P, Pujol P, Lucas A, Baldet P, Rochefort H 1998 Increased immunostaining of fibulin-1, an estrogen-

- regulated protein in the stroma of human ovarian epithelial tumors. *Am J Pathol* 153:1579–1588
61. Grant DS, Kibbey MC, Kinsella JL, Cid MC, Kleinman HK 1994 The role of basement membrane in angiogenesis and tumor growth. *Pathol Res Pract* 190:854–863
 62. Kibbey MC, Yamamura K, Jun SH, Grant DS, Kleinman HK 1994 Enhancement of tumor growth by basement membrane: modulation of growth and angiogenesis by laminin-derived synthetic peptides. *Cancer Treat Res* 71:267–275
 63. de Kretser DM, Meinhardt A, Meehan T, Phillips DJ, O'Bryan MK, Loveland KA 2000 The roles of inhibin and related peptides in gonadal function. *Mol Cell Endocrinol* 161:43–46
 64. Beamer WG, Hoppe PC, Whitten WK 1985 Spontaneous malignant granulosa cell tumors in ovaries of young SWR mice. *Cancer Res* 45:5575–5581
 65. Beamer WG 1986 Gonadotropin, steroid, and thyroid hormone milieu of young SWR mice bearing spontaneous granulosa cell tumors. *J Natl Cancer Inst* 77:1117–1123
 66. Stouffer RL, Grodin MS, Davis JR, Surwit EA 1984 Investigation of binding sites for follicle-stimulating hormone and chorionic gonadotropin in human ovarian cancers. *J Clin Endocrinol Metab* 59:441–446
 67. Stocco DM 2000 The role of the StAR protein in steroidogenesis: challenges for the future. *J Endocrinol* 164:247–253
 68. Pollack SE, Furth EE, Kallen CB, Arakane F, Kiriakidou M, Kozarsky KF, Strauss III JF 1997 Localization of the steroidogenic acute regulatory protein in human tissues. *J Clin Endocrinol Metab* 82:4243–4251
 69. Zhang FP, Poutanen M, Wilbertz J, Huhtaniemi I 2001 Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. *Mol Endocrinol* 15:172–183
 70. Ronen-Fuhrmann T, Timberg R, King SR, Hales KH, Hales DB, Stocco DM, Orly J 1998 Spatio-temporal expression patterns of steroidogenic acute regulatory protein (StAR) during follicular development in the rat ovary. *Endocrinology* 139:303–315
 71. Huang TJ, Shirley LP 2001 Dexamethasone inhibits luteinizing hormone-induced synthesis of steroidogenic acute regulatory protein in cultured rat preovulatory follicles. *Biol Reprod* 64:163–170
 72. Clarke DL, Arey BJ, Linzer DI 1993 Prolactin receptor messenger ribonucleic acid expression in the ovary during the rat estrous cycle. *Endocrinology* 133:2594–2603
 73. Clarke DL, Linzer DI 1993 Changes in prolactin receptor expression during pregnancy in the mouse ovary. *Endocrinology* 133:224–232
 74. Akiyama T 2000 Wnt/ β -catenin signaling. *Cytokine Growth Factor Rev* 11:273–282
 75. Sakanaka C, Sun TQ, Williams LT 2000 New steps in the Wnt/ β -catenin signal transduction pathway. *Recent Prog Horm Res* 55:225–236
 76. Polakis P 2000 Wnt signaling and cancer. *Genes Dev* 14:1837–1851
 77. Taipale J, Beachy PA 2001 The hedgehog and Wnt signalling pathways in cancer. *Nature* 411:349–354
 78. Vainio S, Heikkila M, Kispert A, Chin N, McMahon AP 1999 Female development in mammals is regulated by Wnt-4 signalling. *Nature* 397:405–409
 79. Hsieh M, Johnson MA, Greenberg NM, Richards JS 2002 Regulated expression of Wnts and frizzleds at specific stages of follicular development in the rodent ovary. *Endocrinology* 143:898–908
 80. Koike J, Takagi A, Miwa T, Hirai M, Terada M, Katoh M 1999 Molecular cloning of Frizzled-10, a novel member of the Frizzled gene family. *Biochem Biophys Res Commun* 262:39–43
 81. Duncan WC, McNeilly AS, Fraser HM, Illingworth PJ 1996 Luteinizing hormone receptor in the human corpus luteum: lack of down-regulation during maternal recognition of pregnancy. *Hum Reprod* 11:2291–2297
 82. Richards JS 1980 Maturation of ovarian follicles: actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. *Physiol Rev* 60:51–89

