Human Ovarian Cancer Stroma Contains Luteinized Theca Cells Harboring Tumor Suppressor Gene GT198 Mutations*

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Background: GT198 is a tumor suppressor gene in breast and ovarian cancer.

Results: Tumor stromal cells in various types of human ovarian cancer carry GT198 mutations and express theca cell-specific enzyme CYP17.

Conclusion: GT198 mutant tumor stromal cells are luteinized theca cells in ovarian cancer.

Significance: Studying mutant tumor stromal cells is crucial for understanding the cause of ovarian cancer.

Ovarian cancer is a highly lethal gynecological cancer, and its causes remain to be understood. Using a recently identified tumor suppressor gene, GT198 (PSMC3IP), as a unique marker, we searched for the identity of GT198 mutant cells in ovarian cancer. GT198 has germ line mutations in familial and early onset breast and ovarian cancers and recurrent somatic mutations in sporadic fallopian tube cancers. GT198 protein has been shown as a steroid hormone receptor coregulator and also as a crucial factor in DNA repair. In this study, using GT198 as a marker for microdissection, we find that ovarian tumor stromal cells harboring GT198 mutations are present in various types of ovarian cancer including high and low grade serous, endometrioid, mucinous, clear cell, and granulosa cell carcinomas and in precursor lesions such as inclusion cysts. The mutant stromal cells consist of a luteinized theca cell lineage at various differentiation stages including CD133+, CD44+, and CD34+ cells, although the vast majority of them are differentiatated overexpressing steroidogenic enzyme CYP17, a theca cell-specific marker. In addition, wild type GT198 suppresses whereas mutant GT198 protein stimulates CYP17 expression. The chromatin-bound GT198 on the human CYP17 promoter is decreased by overexpressing mutant GT198 protein, implicating the loss of wild type suppression in mutant cells. Together, our results suggest that GT198 mutant luteinized theca cells overexpressing CYP17 are common in ovarian cancer stroma. Because first hit cancer gene mutations would specifically mark cancer-inducing cells, the identification of mutant luteinized theca cells may add crucial evidence in understanding the cause of human ovarian cancer.

The precursor lesion of human ovarian cancer is one of the most intriguing gynecological research topics, and the source of cancer precursors has been under debate for decades (1). Based mostly on histological and molecular analyses, there are three main hypothetical theories that have been proposed. One theory involves ovarian surface epithelium (2). It suggests that cortical inclusion cysts formed by invagination of ovarian surface epithelium serve as precursor lesions. The second theory involves the secondary Mullerian system, suggesting Mullerian-originated extraovarian lesions such as endometriosis as precursors of ovarian cancer (3–6). The most recent theory suggests that intraepithelial lesions at fallopian tube fimbrial ends, enriched with TP53 somatic mutations or from BRCA1 carriers (7, 8), serve as precursors of high grade serous ovarian cancer (9–12), and thus epithelial ovarian cancer shares a common origin with distal fallopian tube cancer. Each of the hypotheses has substantial supporting evidence and is potentially interconnected. However, a specific functional cell lineage that induces ovarian cancer steroid hormone deregulation has not been confirmed, and causative molecular alterations are still largely unclear.

A related issue is whether the potential causative molecular defect in ovarian cancer occurs in tumor stroma or in tumor parenchyma. A mutated cancer-initiating gene is presumably a specific marker for precursor lesions. Breast and ovarian cancer genes such as BRCA1 and BRCA2 are identified through analyzing germline mutations in familial cases (13, 14), and their somatic mutations are largely undetectable in advanced tumors (15), which is evidence against the possibility for clonal expansion of a mutant progenitor into tumor parenchyma. In contrast, mounting evidence supports a link of cancer initiation to altered tumor microenvironment in tumor stroma (16, 17); stroma is the particular natural habitat of progenitor cells. In ovarian cancer, steroid hormone-producing cells have been observed in tumor stroma promoting inclusion cysts and epithelial tumor cells (18). It remains to be elucidated how ovarian cancer stromal cells alter the tumor microenvironment and lead to the growth of tumor parenchyma.

In this study, we have revealed that ovarian cancer stroma contains luteinized theca cells carrying somatic mutations in tumor suppressor gene GT198. GT198 (gene symbol PSMC3IP,
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also known as TBPIP or Hop2) is a nuclear receptor coregulator participating in estrogen, androgen, glucocorticoid, and progesterone receptor-mediated gene regulation (19, 20). GT198 has also been extensively shown to regulate homologous recombination in DNA repair (21, 22) and to stimulate Rad51-mediated DNA strand exchange (23). A homozygous germ line deletion mutation in GT198 is identified in familial ovarian dysgenesis disease (24). We have recently identified GT198 germ line mutations in familial and early onset breast and ovarian cancers (25) and prevalent GT198 somatic mutations in sporadic fallopian tube cancers (26). Loss of GT198 function is associated with its mutations (26). The collective existing evidence supports a functional role of GT198 in ovary and signifies the potential of its mutations in studying ovarian cancer.

Using GT198 expression as a marker, we have identified GT198 mutant cells as luteinized theca cell lineage in human ovarian cancer. GT198 mutant cells are located in tumor stroma, and most of them strongly express CYP17 (cytochrome P450 17α), a key enzyme catalyzing steroid hormone biosynthesis and exclusively expressed in theca or luteinized theca cells in ovaries (27, 28). GT198 mutant cells are common lesions in various types of ovarian cancer and in early lesion inclusion cysts. Molecular analyses show that GT198 suppression induces the expression of CYP17. Our study reveals that luteinized theca cell lineage in ovarian tumor stroma carries tumor suppressor gene mutations and provides crucial evidence to strengthen the link between steroid hormone and human ovarian cancer initiation.

MATERIALS AND METHODS

Human Tumor Materials and Laser Capture Microdissection—Formalin-fixed paraffin-embedded ovarian tumor tissue sections and tumor microarrays were screened by immunohistochemistry using anti-GT198 for microdissection. Frozen sections were not used in this study because they are incompatible with immunohistochemical staining. Freshly cut ovarian tumor sections and microarrays containing a total of 246 cases of ovarian cancer (Imgenex, US Biomax, Georgia Regents University and Shantou University Medical College) were immunostained to select cases with sufficient GT198+ cells for microdissection (see Table 1). The serial cut adjacent sections or arrays were then immunostained prior to microdissection using standard immunohistochemical procedures followed by dehydration in ethanol and xylene series. The slides were imaged with coverslips after the microdissection. Laser capture microdissection was performed using a Pix-Cell II laser capture microscope and CapSure macro LCM caps according to the manufacturer’s protocol (Arcturus Engineering). Genomic DNA from dissected cells (~50–200 cells) was isolated by DNeasy tissue kit reagents using 1:10 scaling down quantity 109C to c.34/H11002. DNA samples were sequence-analyzed using Pfudy DNA polymerase (Stratagene) with repeats in two pre-
kit (Qiagen). The primers are; CYP17, actccactgtctatctgcctg and ccttacggtgttggacgcgatg; endogenous GT198, cttccccctcagccaatcac and gtgggcccctcaggggtctg; and GAPDH, accacagtcgactc and tccaccacccctgttgctgta. p values are calculated by unpaired t test.

**ChIP and Real Time PCR Analyses—**293 cells were transfected with FLAG-tagged GT198 (WT, 1–217, 1 μg), together with nontagged mutant GT198 (Mut, 126–217, 3 μg), or siRNA (100 μM) in a 60-mm dish for overnight. The cells were treated with 1% formaldehyde for 10 min, and the cross-linking was stopped by 125 mM glycine. The cells were lysed and sonicated in the buffer containing 20 mM Tris, pH 8.0, 75 mM NaCl, 75 mM KCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM DTT, and protease inhibitors. Immunoprecipitation was carried out using anti-FLAG M2 beads (Sigma) preblocked with salmon sperm DNA (0.1 mg/ml). The vector transfected cells and beads only were served as controls. The cross-linking was reversed by eluting with 0.1M NaHCO3, 1% SDS, 0.3M NaCl at 65 °C for 4 h. Purified DNA (Qiagen kit) was subjected to quantitative real time PCR (Mx3000P; Stratagene) using Sybr Green dye. GAPDH was measured and used for internal normalization. Serial diluted normal human genomic DNA was used for standard curves. The CYP17 promoter primers are c.–1897, aaagaaagggagagatgttgcgg and gacaaacatcctcagcttacaaag; c.–1404, ggggaggtcctataacacgccgc and ageggtgccatacgcgtcctgtgata; and c.–272, caacgtacctcctctcctgctgctgct and cagggatagacagcagtggtggt. p values are calculated by unpaired t test.

**RESULTS**

**Cytoplasmic GT198 Expression in Ovarian Cancer Stromal Cells—**The normal expression of GT198 in vivo in mice is tissue-specific, mostly in embryonic tissues, and also in adult testis and ovary (19). The overall protein expression pattern in mouse is remarkably similar between GT198 and BRCA1 or BRCA2 (30). Wild type GT198 is a nuclear protein and the expression becomes cytoplasmic when the GT198 gene is mutated (26). Using cytoplasmic GT198 as a marker, we screened 246 cases of human ovarian cancer by immunohistochemistry. The results showed that GT198 expression was largely cytoplasmic (Fig. 1) and was present in tumor stromal cells in various types of ovarian cancer including high and low grade serous, mucinous, endometrioid, clear cell, müllerian, and granulosa-theca cell carcinomas, but not in metastatic tumors originated from other sites (Fig. 1 and Table 1). Nuclear GT198 expression was absent in epithelial types but was significant in benign fibroma-thecoma (not shown). The overall positive rate could not be assessed, however, because of the lack of stromal area in many small sized microarray tumor samples. GT198⁺ cells with cytoplasmic expression often lined the tumor epithelia (Fig. 1, B–E). In a sample containing ovarian cancer precursor lesions, which resemble inclusion cysts, GT198⁺ cells clustered in stroma surrounding the inclusion cysts (Fig. 1G). They appeared to be more recruited to the irregularly shaped cysts containing proliferating tumor cells, implicating a positive influence of the cysts by GT198⁺ cells (Fig.
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Our data together suggest that cytoplasmic GT198 in tumor stroma is present in various types of ovarian cancer including early lesions.

GT198+ Tumor Stromal Cells Carry Genetic Mutations—

We previously found that GT198 mutations induce GT198 cytoplasmic expression (26). To detect mutation in GT198+
stromal cells, we performed laser microdissection and analyzed GT198 mutations in two previously identified mutation hot spot sequences around exon 1 and intron 4 (26). GT198 mutations were identified in eight of twelve (66.6%) dissected cases of ovarian cancer including serous, mucinous, clear cell, endometrioid, and granulosa carcinomas and inclusion cysts (Fig. 2A and Table 2). Mutations were identified in GT198+ tumor stromal cells but not in nearby control epithelial tumor cells negative for GT198 staining, suggesting a somatic origin of the mutations and confirming an association of cytoplasmic GT198 expression with the mutations (Fig. 2A). Some mutations were identical to those we previously found in fallopian tube cancers (26); the c.284C>T, c.337 + 33A>G, and c.289A>G mutations were further verified through the cloning of mutant alleles (Table 2). The four negative samples could be due to missed detection of mutation outside the hot spots or due to the impurity of dissected cells below detection threshold. We also performed sequencing analysis using ovarian cancer whole tissues without microdissection; no GT198 mutation was detected (0 of 21), suggesting the bulk of tumor lacked GT198 mutation. As additional controls, analysis of tumor samples with nuclear GT198 expression did not reveal GT198 mutation (Fig. 2B and Table 2). In conclusion, our data indicate that somatic muta-

### TABLE 1

Cytoplasmic expression of GT198 in stromal cells of ovarian cancer

<table>
<thead>
<tr>
<th>Ovarian cancer type</th>
<th>Case (N)</th>
<th>Positive expression % (n/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High grade serous papillary</td>
<td>29</td>
<td>6.8 (2/29)</td>
</tr>
<tr>
<td>Low grade serous papillary</td>
<td>93</td>
<td>9.6 (9/93)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>25</td>
<td>32.0 (8/25)</td>
</tr>
<tr>
<td>Endometrioid</td>
<td>12</td>
<td>41.5 (5/12)</td>
</tr>
<tr>
<td>Clear cell</td>
<td>18</td>
<td>16.6 (3/18)</td>
</tr>
<tr>
<td>Brenner</td>
<td>2</td>
<td>50.0 (1/2)</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>9</td>
<td>0 (0/9)</td>
</tr>
<tr>
<td>Granulosa-theca</td>
<td>12</td>
<td>33.3 (4/12)</td>
</tr>
<tr>
<td>Sertoli-Leydig</td>
<td>3</td>
<td>33.3 (1/3)</td>
</tr>
<tr>
<td>Fibroma-thecoma</td>
<td>12</td>
<td>0 (0/12)</td>
</tr>
<tr>
<td>Dysgerminoma</td>
<td>10</td>
<td>0 (0/10)</td>
</tr>
<tr>
<td>Embryonal</td>
<td>2</td>
<td>0 (0/2)</td>
</tr>
<tr>
<td>Mullerian</td>
<td>4</td>
<td>25.0 (1/4)</td>
</tr>
<tr>
<td>Metastasis from distance</td>
<td>15</td>
<td>0 (0/15)</td>
</tr>
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immunofluorescent double staining (Fig. 3B) in theca cells, indicated by theca-specific marker CYP17 using immunohistochemistry. In mouse ovary, GT198 expression was low in primary or secondary follicles (Fig. 3A). In antral follicles, a high level of GT198 expression was nuclear with strong expression in granulosa cells and weak expression in theca cells. In contrast, GT198 expression became cytoplasmic in corpus luteum (Fig. 3D). Because primates closely resemble humans, we also analyzed ovary in baboons. The GT198 expression pattern in normal baboon ovary was largely consistent with the expression pattern in mouse (Fig. 3A). It appeared that GT198 expression is predominantly nuclear in normal granulosa and theca cells until luteinized. Previously, we found that cytoplasmic GT198 induces apoptosis in cultured cells (26). Because apoptosis is a naturally occurring process in corpus luteum and luteinized theca cells are located in stroma, we speculated that mutant stromal cells with cytoplasmic GT198 in cancer might have a link to luteinized theca cells that escaped the fate of apoptosis. We then examined the identity of GT198 mutant cells in cancer using CYP17 as a marker.

The results showed that GT198 mutant cells largely (>95%) overlap with the cell population positive for CYP17 in a mucinous carcinoma and a granulosa cell carcinoma examined by immunofluorescent double staining (Fig. 4A). This conclusion was further confirmed using tumor microarrays containing various types of ovarian cancer carrying GT198 mutations (Fig. 5 and Table 2). CYP17 appeared to be overexpressed in tumor stromal GT198+ cells in every sample we analyzed containing GT198+ cells. Detailed morphological examination suggested that these cells indeed retain luteinized theca cell features including their localization in highly vascularized tissues and the presence of potential lipid vesicles in large cytoplasm for steroid secretion (Fig. 4, B and C). Expression levels of GT198 and CYP17 in each cell varied considerably but the two proteins mostly coexisted, suggesting a theca origin of GT198+ stromal cells (Figs. 4A and 5).

A Subpopulation of GT198+ Stromal Cells Express Stem/Progenitor Cell Markers—The nature of GT198+ luteinized theca cells was further studied by immunofluorescent double staining using stem/progenitor cell surface markers. The overlapping subpopulations of GT198+ cells were found to be positive for stem cell marker CD133 (~1–2%) and for progenitor cell markers CD44 (~15–25%) and CD34 (~30–40%) in a granulosa cell carcinoma carrying GT198 mutation (Fig. 6, A and B). However, CYP17 marker did not overlap with CD133 (Fig. 6, A and C), possibly because CYP17 is expressed in differentiating cells only. CD133+ cells are very small in size with undifferentiated morphology and are always located in tumor stroma. Consistently, small sized GT198+ cells are found deeply in tumor stroma, and large sized GT198+ cells with differentiated morphology, mostly CYP17+, are found to be lining the tumor layer (Fig. 6B). Thus, GT198+ cells are not homogenous and they undergo differentiation at various stages (Fig. 6C). In a given tumor, these cells carry the same GT198 mutation so that they are potentially a cell lineage originated from a single progenitor harboring the initial mutation. Mutant theca precursors may replenish mutant luteinized theca progenies that overexpress CYP17 to promote steroid hormone production, thereby potentially modifying the tumor stromal environment in ovarian cancer. The identity of mutant progenitors requires further

### Table 2

GT198 Mutations in Ovarian Cancer Stroma

Sanger sequencing analysis of genomic DNA from microdissected ovarian cancer stromal or tumor cells in two GT198 mutation hot spots at c. −109C to c.34 + 69C and c.265C to c.337 + 116T. Dashes indicate the absence of mutation or amino acid change. NA denotes not applicable.

<table>
<thead>
<tr>
<th>Tumor ID</th>
<th>Age</th>
<th>Pathology diagnosis</th>
<th>Nucleotide change</th>
<th>Amino acid change</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>OV-1</td>
<td>43</td>
<td>High grade serous</td>
<td>c.284C&gt;T</td>
<td>A95V</td>
<td>Exon 4</td>
</tr>
<tr>
<td>OV-2</td>
<td>79</td>
<td>Inclusion cyst</td>
<td>c.337 + 13A&gt;G</td>
<td>—</td>
<td>Intron 4</td>
</tr>
<tr>
<td>OV-3</td>
<td>48</td>
<td>Mucinous</td>
<td>c.377 + 33A&gt;G</td>
<td>—</td>
<td>Intron 4</td>
</tr>
<tr>
<td>OV-4</td>
<td>37</td>
<td>Clear cell</td>
<td>c.74A&gt;C</td>
<td>5′-UTR</td>
<td></td>
</tr>
<tr>
<td>OV-5</td>
<td>34</td>
<td>Endometrioid</td>
<td>c.289A&gt;G</td>
<td>T97A</td>
<td>Exon 4</td>
</tr>
<tr>
<td>OV-6</td>
<td>35</td>
<td>Low grade serous</td>
<td>—</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>OV-7</td>
<td>44</td>
<td>Granulosa cell</td>
<td>c.337 + 67A&gt;G</td>
<td>—</td>
<td>Intron 4</td>
</tr>
<tr>
<td>OV-8</td>
<td>47</td>
<td>Low grade serous</td>
<td>—</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>OV-9</td>
<td>18</td>
<td>Hyperplasia</td>
<td>c.284C&gt;T</td>
<td>—</td>
<td>Intron 4</td>
</tr>
<tr>
<td>OV-10</td>
<td>38</td>
<td>Mucinous</td>
<td>—</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>OV-11</td>
<td>51</td>
<td>Endometrioid</td>
<td>—</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>OV-12</td>
<td>75</td>
<td>Low grade serous</td>
<td>c.37A&gt;T</td>
<td>5′-UTR</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c.−18T&gt;C</td>
<td>—</td>
<td>5′-UTR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>c.337 + 19T&gt;C</td>
<td>—</td>
<td>Intron 4</td>
</tr>
</tbody>
</table>

In normal human ovary, GT198 expression was also consistent with the expression pattern in various types of ovarian cancer.

GT198 Mutant Cells in Ovarian Cancer Are Luteinized Theca Cells Overexpressing CYP17—To gain insight into the identity of GT198 mutant stromal cells in ovarian cancer, we first analyzed GT198 expression in normal ovary by immunohistochemistry. In mouse ovary, GT198 expression was low in theca cells, indicated by theca-specific marker CYP17 using immunofluorescent double staining (Fig. 3A). In antral follicles, a high level of GT198 expression was nuclear with strong expression in granulosa cells and weak expression in theca cells. In contrast, GT198 expression became cytoplasmic in corpus luteum (Fig. 3A). Because primates closely resemble humans, we also analyzed ovary in baboons. The GT198 expression pattern in normal baboon ovary was largely consistent with the expression pattern in mouse (Fig. 3B). Strong nuclear GT198 was present in granulosa cells, and weak nuclear GT198 was present in theca cells, indicated by theca-specific marker CYP17 using immunofluorescent double staining (Fig. 3B). CYP17 is a steroid synthesis enzyme exclusively expressed in theca or luteinized theca cells in ovary (27, 28). Baboon corpus luteum contained CYP17+ luteinized theca cells with cytoplasmic GT198. In normal human ovary, GT198 expression was also consistent with the expression in baboon and mouse (Fig. 3C), except that normal human antral follicle was too rare to obtain and was unavailable for analysis. In a human antral follicle adjacent to ovarian cancer, however, GT198 expression in theca cells became cytoplasmic (Fig. 3D).

The results showed that GT198 mutant cells largely (>95%) overlap with the cell population positive for CYP17 in a mucinous carcinoma and a granulosa cell carcinoma examined by immunofluorescent double staining (Fig. 4A). This conclusion was further confirmed using tumor microarrays containing various types of ovarian cancer carrying GT198 mutations (Fig. 5 and Table 2). CYP17 appeared to be overexpressed in tumor stromal GT198+ cells in every sample we analyzed containing GT198+ cells. Detailed morphological examination suggested that these cells indeed retain luteinized theca cell features including their localization in highly vascularized tissues and the presence of potential lipid vesicles in large cytoplasm for steroid secretion (Fig. 4, B and C). Expression levels of GT198 and CYP17 in each cell varied considerably but the two proteins mostly coexisted, suggesting a theca origin of GT198+ stromal cells (Figs. 4A and 5).

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Our data indicate that GT198 mutant cell populations are not homogenous but differentiating and are located in tumor stroma. This finding provides critical evidence to confirm the notion that tumor stromal cells harbor tumor suppressor gene mutations. In ovarian cancer, they are the luteinized theca cells.

GT198 Suppresses Whereas Its Mutant Protein Stimulates CYP17 Expression—The above observation raised a question on whether GT198 or its mutant protein would regulate CYP17, given that GT198 is a steroid hormone receptor coregulator (19), and CYP17 promoter is regulated by steroid hormone receptor SF1 (31). We performed ChIP analysis in 293 cells that express endogenous CYP17, and found that wild type GT198 protein binds to the human CYP17 promoter (Fig. 7A). The binding activity was the highest at ~1.8 kb upstream of the CYP17 translation initiation site, although lower amount of binding was detectable at ~1.4 and 0.2 kb (Fig. 7A). This is consistent with a previous promoter deletion study from the other group indicating that the 1.8-kb CYP17 promoter had higher activity than shortened promoter (32). We have previously found that GT198 mutations are able to alter alternative splicing to produce a truncated mutant protein (amino acids 126–217) acting as a dominant negative (26). Two intron 4 mutations, c.337 + 13A>G and c.337 + 19T>C, identified both in this study and in a previous study, altered exon 4 alternative splicing, resulting in a frameshift to produce the mutant protein (25). Exon 4 mutations, such as missense mutations c.284C>T and c.289A>G, may affect siRNA regulation on alternative splicing as we previously proposed (26). Thus, we tested the effect of GT198 mutant protein and the GT198

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**FIGURE 3.** GT198 expression in mouse, baboon, and human ovaries. Immunohistochemical staining of GT198 and immunofluorescent double staining of GT198 (red) and CYP17 (green). Boxed areas are enlarged. A, in normal mouse ovary antral follicles, nuclear staining is present in granulosa and theca cells, which are enlarged in the insets. Cytoplasmic GT198 is present in corpus luteum. Sections are counterstained with hematoxylin. B, in normal baboon ovary, GT198 nuclear staining is present in granulosa and theca cells. Cytoplasmic GT198 is present in corpus luteum, which is from the same slide outside the low power view. An adjacent slide was fluorescence-stained to show GT198 expression (red) in theca or luteinized theca cells expressing CYP17 (green). C, GT198 expression in normal human ovary, age 20. Boxed areas are enlarged, showing negative staining in primordial follicular cells and positive cytoplasmic staining in corpus luteum. D, cytoplasmic GT198 expression in theca cells in an antral follicle adjacent to ovarian cancer, age 45. Three serial cut sections were hematoxylin/eosin-, immuno-, and fluorescence-stained. Scale bars, 50 μm in A–C and 25 μm in D.
siRNA in the ChIP assay. When cotransfected with wild type GT198, the mutant protein and siRNA significantly reduced wild type GT198 occupancy on the CYP17 promoter at 1.8 kb upstream (Fig. 7A). In addition, using a luciferase reporter carrying a 1.8-kb human CYP17 promoter, we confirmed that wild type GT198 suppresses whereas the mutant protein or siRNA stimulates CYP17 promoter activity (Fig. 7B). Similar effects were observed when endogenous CYP17 expression was examined by RT-PCR in 293 cells (Fig. 7C). We further tested the subcellular localization of wild type GT198 in the presence of mutant protein or siRNA. The results showed that wild type GT198 localizes to the nucleus while FLAG-tagged mutant protein is present in the cytoplasm (Fig. 7D). This is consistent with our previous study that GT198 nuclear-cytoplasmic shuttling is reduced when the mutant protein is present (25). The mechanism of GT198 nuclear-cytoplasmic shuttling, however, remains to be further studied. We suggest that GT198 suppresses and silences the CYP17 promoter. GT198 mutant protein stimulates CYP17 expression through the decrease of wild type promoter occupancy and the increase of its cytoplasmic localization (Fig. 7E). In conclusion, our studies together suggest that GT198 mutant stromal cells in ovarian cancer are luteinized theca cells in origin and that they overexpress CYP17.

**DISCUSSION**

Cancer cell development often mirrors normal cell differentiation. In normal ovary, the follicle is a basic functional unit containing an oocyte and granulosa cells enclosed by a basal lamina. Theca cells are thought to be recruited from stroma and differentiate only surrounding the lamina of developing follicles. After ovulation, theca cells are luteinized in corpus luteum. At a functional level, theca or luteinized theca cells express steroid hormone synthesis enzymes including CYP17, which converts cholesterol into androgens.
Androgens serve as substrates for estrogens synthesis by granulosa cells. Theca and granulosa cells are two principle steroid hormone-producing cells and are mutually dependent in differentiation with robust paracrine communications (34). Theca cells are originated from stromal stem cells that differentiate under the stimulation by a number of peptide factors secreted from granulosa cells (33–35).

During ovarian cancer development, ovarian surface epithelium undergoes ovulation-induced rupture and forms cortical inclusion cysts as precursor lesions (Fig. 8) (6, 10). Hormone

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**FIGURE 5.** GT198 mutant stromal cells express CYP17. Three serial cut paraffin sections of ovarian endometrioid carcinoma OV-5 were immunostained with GT198, microdissected for mutation analysis using positive stromal cells (red dots), and immunofluorescent double stained with GT198 (red) and CYP17 (green). Sequence trace shows cloned mutant allele. Scale bars, 50 μm.

**FIGURE 6.** GT198+ cells express stem/progenitor cell markers. A, immunofluorescent double staining of GT198, CYP17, and cell surface markers CD133, CD44, and CD34 in ovarian granulosa cell tumor OV-7 carrying mutation c.337 + 67A>G. Boxed areas are enlarged at the right. Slides are counterstained with DAPI (blue). B, immunohistochemistry staining of GT198 in OV-7 to be compared with the fluorescent staining in A. GT198+ cells with strong expression (black arrows) line the tumor, whereas small undifferentiated cells with weak expression (white arrows) are located deeply in the tumor stroma. C, a diagram illustrates the observed differentiating GT198+ subpopulations. GT198+ cells overlap with CYP17+ cells the most and with CD133+ cells the least. CD133+ and CYP17+ cells do not overlap, as shown in A. Scale bars, 50 μm.
stimuli are thought to be responsible for subsequent transformation into epithelial ovarian carcinomas. Our data show that mutant luteinized theca cells are recruited to the inclusion cysts (Fig. 1G), as well as to the stroma in ovarian cancer (Fig. 1B–F). Increased CYP17 in these mutant cells potentially provides required hormone stimuli for cancer cell transformation. Our data also show not only epithelial types but also other types of ovarian cancer, including granulosa cell tumors, contain GT198 cells in tumor stroma (Table 1). Hence, mutant luteinized theca cells may be a common source of hormone stimuli in different types of ovarian cancer (Fig. 8), although more evidence is needed to clarify whether they share similar pathways in cancer development.

Our finding is consistent with multiple lines of evidence previously shown by others. It was suggested that inclusion cysts in ovarian cortex lie in proximity to steroid hormone-producing cells in the vasculature and are exposed to paracrine ovarian androgens (36, 37). Consistently, high androgen level is a significant risk factor in ovarian cancer (36, 38), indicating the involvement of theca cell activity. In addition, enzymatically active stromal cells have been observed near inclusion cysts and epithelial tumor cells (18). The stromal luteinization in ovarian cancer has also long been observed in the history (39, 40), although these stromal cells have been labeled by various names such as stromal theca cells, luteinized stromal cells, and enzymatically active stromal cells (41). Thus, steroid hormone-producing tumor stromal cells are significant characteristic in ovarian cancer.

In addition, previous studies have been shown that sorted CD133+ and CD44+ cells from human ovarian cancer have increased tumorigenic capacity in mice (42–44). This is consistent with our results that GT198 mutant cells consist of a cell
lineage from undifferentiated CD133+ or CD44+ cells to differentiated CYP17+ luteinized theca cells. In this regard, a theca progenitor cell harboring mutation may be present in tumor stroma, whereas only its differentiated luteinized theca progenies are functionally capable of producing hormones. It is important to note that cytoplasmic GT198 or CYP17 proteins are more specific markers for mutant stromal cells than the CD markers that are also present on normal but not GT198+ cells as we observed (Fig. 6).

Finally, GT198 has dual functions in steroid hormone regulation (19, 20) and in DNA repair (21). It is possible that mutations in other DNA repair genes would affect the entire DNA repair pathway involving GT198 function and hence steroid hormone regulation. Thus, mutations in individual DNA repair genes may together increase the risk of breast and ovarian cancer.

In conclusion, using a tumor suppressor gene GT198 as marker, we have identified that luteinized theca cells carry GT198 mutations in multiple types of ovary cancer stroma. The finding provides critical evidence in the understanding of steroid hormone regulation in ovarian cancer. Future studies on GT198 mutant tumor stromal cells may provide a potential new target for ovarian cancer treatment.

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