Oncogenic transformation of human ovarian surface epithelial cells with defined cellular oncogenes

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Ovarian surface epithelium (OSE) is considered to give rise to epithelial ovarian carcinomas (EOCs). To elucidate early processes contributing to the development of EOCs from the OSE, two batches of primary human OSE cells were transduced with non-viral human genes (mutant Cdk4, cyclinD1 and hTERT) so as to efficiently establish normal diploid OSE cells without chromosomal instability. Then defined genetic alterations frequently observed in EOCs were transduced into the OSE cells. A combination of p53 inactivation and oncogenic Kras transduction did not confer tumor-forming ability in immunodeficient mice, though additional transduction of Akt or combined transduction of c-myc with bcl-2 did result in tumor formation. In the latter case, tumors demonstrated phenotypes reminiscent of human EOCs, including cytokeratin expression, a highly aggressive phenotype, metastatic behavior and formation of ascites. These results indicate that inactivation of p53 and activation of the Ras pathway play critical roles in ovarian carcinogenesis in co-operation with genes of Akt or c-myc pathways. This first in vitro model system faithfully recapitulating the development of EOCs using normal human OSE cells should greatly facilitate further studies of EOCs.

Introduction

Epithelial ovarian carcinomas (EOCs) are the fourth to fifth most common cause of cancer death in females worldwide and the primary cause of death from gynecological malignancies. Lack of an adequate screening test for early disease detection and the rapid progression to chemotherapyresistance have prevented appreciable improvement in the 5 years of survival rate for patients with the disease. In spite of the clinical importance, we have a far from complete understanding of the cell of origin and molecular mechanisms of initiation and progression of EOCs.

All histologic types of EOCs can be divided into two categories, designated type I and type II, which correspond to two pathways of tumorigenesis (1). Type I tumors include low-grade serous carcinoma, mucinous carcinoma, endometrioid carcinoma and clear cell carcinoma that develop slowly in a stepwise manner from premalignant conditions or borderline tumors. Type II tumors include the high-grade serous carcinoma, carcinosarcomas and undifferentiated carcinomas, which grow rapidly and typically have spread beyond the ovaries at presentation. These tumors are rarely associated with morphologically recognizable precursor lesions and it has been proposed that they develop de novo from ovarian inclusion cysts or the surface epithelium (2,3). In addition, type II tumors with pelvic dissemination include carcinomas arising from the epithelium of fimbra.

Abbreviations: Cdk, cyclin-dependent kinase; EOC, epithelial ovarian carcinoma; HPV, human papillomavirus; hTERT, human telomerase reverse transcriptase; OSE, ovarian surface epithelium; SCID, severely compromised immunodeficient; shRNA, short hairpin RNA.

The ovarian surface epithelium (OSE), a single continuous layer of flat-to-cuboidal mesothelial cells covering the ovary, is considered to be the tissue of origin of EOCs (3,4). The ‘incessant ovulation’ hypothesis (5) and transgenic mouse models of serous adenocarcinoma or endometrioid adenocarcinoma (6–12) are in line with precursor cells of not only type I but also some type II EOCs being located in the OSE. However, recent studies proposed that the fallopian tubal fimbra is a site for early serous adenocarcinomas, especially in women with mutations in BRCA1 or BRCA2 (13).

Type I tumors are associated with distinct molecular changes that are rarely present in type II tumors, such as BRAF and Kras mutations for serous tumors, Kras mutations for mucinous tumors and beta-catenin and PTEN mutations and microsatellite instability for endometrioid tumors. However, there are very limited data on the molecular alterations associated with type II tumors except for frequent p53 mutations. As in vivo models that accurately represent the cellular and molecular changes associated with the initiation and progression of human EOCs are lacking, establishment of in vitro models using normal human OSE cells might provide alternative tools to analyze the molecular events associated with development of EOCs.

Numerous studies have revealed that oncogenes such as Kras, c-erbB-2, c-myc, bcl-2, PIK3CA and Akt are often amplified, overexpressed and/or mutated (14–19) in EOCs. Activation of the catalytic subunit of human telomerase reverse transcriptase (hTERT) is also common (20). In addition, mutations of tumor suppressor genes including p53 (21) and PTEN and disruption of the Rb pathway (p16-CDK4/cyclinD1-pRb) are frequently observed (18,22). Although such genetic changes have been identified, how they contribute to transformation of OSE cells has yet to be clarified in detail.

To address this question, several investigators have cultured OSE cells for use in model systems to study development of EOCs. To avoid the limitation that primary human OSE cells have a very limited life span in culture, immortalized OSE cell lines have been established with introduction of human papillomavirus (HPV) type 16 E6/E7 and SV40 large T (23–25). Owing to the viral oncogenes used, unfortunately, these cell lines show genetic instability. Furthermore, involvement of these viruses in the development of EOCs has yet to be clarified, they are clearly not ideal for establishment of an in vitro carcinogenesis model. Recently, we have generated an immortalized OSE cell line with HPV16 E7 and hTERT, which retains a normal diploid genome without genetic instability (26). The results indicated that p53 inactivation is not required, whereas inactivation of the pRb pathway and activation of telomerase are sufficient for OSE immortalization. From this base, we first tried to establish genetically stable, non-transformed immortalized OSE cells without viral oncogenes and found we could reproducibly obtain such cells by expressing mutant cyclin-dependent kinase (Cdk) 4, cyclinD1 and hTERT. Using the immortalized OSE cells, we could successfully establish an in vitro carcinogenesis model of EOCs with defined genetic elements.

Materials and methods

Isolation of human OSE

Ovaries were obtained from a post-menopausal patient (26) and a premenopausal patient undergoing abdominal total hysterectomy and bilateral salpingo-oophorectomy for a gynecological disease other than ovarian cancer at Kumamoto University Hospital. The Ethics Committees of Kumamoto University and National Cancer Center approved this study and the subjects gave their informed consent for participation. The ovaries were grossly normal and no pathological lesions were observed on subsequent histological examination. After collagenase digestion under aseptic conditions, OSE cells were obtained by scraping with a surgical blade as described previously (27) and maintained in Dulbecco’s modified Eagle’s medium:F12 (1:1 mixture) (WAKO, Osaka, Japan) supplemented with 10% fetal bovine serum (26).

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Viral vector construction and viral transduction
Lentiviral vector plasmids were constructed by recombination using the Gateway system (Invitrogen, Carlsbad, CA). Briefly, hTERT, human cyclinD1 and human mutant Cdk4 (Cdk4R24C; an inhibitor resistant form of Cdk4, generously provided by Dr Hara) were first recombined into entry vectors by BP reaction (Invitrogen). Then these segments were recombined with a lentiviral vector, CSII-CMV-RfA (a gift from Dr Miyoshi), by LR reaction (Invitrogen) to generate CSII-CMV-hTERT, -cyclinD1 and -Cdk4R24C. The production of recombinant lentiviruses with the vesicular stomatitis virus G glycoprotein was as described previously (28). Mutant Kras (KrasV12), a constitutive active form of Akt1 (myristylation signal-fused human Akt1, a gift from Dr Goto), c-myc, a mutant form of c-myc (c-mycT58A) generated by site-directed mutagenesis, a dominant-negative form of p53 (Dnp53) (29), bcl-2 and mutant PIK3CA (PIK3CAH1047R, a gift from Dr Kanai) (30) were cloned into retroviral vector plasmids by recombination using the Gateway system (Invitrogen). Then these segments were recombined with hTERT, human cyclinD1 and human mutant Cdk4 (Cdk4R24C; an inhibitor resistant form of Cdk4, generously provided by Dr Hara) were first recombined into entry vectors by BP reaction (Invitrogen). Then these segments were recombined with a lentiviral vector, CSII-CMV-RfA (a gift from Dr Miyoshi), by LR reaction (Invitrogen) to generate CSII-CMV-hTERT, -cyclinD1 and -Cdk4R24C. The production of recombinant lentiviruses with the vesicular stomatitis virus G glycoprotein was as described previously (28). Mutant Kras (KrasV12), a constitutive active form of Akt1 (myristylation signal-fused human Akt1, a gift from Dr Goto), c-myc, a mutant form of c-myc (c-mycT58A) generated by site-directed mutagenesis, a dominant-negative form of p53 (Dnp53) (29), bcl-2 and mutant PIK3CA (PIK3CAH1047R, a gift from Dr Kanai) (30) were cloned into retroviral vector plasmids by recombination using the Gateway system (Invitrogen). Briefly, after recombination with pDONR201 or pDONR221 by BP reaction to generate entry vectors, they were recombined with the retroviral vector, CSII-CMV-RfA (a gift from Dr Miyoshi), by LR reaction (Invitrogen) to generate CSII-CMV-hTERT, -cyclinD1 and -Cdk4R24C. The production of recombinant lentiviruses with the vesicular stomatitis virus G glycoprotein was as described previously (28). Mutant Kras (KrasV12), a constitutive active form of Akt1 (myristylation signal-fused human Akt1, a gift from Dr Goto), c-myc, a mutant form of c-myc (c-mycT58A) generated by site-directed mutagenesis, a dominant-negative form of p53 (Dnp53) (29), bcl-2 and mutant PIK3CA (PIK3CAH1047R, a gift from Dr Kanai) (30) were cloned into retroviral vector plasmids by recombination using the Gateway system (Invitrogen). Then these segments were recombined with a lentiviral vector, CSII-CMV-RfA (a gift from Dr Miyoshi), by LR reaction (Invitrogen) to generate CSII-CMV-hTERT, -cyclinD1 and -Cdk4R24C. The production of recombinant lentiviruses with the vesicular stomatitis virus G glycoprotein was as described previously (28).
In diameter were counted after a lapse of 3 weeks. Five photographs of randomly selected areas in each dish were taken at the magnification of 40. The numbers of colonies were assessed with the COLONY program (Fujifilm, Tokyo, Japan). The experiments were performed in triplicate.

<table>
<thead>
<tr>
<th>Cells</th>
<th>No. of tumors per subcutaneous injection</th>
<th>No. of tumors per intraperitoneal injection</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOSE2C-Dnp53-KrasV12</td>
<td>$1 \times 10^7$</td>
<td>$1 \times 10^6$</td>
</tr>
<tr>
<td>Vector</td>
<td>exp 1</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>exp 2</td>
<td>0/6</td>
</tr>
<tr>
<td>c-myc wt</td>
<td></td>
<td></td>
</tr>
<tr>
<td>c-mycT58A</td>
<td>exp 1</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>exp 2</td>
<td>0/7</td>
</tr>
<tr>
<td>Akt</td>
<td>exp 1</td>
<td>3/3 (8)</td>
</tr>
<tr>
<td></td>
<td>exp 2</td>
<td>2/6 (14)</td>
</tr>
<tr>
<td>PIK3CAH1047R</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>shPTEN</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>bcl-2</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>c-myc wt-vector</td>
<td></td>
<td>0/3</td>
</tr>
<tr>
<td>c-myc wt-bcl-2</td>
<td>5/7 (10)</td>
<td></td>
</tr>
<tr>
<td>c-mycT58A-vector</td>
<td>exp 1</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>exp 2</td>
<td>0/3</td>
</tr>
<tr>
<td>c-mycT58A-bcl-2</td>
<td>exp 1</td>
<td>3/3 (11)</td>
</tr>
<tr>
<td></td>
<td>exp 2</td>
<td>6/6 (6)</td>
</tr>
<tr>
<td>HOSE1C-Dnp53-KrasV12</td>
<td>$1 \times 10^6$</td>
<td>$1 \times 10^7$</td>
</tr>
<tr>
<td>Vector</td>
<td>2/6 (18)</td>
<td></td>
</tr>
<tr>
<td>c-mycT58A-bcl-2</td>
<td>6/6 (4)</td>
<td>3/3 (7–9)</td>
</tr>
</tbody>
</table>

Incidence of tumor formation within 16 weeks of observation period was scored. Number in parentheses indicates observation period (weeks) when mice were killed because of the faster growth of one or more subcutaneous tumors in the same mouse or any signs of intraperitoneal tumors. Some cell lines were repeatedly established. exp 1, 1st experiment; exp 2, 2nd experiment.

**Tumorigenesis in nude mice**

All surgical procedures and care administered to the animals were in accordance with institutional guidelines. Cells were resuspended in 50% Matrigel (BD Biosciences) and injected subcutaneously into a flank or intraperitoneally into female 6- to 7-week-old BALB/c nude or nonobese diabetic/severely compromised immunodeficient (NOD/SCID) mice (Clea Japan, Tokyo, Japan).

**Immunofluorescence**

Paraffin-embedded sections (3 μm) were incubated with 5% normal goat serum in 1% bovine serum albumin/phosphate-buffered saline to block non-specific staining, then exposed to antibodies against cytokeratin 18 (ab52948, rabbit polyclonal antibody; abcam). After incubation with appropriate secondary antibodies, fluorescence was visualized by fluorescence microscopy.

**Fluorescence-activated cell sorting**

Cells were stained with propidium iodide using a CyteTest Plus DNA Reagent kit (BD Biosciences) and analyzed with a FACSCalibur flow cytometer (BD Biosciences).

**Results**

**Immortalization of human OSE cells without viral oncogenes**

Previously we have shown that HOSE cells can be immortalized with the E7 gene of HPV16 and hTERT, indicating that disruption of the Rb pathway and activation of telomerase are sufficient for immortalization (26). However, since E7 is not involved in development of EOCs, we chose a core set of genes that might replace it to immortalize OSE cells. Human mutant Cdk4 (Cdk4R24C, an inhibitor resistant form of Cdk4) and cyclin D1, which can inactivate Rb by phosphorylation and is frequently deregulated in EOCs, were serially transduced into two independent batches of primary human OSE cells (HOSE1 and HOSE2) with lentiviral vectors. Expression of the transduced proteins was confirmed by immunoblotting (Figure 1A). As expected, the combination of Cdk4R24C, cyclin D1 and hTERT resulted in efficient immortalization of the cells, whereas neither Cdk4R24C nor cyclin D1 alone in combination with hTERT could efficiently extend the life span of primary HOSE cells, which consistently stopped growing at around population doublings 20 (Figure 1B). Pooled populations of immortalized OSE cells were named HOSE1C and HOSE2C, respectively. Both the primary and immortalized cell lines expressed cytokeratin 18, a marker of epithelial cells, as revealed by western blotting (Figure 1C). However, the expression levels of cytokeratin 18 in immortalized HOSE cells, especially in HOSE2C cells, were significantly lower than those in primary cells for unknown reasons. The HOSE cells showed high telomerase activity, as measured by TRAP assay, clearly contrasting with the lack in primary cells (Figure 1D). As described previously, human OSE cells immortalized by introduction of SV40 T antigen (23,24) or HPV16 E6 and E7 (25) showed obvious chromosomal abnormalities, including translocations and aneuploidy. On the other hand, HOSE1 cells immortalized with E7 and hTERT were normal (26). Approximately, 92% (46/50) of HOSE1C cells and 100% (50/50) of HOSE2C cells around population doublings 60 showed a normal diploid genotype without structural alteration (supplementary Table 1 is available at Carcinogenesis Online). The results support the notion that an intact p53 pathway, but not an intact Rb pathway, is critical for genome stability.

**Combined transduction of mutant p53 and Kras into HOSE2C cells induces anchorage-independent growth but not tumor-forming ability in nude mice**

Since HOSE2C cells were established before HOSE1C cells during the course of the experiment, they were employed to examine for effects of several oncogenes or tumor suppressor genes relevant to ovarian carcinogenesis. First, we tested for two genetic alterations frequently observed in EOCs; mutation of p53 that is observed in >50% of cases and change of Kras that is generally apparent in ~75% of mucinous adenocarcinomas, while being less frequent in other types (17). In preliminary experiments, transduction of oncogenic Kras (KrasV12) into HOSE2C cells resulted in massive cell death with vacuolation (data not shown). Thus, a dominant-negative form of p53 (Dnp53) and Kras V12 were serially transduced into HOSE2C cells. Expression of exogenous KrasV12 and Dnp53, as well as downregulation of p14ARF was confirmed by western blotting (Figure 2A). HOSE2C expressing both Dnp53 and KrasV12 grew faster than cells with an empty vector or carrying Dnp53 alone (Figure 2B), with increase in cell density after reaching confluence (Figure 2C). In anchorage-independent growth assays, HOSE2C expressing Dnp53 and KrasV12 formed numerous large colonies, whereas cells with empty vector or expressing Dnp53 alone (Figure 2D). When tested for tumorigrenic ability in nude mice, HOSE2C-Dnp53-KrasV12 cells failed to form tumors even when $1 \times 10^7$ cells were injected together with Matrigel (Table 1). These results indicate that a combination of disrupted Rb function, impaired p53 function and oncogenic Kras mutation can confer anchorage-independent growth but not tumorigenicity on HOSE cells.

**Additional introduction of Akt, but not c-myc, PIK3CA or PTEN knockdown, confers tumorigenic potential on HOSE2C-Dnp53-KrasV12 cells**

To explore further genetic alterations required for full transformation of OSE cells, we additionally transduced several oncogenes into HOSE2C-Dnp53-KrasV12 cells. As activation of oncogenes such as c-myc, Akt and PIK3CA, as well as inactivation of tumor suppressor genes like PTEN has been frequently found in EOCs, HOSE2C cells expressing Dnp53 and KrasV12 were infected with retroviruses expressing c-mycT58A (a stable mutant), myr-Akt1 (Akt), PIK3CAH1047R (with a hotspot mutation in the kinase domain of PIK3CA found in EOCs) (14) or a PTEN-specific shRNA (shPTEN).

Exogenous expression of the genes and shRNA effects were...
confirmed by western blotting (Figure 3A). PTEN protein levels were reduced to ~30% of the control level by the shRNA expression (Figure 3A). Morphologically, HOSE2C-Dnp53-KrasV12- expressing c-mycT58A or PIK3CAH1047R were small and round in shape, whereas Akt-transduced cells appeared more fibroblastic. Transduction of shPTEN did not alter the morphology (Figure 3B).

In culture dishes, c-mycT58A- and PIK3CAH1047R-expressing HOSE2C-Dnp53-KrasV12 cells demonstrated the fastest growth rate. Cells (2 × 10^4) were cultured in triplicate 12-well plates and counted every 2 days. The data in the graphs are means ± SD. (C) Dnp53-KrasV12-expressing HOSE2C cells continue growing after reaching confluence. Cells were grown as described in (B) and pictures were taken on day 8. Scale bars, 100 μm. (D) Anchorage-independent growth of HOSE2C cells. Cells (5 × 10^4) were seeded in 35 mm plates. After 3 weeks, colonies were counted when sized >50 μm in diameter. The experiments were performed in triplicate and the total number of colonies in a 15 mm² area was counted. Scale bars, 250 μm.

HOSE2C-Dnp53-KrasV12-Akt cells give rise to tumors in the peritoneal cavity of immunodeficient mice

With gross examination, isolated subcutaneous HOSE2C-Dnp53-KrasV12-Akt tumors featured abundant hemorrhage and neovascularization, as confirmed by histological examination (Figure 4A-a). Histologically, major components were sarcomatous, but one which was positive for cytokeratin 18 expression proved to be carcinomatous in structure (Figure 4A-b). The origin of the tumor cells was confirmed by the presence of myristylated forms of Akt, the epithelial marker cytokeratin 18 and the mesenchymal marker vimentin, with antibodies that do not react with mouse proteins (Figure 4B). Then the cells were injected intraperitoneally into SCID mice to examine whether they might give rise to tumors in the peritoneal cavity where human EOCs develop and disseminate. Within 10 weeks, they formed large solid masses (3/3, 100%) but without dissemination in the peritoneal cavity (Figure 4C). Only one of three mice exhibited a small volume of ascites. Histological examination revealed that the
intraperitoneal tumors were similar to their subcutaneous counterparts (Figure 4A-a–c and data not shown), with abundant neovascularization and invasion to the pancreas (Figure 4A-d).

Additional bcl-2 transduction into HOSE2C-Dnp53-KrasV12 cells confers tumor-forming ability with resemblance of lesions to human EOCs

Overexpression of bcl-2 is a feature of 24–80% of EOCs (35–38). It is also well known that c-myc and bcl-2 co-operatively transform hematological cells in which bcl-2 can suppress c-myc-induced apoptosis (39). Thus, we introduced bcl-2 into HOSE2C-Dnp53-KrasV12-c-mycT58A cells to examine whether such co-operation might also be observed in this model. When expression of transduced bcl-2 was examined by immunoblotting, we noted that the Bcl-2 protein level was decreased in c-mycT58A-expressing cells (Figure 5A), as reported previously for myeloid cells (40). Increased levels of Bcl-2 were confirmed in HOSE2C-Dnp53-KrasV12-c-mycT58A-bcl-2 cells. In soft agar assays, only a limited increase in colony number was observed with HOSE2C-Dnp53-KrasV12-c-mycT58A-bcl-2 cells compared with HOSE2C-Dnp53-KrasV12-c-mycT58A (supplementary Figure 1A is available at Carcinogenesis Online). However, additional transduction of bcl-2 suppressed apoptosis induced by c-myc transduction alone (Figure 5B) and conferred tumor-forming ability in nude mice (3/3, within 8 weeks) (Table I). More interestingly, dissemination was evident in the peritoneal cavity of SCID mice (3/3, 100%). Tumor spread sites included the peritoneum, omentum, intestines, pancreas, diaphragm, liver, uterus and the bursa surrounding the ovaries. Some tumors consolidated with the intestine, pancreas and spleen (Figure 5C-a–c). Histological examination showed HOSE2C-Dnp53-KrasV12-c-mycT58A-bcl-2 tumors to be comprised mainly of poorly differentiated epithelial cells with cytokeratin 18 expression (Figure 5C-d and e) and also confirmed cell infiltration into the abdominal organs (Figure 5C-f and data not shown). Tumor cells showed a high nuclear:cytoplasmic ratio with pleomorphic large nuclei and frequent mitotic figures. Expression of cytokeratin 18 and vimentin was also confirmed by immunoblotting (Figure 5D). The overall characteristics of the HOSE2C-Dnp53-KrasV12-c-mycT58A-bcl-2 tumors are reminiscent of human EOCs.

We confirmed that the bcl-2 transgene alone is not sufficient for transformation of HOSE2C-Dnp53-KrasV12 cells (Table I; supplementary Figure 1B is available at Carcinogenesis Online). Without exogenous expression of KrasV12, HOSE2C-Dnp53-c-mycT58A-bcl-2 cells did not form tumors in nude mice (supplementary Table 2 is available at Carcinogenesis Online).

Confirmation of the multistage nature of carcinogenesis with HOSE2C-HOSE1 cell lines

It is possible that additional alterations (genetic and/or epigenetic) occurring during the process of introducing Akt or c-mycT58A and
bcl-2 could contribute to the tumorigenic phenotype. To address this possibility, we repeatedly transduced Akt- or c-mycT58A plus bcl-2 into HOSE2C-Dnp53-KrasV12 cells (Table I, exp 2). Additionally, we transduced wild-type c-myc (c-myc wt) and bcl-2 as c-myc amplification is frequently observed without mutation in EOCs (Table I; supplementary Figure 1B is available at Carcinogenesis Online). All the resultant cells reproducibly developed into subcutaneous tumors in nude mice, whereas those carrying empty vectors failed to form tumors. Histologically, tumors of HOSE2C-Dnp53-KrasV12-c-myc wt-bcl-2 cells were found to be composed of poorly differentiated epithelial cells, with as HOSE2C-Dnp53-KrasV12-c-mycT58A-bcl-2.

We furthermore tested the tumorigenic effects of Dnp53, KrasV12, c-mycT58A and bcl-2 using HOSE1C cells, another independent batch of HOSEC derived from a different patient (Table I; supplementary Figure 2 is available at Carcinogenesis Online). The resultant cells formed tumors (6/6) within 4 weeks after subcutaneous injection into nude mice and intraperitoneal tumors (3/3) after intraperitoneal injection into SCID mice, whereas HOSE1C-Dnp53-KrasV12-vector cells developed only small tumors (2/6) with a longer latent period (Table I). Dissemination in the peritoneal cavity and the histological appearance of HOSE1C-Dnp53-KrasV12-c-mycT58A-bcl-2 tumors were similar to those with HOSE2C (data not shown), but accumulation of ascesis was more evident (3/3, 100%). Finally, we examined whether Dnp53 is essential for tumor formation. Though KrasV12 transduction into HOSE1C cells induced massive cell death, sequential transduction of the other oncogenes allowed establishment of HOSE1C-KrasV12-c-mycT58A-bcl-2 cells with intact p53 functions, which repeatedly developed tumors (supplementary Figure 3 is available at Carcinogenesis Online).

These results indicate that the combination of multiple genetic elements we applied can reproducibly fully transform human OSE cells.

**Discussion**

Our goal is to develop an appropriate model for recapitulating development and progression of human EOCs of both type I and type II. Although recent studies in women with germline mutations in BRCA1 or BRCA2 following prophylactic salpingo-oophorectomy have indicated the fimbrial portion as a site of origin of the serous type (13), we chose OSE as a source of cells to examine processes of ovarian carcinogenesis in this study. The OSE is believed to arise from the same coelomic epithelial cells that give rise to the Mullerian duct, the progenitor of the epithelia of the structures of the female reproductive tract, including the Fallopian tube, uterus, and upper vagina (4). It is thought that OSE cells retain the potential to differentiate into epithelia similar to each of those structures (3), explaining how the distinct histological subtypes observed in EOCs arise. At least some of the morphological heterogeneity of EOCs may be due to aberrant expression of HOX genes, which regulate Mullerian duct differentiation and specify morphological identity within the female reproductive tract (12).

A recently described genetically engineered mouse model of type I, involving conditional deletion of PTEN and activation of Kras or inactivation of Apc (Wnt/β-caten defects) and PTEN in mouse OSE, features tumors similar to endometrioid adenocarcinomas (8,9). Other reported serous adenocarcinoma models are mostly associated with development of type II tumors (6,10,11). One showed conditional deletion of p53 and activation of c-myc to be sufficient for transformation of BRCA1-deficient mouse OSE cells (11). Although these models of each major subtype of EOCs have undoubtedly improved our knowledge of EOCs’ biology, we have no direct evidence for the contribution of human OSE.

It has been shown that human OSE cells can be immortalized and converted to a tumorigenic state by enforced expression of the SV40
early region in combination with hTERT and oncogenic Ras (41,42). The SV40 T antigen is known to inactivate p53 and Rb pathways and interact with a wide range of cellular proteins, but it is not generally involved in EOC etiology. We therefore sought to identify a core set of genes that could replace viral oncogenes. By Cdk4 and cyclinD1 transduction in combination with hTERT, we here established novel OSE cell lines, termed HOSE1C and HOSE2C, which have neither major chromosomal alterations nor a transformed phenotype. The Rb pathway is frequently disrupted in EOCs and abnormal expression of Cdk4/cyclinD1 is often observed (18,22). EOCs, like many other carcinomas, maintain telomere length with telomerase activation (20). Immortality is one of the important characteristics of malignancy and ectopic expression of these genes thus could mimic the events that occur during progression of EOCs.

Alterations of p53 have been detected in 50% of EOCs (17) and some authors suggest that p53 alterations might represent an early step in ovarian carcinogenesis. Mutations in Kras are also relatively common in type I tumors (17). Even though they are much less common in type II lesions, the Ras pathway is activated in 41% of serous adenocarcinomas (43). Here, transduction of genetic elements with these two alterations into immortalized HOSE cells resulted in enhanced anchorage-independent growth but only weak or no tumour-forming ability. Therefore, we tried to define essential genetic alterations that co-operate with inactivation of p53 and activation of Ras pathway to induce a fully transformed phenotype.

The PI3K pathway is frequently activated in EOCs. In our HOSE2C cell lines, while neither additive introduction of shPTEN nor PIK3CA H1047R stimulation of Dnp53-KrasV12 cells was insufficient for full transformation, HOSE2C-Dnp53-KrasV12 cells transduced with Akt displayed fibroblastic morphology and reproducibly formed tumors consisting of mixtures of mainly sarcomatous and minor carcinomatous components. Amplification and/or overexpression of the c-myc gene, an oncogene but also a strong inducer of apoptosis, has been documented in 26-40% of all ovarian tumors (17). Furthermore, amplification of c-myc is a common finding in advanced stages, which may suggest

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**Fig. 5.** EOC-like features of HOSE2C-Dnp53-KrasV12-c-mycT58A-bcl-2 cells. (A) Expression of c-myc and bcl-2 confirmed after bcl-2 transduction into HOSE2C-Dnp53-krasV12-c-mycT58A cells. (B) Resistance of HOSE2C-Dnp53-KrasV12-c-mycT58A-bcl-2 cells to apoptosis in low-serum growth medium. A total of 1 x 10⁶ cells of each cell line were seeded on 10 mm dishes with complete growth medium containing 10% fetal bovine serum and cultivated for 10 h, then were washed with phosphate-buffered saline and fed with medium supplemented with 1% fetal bovine serum and cultivated for 90 h. Cells were collected and DNA content was analyzed by flow cytometry. The number inside each panel refers to the percentage of sub-G1 population (apoptotic cells). Scale bars, 250 μm. (C) Macroscopic appearance of HOSE2C-Dnp53-krasV12-c-mycT58A-bcl-2 tumors spreading and disseminating in the peritoneal cavity (a-c, arrows). Histopathology of intraperitoneal tumors, paraffin embedded, sectioned and stained with hematoxylin and eosin (d-f). (d and e) Tumors consist mostly of sheets of poorly differentiated epithelial cells. Cells show a high nuclear:cytoplasmic ratio with pleomorphic large nuclei. The inset in (e) illustrates immunofluorescence staining for cytokeratin 18. As all the tumors examined show strong cytokeratin 18 staining, representative staining is illustrated. (f) Invasion to liver (l). Scale bars, 40 μm. (D) Expression of cytokeratin 18 (CK18), vimentin and c-myc in HOSE2C-Dnp53-KrasV12-c-mycT58A-bcl-2 cells under monolayer culture conditions (culture), in subcutaneous tumors (sc tumor) and in intraperitoneal tumors (ip tumor).
a critical role in progression. Transduction of wild-type or a mutant c-myc here strongly enhanced anchorage-independent growth of HOSEC-Dnp53-KrasV12 but still did not result in tumor formation, probably because of enhanced sensitivity to apoptosis. Indeed, transduction of bcl-2, an anti-apoptotic gene, to c-myc-expressing cells conferred resistance to apoptosis and induced tumor formation with 100% efficiency. Recent studies suggest that bcl-2 can be upregulated by p-Akt (44), and Bcl-2 activates Akt by direct interaction (45). However, we failed to detect any significant upregulation of Bcl-2 in Akt-expressing cells (data not shown). The Bcl-2-Myc-expressing cells demonstrated a more aggressive phenotype than Akt-expressing cells and also exhibited metastatic behavior initiating human EOCs. Interestingly, c-myc transduction conferred epithelial morphology to the cells and, in co-operation with bcl-2, gave rise to carcinomas with cytokeratin 18 expression, though CA125, a tumor marker often elevated in EOCs, was not detected in the culture supernatant, tumors or ascites in mice (data not shown). Bcl-2 is overexpressed in 24–80% of EOCs (35–38) and is associated with resistance to chemotherapy (46,47). Antisense oligonucleotides and small molecules to inhibit bcl-2 have been developed at the preclinical and clinical levels for chronic lymphocytic leukemia, myelomas and non-small cell lung carcinomas (48). Our results provide evidence that such therapy targeting bcl-2 could be also applied to some EOCs. 

We attempted to clarify critical pathways in the carcinogenesis of each histological subtype, especially the type II tumor for which there are limited data on molecular alterations. Our cell lines co-operatively resulted in carcinosarcomas and poorly differentiated adenocarcinomas that should be classified as type II tumors based on their histology. Further challenges include establishment of type I and type II EOCs with different histologies by investigating the tumor microenvironment, such as interactions with stromal cells, cytokines, sex steroids and hormones. It will be also of interest to explore fallopian tube epithelial cells as the target population. Our experimental model should facilitate further studies to understand genesis of EOCs and hopefully will assist in the evaluation of new therapies targeting ovarian cancer-initiating cells (49) existing in our transformed OSE cells.

Supplementary materials

Supplementary Tables 1 and 2 and Figures 1–3 can be found at http://ian.carcin.oxfordjournals.org.

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References


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