**Molecular and Cellular Pathobiology**

**Wnt5a Suppresses Epithelial Ovarian Cancer by Promoting Cellular Senescence**

Benjamin G. Bitler¹, Jasmine P. Nicodemus¹, Hua Li¹, Qi Cai², Hong Wu³, Xiang Hua⁴, Tianyu Li⁵, Michael J. Birrer⁶, Andrew K. Godwin⁸, Paul Cairns⁶, and Rugang Zhang¹

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**Abstract**

Epithelial ovarian cancer (EOC) remains the most lethal gynecologic malignancy in the United States. Thus, there is an urgent need to develop novel therapeutics for this disease. Cellular senescence is an important tumor suppression mechanism that has recently been suggested as a novel mechanism to target for developing cancer therapeutics. Wnt5a is a noncanonical Wnt ligand that plays a context-dependent role in human cancers. Here, we investigate the role of Wnt5a in regulating senescence of EOC cells. We show that Wnt5a is expressed at significantly lower levels in human EOC cell lines and in primary human EOCs (n = 130) compared with either normal ovarian surface epithelium (n = 31; P = 0.039) or fallopian tube epithelium (n = 28; P < 0.001). Notably, a lower level of Wnt5a expression correlates with tumor stage (P = 0.003) and predicts shorter overall survival in EOC patients (P = 0.003). Significantly, restoration of Wnt5a expression inhibits the proliferation of human EOC cells both in vitro and in vivo in an orthotopic EOC mouse model. Mechanistically, Wnt5a antagonizes canonical Wnt/β-catenin signaling and induces cellular senescence by activating the histone repressor A/promyelocytic leukemia senescence pathway. In summary, we show that loss of Wnt5a predicts poor outcome in EOC patients and Wnt5a suppresses the growth of EOC cells by triggering cellular senescence. We suggest that strategies to drive senescence in EOC cells by reconstituting Wnt5a signaling may offer an effective new strategy for EOC therapy.

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**Introduction**

Cellular senescence is an important tumor suppression mechanism in vivo (1). In primary mammalian cells, cellular senescence can be triggered by various inducers including critically shortened telomeres and activated oncogenes (such as oncogenic RAS; ref. 1). Senescent cells are viable but non-dividing (2). Senescent cells also exhibit several distinctive morphologic characteristics and molecular markers, including a large flat cellular morphology and expression of senescence-associated β-galactosidase (SA-β-gal) activity (3). In murine liver carcinoma and sarcoma models, reactivation of the tumor suppressor p53 induces senescence and is associated with tumor regression (4, 5). Hence, driving cancer cells to undergo cellular senescence represents a novel mechanism for developing cancer therapeutics (6, 7).

More than 85% of ovarian cancers are of epithelial origin (8). Epithelial ovarian cancers (EOC) are classified into distinct histologic types including serous, mucinous, endometrioid, and clear cell (9). The most common histology of EOC is serous (~60% of all cancers) and less common histologies include endometrioid, clear cell, and mucinous (9). Recently, an alternative classification has been proposed, in which EOC is broadly divided into 2 types (10). Type I EOC includes endometrioid, mucinous, low-grade serous, and clear-cell carcinomas, and type II EOC includes high-grade serous carcinomas (10). EOC remains the most lethal gynecologic malignancy in the United States (8). Thus, there is an urgent need to better understand the etiology of EOC to develop novel therapeutics for this devastating disease.

Wnt signaling is initiated by binding of the Wnt ligand to its cognate frizzled receptor (11). Canonical Wnt signaling results in stabilization of the key transcription factor β-catenin, which then translocates into the nucleus and drives expression of its target genes, such as _CCND1_ (cyclin D1), _FOSL1_, and _c-MYC_ (12, 13). Canonical Wnt signaling is active in the putative somatic stem/progenitor cells of the coelomic epithelium of the mouse ovary (14). Underscoring the importance of Wnt signaling in EOC, in a murine ovarian cancer model, activation of canonical Wnt signaling cooperates with inactivation of the tumor suppressor PTEN in driving ovarian carcinogenesis (15). However, the role of Wnt signaling in EOC is not fully understood.

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**Authors’ Affiliations:** ¹Women’s Cancer Program; ²Biosample Repository Facility; ³Department of Pathology; ⁴Transgenic Facility; ⁵Department of Bioinformatics and Biostatistics; and ⁶Department of Surgical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania; ⁷Massachusetts General Hospital Cancer Center, Boston, Massachusetts; and ⁸University of Kansas Medical Center, Kansas City, Kansas

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**Corresponding Author:** Rugang Zhang, W446, Fox Chase Cancer Center, 330 Cottman Avenue, Philadelphia, PA 19111. Phone: 215-728-7108; Fax: 215-728-3616; E-mail: rugang.zhang@fccc.edu

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Wnt5a is a noncanonical Wnt ligand that plays opposing roles in different types of cancer and has variable expression dependent on the cancer context (16). Specifically, in EOC the role of Wnt5a remains unclear. Thus, in this study, we investigated Wnt5a expression and its potential function in human EOC cells. We discovered that Wnt5a was expressed at significantly lower levels in primary human EOC compared with either primary human ovarian surface epithelium or fallopian tube epithelium. Notably, loss of Wnt5a expression was associated with tumor stage and predicted shorter overall survival in EOC patients. Significantly, Wnt5a reconstitution inhibited the growth of EOC cells both in vitro and in vivo in an orthotopic EOC mouse model by promoting cellular senescence. These studies show, for the first time, a functional role of the noncanonical Wnt ligand, Wnt5a, in promoting senescence. Importantly, they also suggest that promoting EOC cells to undergo senescence represents a potential novel strategy for developing urgently needed EOC therapeutics.

Materials and Methods

Cells and culture conditions
Primary human ovarian surface epithelial (HOSE) cells were isolated and cultured as previously described (17). Human EOC cell lines were obtained from American Type Culture Collection (ATCC) and were passaged for less than 6 months. EOC cell line identification was further confirmed by DNA Diagnostic Center (www.dnacenter.com). EOC cell lines were cultured according to ATCC in RPMI-1640 medium supplemented with 10% FBS. 5-Aza-cytidine (Aza-C; Sigma) was used at 0.005% crystal violet, and the number of colonies was counted by using a dissecting microscope.

Retrovirus production, infection, and drug selection
The following retrovirus constructs were used: pBABE-puro was obtained from Addgene, hygro-pWZL-luciferase was a kind gift of Dr. Denise Connolly, and pBABE-Wnt5a was generated by using standard cloning protocol. Retrovirus packaging was done as previously described by using Phoenix packaging cells (19, 20). To increase infection efficacy, double virus infection was carried out. For drug selection, 3 μg/mL of puromycin was used for the OVCAR5 human EOC cell line.

Reverse transcriptase PCR, quantitative reverse transcriptase PCR, and immunoblotting
RNA from cultured primary HOSE cells or human EOC cell lines was isolated by using TRIzol (Invitrogen) according to manufacturer’s instruction. For quantitative reverse transcriptase PCR (qRT-PCR), TRizol-isolated RNA was further purified by using an RNeasy kit (QIAGEN) following manufacturer’s instruction. The Wnt5a, CCND1, FOSL1, and c-MYC primers used for qRT-PCR were purchased from SABiosciences. mRNA expression of the housekeeping gene β-2-microglobulin (B2M) was used to normalize mRNA expression. Soluble β-catenin was extracted, using a buffer that consisting of 10 mmol/L Tris-HCl (pH 7.5), 0.05% NP-40, 10 mmol/L NaCl, 3 mmol/L MgCl₂, 1 mmol/L EDTA, and protease inhibitors (Roche) as previously described (21, 22). The following antibodies were used for immunoblotting from the indicated suppliers, goat anti-Wnt5a (R&D Systems), mouse anti-β-catenin (Sigma), mouse anti-GAPDH (Millipore), mouse anti-β-catenin, mouse anti-Rb (BD Biosciences), and rabbit anti-pRBpS780 (Cell Signaling).

Immunofluorescence and SA-β-gal staining
Indirect immunofluorescence staining was carried out as previously described (19, 20, 22). The following antibodies were used for immunofluorescence: a cocktail of mouse anti-HIRA monoclonal antibodies (WC19, WC117, and WC119; I:10; ref. 20) and a rabbit anti-PML antibody (Chemicon, I:5,000). Images were captured by a DS-QiMc camera on a Nikon Eclipse 80i microscope and processed by NIS-Elements BR3.0 software (Nikon). SA-β-gal staining was carried out as described previously (3, 23). For SA-β-gal staining in sections from xenografted tumors, 8 separate fields were examined from 2 individual tumors for each of the groups.

In vivo orthotopic xenograft tumorigenesis study
The protocol was approved by the FCCC Institutional Animal Care and Use Committee. OVCAR5 cells were infected with a luciferase-encoding retrovirus (hygro-pWZL-luciferase) and infected cells were selected with 50 μg/mL hygromycin. Drug-selected cells were then infected with control or Wnt5a-encoding retrovirus and subsequently selected with 3 μg/mL puromycin and 50 μg/mL hygromycin. A total of 3 × 10⁶ drug-selected cells were unilaterally injected into the ovarian bursa sac of immunocompromised mice (6 mice per group; ref. 24). From day 10 postinfection, tumors were visualized by injecting luciferin (intraperitoneal, 4 mg/mice) resuspended in PBS and...
imaged with an IVIS Spectrum imaging system every 5 days until day 30. Images were analyzed by Live Imaging 4.0 software. At day 30, tumors were surgically dissected and either fixed in 10% formalin or fresh-frozen in Optimal Cutting Temperature compound (Tissue-Tek). Sections of the dissected tumors were processed by the FCCC Histopathology Core Facility.

Statistical analysis
Quantitative data are expressed as mean ± SD, unless otherwise indicated. ANOVA with Student’s t test was used to identify significant differences in multiple comparisons. The Pearson $\chi^2$ test was used to analyze the relationship between categorical variables. Overall survival was defined as the time elapsed from the date of diagnosis and the date of death from any cause or the date of last follow-up. Kaplan–Meier survival plots were generated and comparisons were made by using the log-rank sum statistic. For all statistical analyses, the level of significance was set at 0.05.

Results

Wnt5a is expressed at significantly lower levels in human EOC cell lines and primary human EOCs compared with normal human ovarian surface epithelium or fallopian tube epithelium

To determine Wnt5a expression in human EOC cell lines and primary HOSE cells, we examined the relative Wnt5a mRNA levels by carrying out semiquantitative RT-PCR. We observed that Wnt5a mRNA levels were greatly diminished in human EOC cell lines compared with primary HOSE cells (Fig. 1A). This finding was further confirmed through qRT-PCR analysis of Wnt5a mRNA in multiple isolations of primary HOSE cells and human EOC cell lines, showing that the levels of Wnt5a mRNA were significantly lower in human EOC cell lines compared with primary HOSE cells (Fig. 1B; $P = 0.008$). Consistently, we observed that Wnt5a protein levels were also lower in human EOC cell lines compared with primary HOSE cells as determined by immunoblotting (Fig. 1C). On the basis of these results, we conclude that Wnt5a is expressed at lower levels in human EOC cell lines compared with primary HOSE cells.

We next determined whether the loss of Wnt5a expression found in human EOC cell lines was also observed in primary human EOCs. We examined Wnt5a expression in 130 cases of primary human EOC specimens and 31 cases of normal human ovary with surface epithelium by IHC, using an antibody against Wnt5a (Table 1). In addition, there is recent evidence to suggest that a proportion of high-grade serous EOC may arise from distant fallopian tube epithelium (25). Thus, we also included 28 cases of normal human fallopian tube specimens in our IHC analysis (Table 1). The specificity of the anti-Wnt5a antibody was confirmed in our study (Supplementary Fig. S1). A single band at predicted molecular weight (~42 kDa) was detected in OVCA5 cells with ectopically expressed Wnt5a and was absent after expression of a short hairpin RNA to the human Wnt5a gene (shWnt5a), which effectively knocked down Wnt5a mRNA expression (Supplementary Fig. S1A and data not shown). In addition, Wnt5a staining was lost when primary anti-Wnt5a antibody was replaced with an isotype-matched IgG control (Supplementary Fig. S1B).

As shown in Figure 1D, in normal human ovarian surface epithelial cells and fallopian tube epithelial cells, both cytoplasm and cell membrane were positive for Wnt5a IHC staining (black arrows, Fig. 1D). In contrast, Wnt5a staining in EOC cells was dramatically decreased (Fig. 1D). We scored expression of Wnt5a as high ($H$-score ≥ 30) or low ($H$ score < 30) on the basis of a histological score ($H$ score; 26), which considers both intensity of staining and percentage of positively stained cells, as previously described (17). Wnt5a expression was scored as high in 58.1% (18/31) cases of normal human ovarian surface epithelium and 82.1% (23/28) cases of normal human fallopian tube epithelium (Table 1). In contrast, Wnt5a expression was scored as high in 37.7% (49/130) cases of primary human EOCs (Table 1). Statistical analysis revealed that Wnt5a was expressed at significantly lower levels in primary human EOCs compared with either normal human ovarian surface epithelium ($P = 0.039$) or normal human fallopian tube epithelium ($P < 0.001$; Table 1). On the basis of these studies, we conclude that Wnt5a is expressed at significantly lower levels in primary human EOCs compared with either normal human ovarian surface epithelium or fallopian tube epithelium.

Wnt5a expression negatively correlates with tumor stage and lower Wnt5a expression predicts shorter overall survival

We next examined the correlation between Wnt5a expression and clinical and pathologic features of human EOCs. Significantly, there was a negative correlation between Wnt5a expression and tumor stage ($P = 0.003$; Table 1). Notably, the majority of examined cases are high-grade serous subtypes that are usually of stage 3/4. In addition, we examined the correlation between expression of Wnt5a and a marker of cell proliferation, Ki-67 (ref. 27; Fig. 1E). There was a significant negative correlation between Wnt5a expression and Ki-67 ($P = 0.039$; Table 1). We next assessed whether Wnt5a expression based on $H$ score might predict prognosis of EOC patients (High, $H$ score ≥ 30; Low, $H$ score < 30; n = 123), for which long-term follow-up data were available. Significantly, lower Wnt5a expression correlated with shorter overall survival in the examined EOC patients ($P = 0.003$; Fig. 1F). Together, we conclude that a lower level of Wnt5a expression correlates with tumor stage and predicts shorter overall survival in human EOC patients.

Wnt5a gene promoter hypermethylation contributes to its downregulation in human EOC cells

Wnt5a gene promoter hypermethylation has been implicated as a mechanism underlying its silencing in several types of human cancers (16). Consistently, we also observed Wnt5a gene promoter hypermethylation in a number of human EOC cell lines (Fig. 2A; Supplementary Table S1). Further supporting a role of promoter hypermethylation in suppression of Wnt5a expression, treatment with a DNA demethylation drug, Aza-C (28), in PEO1 EOC cells resulted in a significant increase in levels of both Wnt5a mRNA and protein (Fig. 2B and C). We
conclude that Wnt5a gene promoter hypermethylation contributes to its downregulation in human EOC cells.

**Wnt5a restoration inhibits the growth of human EOC cells by antagonizing the canonical Wnt/β-catenin signaling**

We next sought to determine the effects of Wnt5a reconstitution in human EOC cells. Wnt5a expression was reconstituted in the OVCAR5 EOC cell line via retroviral transduction. Ectopically expressed Wnt5a was confirmed by both qRT-PCR and immunoblotting in OVCAR5 cells stably expressing Wnt5a or a vector control (Fig. 3A and B). Of note, the levels of ectopically expressed Wnt5a in OVCAR5 cells are comparable with the levels observed in primary HOSE cells (Fig. 3B). Interestingly, Wnt5a reconstitution in OVCAR5 human EOC cells significantly inhibited both anchorage-dependent and anchorage-independent growth in soft agar compared with vector controls (Fig. 3C and D). In addition, similar growth inhibition by Wnt5a reconstitution was also observed in the PEO1 human EOC cell line.
Supplementary Fig. S2A–C) suggesting that this effect is not cell line specific. On the basis of these results, we conclude that Wnt5a reconstitution inhibits the growth of human EOC cells in vitro.

Canonical Wnt signaling promotes cell proliferation and Wnt5a has been shown to antagonize the canonical Wnt/β-catenin signaling in certain cell contexts (16, 29–31). Because Wnt5a expression inversely correlated with expression of Ki-67 (Fig. 1E; Table 1), a cell proliferation marker, we hypothesized that Wnt5a would suppress the growth of human EOC cells by antagonizing canonical Wnt/β-catenin signaling. To test our hypothesis, we examined the effect of Wnt5a reconstitution on expression of markers of active Wnt/β-catenin signaling in human EOC cells, namely the levels of “active” soluble β-catenin (21, 22, 32) and expression of β-catenin target genes such as CCND1, c-MYC, and FOSL1 (12, 13). Indeed, we observed a decrease in soluble β-catenin in Wnt5a-reconstituted OVCAR5 cells compared with vector controls (Fig. 3E). Consistently, we also observed a significant decrease in the levels of β-catenin target genes in these cells, namely CCND1

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<th>Table 1. Wnt5a expression in primary human EOCs and correlation of its expression with clinicopathologic variables</th>
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<td><strong>Patient characteristics</strong></td>
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<td>Age (23–85 y; mean 59.2 y)</td>
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*Compared with type I EOC.  
*Compared with EOC.  
*Compared with stage 1/2.
and the size of PCR product is also indicated. B, PEO1 cells were treated with 5 μmol/L Aza-C for 4 days, and mRNA was isolated from control- and Aza-C-treated cells and examined for Wnt5a mRNA expression by qRT-PCR. Mean of 3 independent experiments with SD. C, same as (B) but examined for Wnt5a protein expression by immunoblotting.

(P = 0.0095), FOSL1 (P = 0.0012), and c-MYC (P = 0.0286; Fig. 3F). Similar effects of Wnt5a reconstitution on expression of markers of active Wnt/β-catenin signaling (such as decreased levels of soluble β-catenin) were also observed in PEO1 human EOC cells (Supplementary Fig. S2D), suggesting that this is not cell line specific. On the basis of these results, we conclude that Wnt5a suppresses the growth of human EOC cells by antagonizing canonical Wnt/β-catenin signaling in human EOC cells.

**Wnt5a reconstitution drives cellular senescence in human EOC cells**

Next, we sought to determine the cellular mechanism whereby Wnt5a inhibits the growth of human EOC cells. We have previously shown that suppression of canonical Wnt signaling promotes cellular senescence in primary human fibroblasts by activating the senescence-promoting histone repressor A (HIRA)/promyelocytic leukemia (PML) pathway (22). PML bodies are 20 to 30 dot-like structures in the nucleus of virtually all human cells. PML bodies are sites of poorly defined tumor suppressor activity and are disrupted in acute PML (33). PML has been implicated in regulating cellular senescence. For example, the foci number and size of PML bodies increase during senescence (33, 34) and inactivation of PML suppresses senescence (35). Activation of the HIRA/PML pathway is reflected by the recruitment of HIRA into PML bodies (36).

To determine whether Wnt5a reconstitution activates the HIRA/PML senescence pathway and induces senescence in EOC cells, we first sought to determine whether the HIRA/PML pathway is conserved in human ovarian epithelial cells. Ectopically expressing activated oncogenes (such as oncogenic RAS) is a standard approach for inducing senescence in a synchronized manner in primary human cells (1, 2, 19, 20). Indeed, ectopic expression of oncogenic H-RAS(B12V) induced senescence of primary HOSE cells, as evident by an increase in SA-β-gal activity, a universal marker of cellular senescence (Supplementary Fig. S3A and B). Notably, the HIRA/PML pathway was activated during senescence of primary HOSE cells induced by oncogenic RAS, as evident by the relocalization of HIRA into PML bodies (Supplementary Fig. S3C and D). This result shows that the senescence-promoting HIRA/PML pathway is conserved in human ovarian epithelial cells. In addition, primary HOSE cells with HIRA foci displayed a marked decrease in BrdU incorporation, a marker of cell proliferation, compared with HIRA foci-negative cells (Supplementary Fig. S3E and F). This result is consistent with the idea that activation of the HIRA/PML pathway is directly correlated with senescence-associated cell growth arrest (37).

We next asked whether Wnt5a expression is regulated during natural senescence of primary HOSE cells. Indeed, we observed an increase in the levels of Wnt5a mRNA in senescent primary HOSE cells compared with young cells (Fig. 4A–C). In addition, we found that ectopic Wnt5a induces senescence of primary HOSE cells (Fig. 4D–F). Together, we conclude that Wnt5a plays a role in regulating senescence of primary HOSE cells.

As Wnt5a antagonizes canonical Wnt signaling in human EOC cells (Fig. 3E and F), we sought to determine whether Wnt5a restoration might activate the senescence-promoting HIRA/PML pathway and induce senescence in human EOC cells. Toward this goal, we examined the localization of HIRA in OVCAR5 EOC cells reconstituted with Wnt5a or vector control. Notably, there was a significant increase in the percentage of cells with HIRA localized to PML bodies in Wnt5a restored human EOC cells compared with controls (Fig. 5A and B; P = 0.004). In addition, we also observed an increase in the number and size of PML bodies in the Wnt5a restored OVCAR5 EOC cells (Fig. 5A), which are also established markers of cellular senescence (35, 38). Similarly, we observed activation of the HIRA/PML pathway by Wnt5a restoration in PEO1 human EOC cells (Supplementary Fig. S4A and B), suggesting that the observed effects are not cell line specific. Together, we conclude that Wnt5a reconstitution activates the HIRA/PML senescence pathway.

The p53 and pRB tumor suppressor pathways play a key role in regulating senescence (1). Thus, we sought to determine whether activation of the HIRA/PML pathway depends on the p53 and pRB pathways. Interestingly, p16INK4a, the upstream repressor of pRB, is deleted in OVCAR5 human EOC cell line (39). In addition, the levels of total phosphorylated pRB were not decreased by Wnt5a, whereas the levels of cyclin D1/Cdk4-mediated Serine 780 phosphorylation on pRB (pRbP780) were decreased by Wnt5a (ref. 40; Fig. 5C and D). Furthermore, p53 is null in OVCAR5 cells (41). We conclude that activation of the HIRA/PML pathway is independent of the p53 and p16INK4a.

We next sought to determine whether Wnt5a restoration induces SA-β-gal activity, a universal marker of cellular senescence (1). Indeed, SA-β-gal activity was notably induced by
Wnt5a reconstitution in both OVCAR5 and PEO1 human EOC cells compared with controls (Fig. 5E and F; Supplementary Fig. S4C and D, respectively). On the basis of these results, we concluded that Wnt5a restoration induced senescence of human EOC cells by activating the HIRA/PML senescence pathway.

**Wnt5a inhibits the growth of human EOC cells* in vivo* by inducing cellular senescence**

We next sought to determine whether Wnt5a would mediate growth inhibition and induce senescence* in vivo* in an orthotopic EOC model in immunocompromised mice. A luciferase gene was retrovirally transduced into control or Wnt5a-reconstituted OVCAR5 cells to monitor the cell growth* in vivo* via noninvasive imaging. These cells were injected unilaterally into the bursa sac covering the ovary in female immunocompromised mice (n = 6 for each of the groups; Supplementary Fig. S5). Tumor growth was monitored every 5 days starting at day 10 postinooculation by measuring luciferase activity, and the growth of the tumor was followed for a total of 30 days (Fig. 6A). Wnt5a significantly suppressed the growth of xenografted OVCAR5 human EOC cells compared with controls (Fig. 6B; P < 0.0012). Consistently, following general pathologic examination during surgical dissection at day 30, we observed that tumor sizes were notably smaller from mice injected with Wnt5a-reconstituted OVCAR5 cells compared with controls (data not shown). The expression of ectopic Wnt5a was confirmed by IHC staining in sections from dissected tumors (Fig. 6C).

We next sought to determine whether cell proliferation was suppressed by Wnt5a reconstitution in dissected tumors. Toward this goal, we examined the expression of Ki-67 by IHC staining in sections from dissected tumors (Fig. 6C). We observed, there was a significant reduction in the number of Ki-67-positive cells in tumors formed by Wnt5a-reconstituted OVCAR5 cells compared with control tumors. Indeed, we observed a significant increase in the number of Ki-67-positive cells (Fig. 6D). On the basis of these results, we conclude that Wnt5a reconstitution inhibits the proliferation of human EOC cells* in vivo* in an orthotopic xenograft EOC model.

We next investigated whether the growth inhibition observed by Wnt5a reconstitution *in vivo* was due to induction of cellular senescence. Toward this goal, we examined the expression of SA-β-gal activity in fresh sections of dissected tumors formed by OVCAR5 cells reconstituted with Wnt5a or control cells. Indeed, we observed a significant increase in the number of cells positive for SA-β-gal activity in OVCAR5 cells reconstituted with Wnt5a compared with control tumors.
Wnt5a promotes senescence of primary HOSE cells. A, young proliferating primary HOSE cells were passaged to senescence (after 7 population doublings). Expression of SA-β-gal activity was measured in young and naturally senescent primary HOSE cells. B, same as A. Quantitation of SA-β-gal–positive cells. **P < 0.001. C, same as (A), but mRNA was isolated and examined for Wnt5a expression by qRT-PCR. Expression of B2M was used as a control. *P = 0.003. D, young primary HOSE cells were transduced with retrovirus encoding human Wnt5a gene or a control. Expression of Wnt5a in indicated cells was determined by qRT-PCR. Expression of B2M was used as a control. E, same as (D), but stained for expression of SA-β-gal activity in drug-selected cells. F, quantitation of (E). Mean of 3 independent experiments with SD. *P < 0.05.

**(Fig. 6F and G; P = 0.003).** Together, we conclude that Wnt5a reconstitution inhibits the growth of human EOC cells in vivo by inducing cellular senescence.

**Discussion**

Driving cancer cells to undergo cellular senescence has recently been proposed to be a novel mechanism to target for developing cancer therapeutics (1, 6). For example, pharmacologic inhibitor of PTEN drives senescence and, consequently, inhibits tumorigenesis in vivo in xenograft models of PTEN heterozygous prostate cancer cells (42, 43). Compared with apoptosis, therapeutics that drive cellular senescence are proposed to have less cytotoxic side effects (6), which makes prosenescence therapy attractive. Herein, we describe that restoration of Wnt5a signaling drives senescence of human EOC cells both in vitro and in vivo in an orthotopic mouse model of EOC (Figs. 5 and 6). Restoring gene expression by gene therapy has had limited success. Therefore, restoring Wnt5a signaling via exogenous ligand could prove to be an alternative approach. Interestingly, it has been previously reported that a Wnt5a-derived hexapeptide is sufficient to restore Wnt5a heterozygous prostate cancer cells (42, 43).
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signaling both in vitro and in vivo in xenograft models of breast cancer (44). It would be interesting to test whether the Wnt5a-derived hexapeptide will be sufficient to reconstitute Wnt5a signaling and drive senescence of EOC cells. Our data suggest that cellular senescence is a potential target for developing EOC therapeutics. In addition, these data imply that restoration of Wnt5a signaling represents a potential novel strategy to drive senescence of EOC cells.

This study is the first to show a role for Wnt5a in regulating senescence. We showed that Wnt5a activated the senescence-promoting HIRA/PML pathway in human EOC cells (Fig. 5A; Supplementary Fig. S4A). In primary human cells, activation of HIRA/PML pathway is sufficient to drive senescence by facilitating epigenetic silencing of proliferation-promoting genes (such as E2F target genes; ref. 19). Herein, we reported for the first time that the key HIRA/PML senescence pathway can be reactivated to drive senescence of human cancer cells. Further studies are warranted to elucidate the molecular basis by which Wnt5a restoration and activation of HIRA/PML pathway drive cellular senescence in human EOC cells.

Interestingly, senescence induced by Wnt5a restoration in human EOC cells was independent of both the p53 and p16INK4A tumor suppressors, which implies that EOC cells that lack p53 and p16INK4A retain the capacity to undergo senescence via HIRA/PML pathway through suppressing the canonical Wnt signaling. This is consistent with previous reports showing that cancer cells that lack p53 and pRB retain the capacity to undergo senescence when treated with anticancer agents or ionizing radiation (6). Notably, although the levels of total phosphorylated pRB were not decreased by Wnt5a, we observed a decrease in the levels of pRBP5780 that is mediated by cyclin D1/CDK4 (Fig. 5C and D). Future studies will determine whether the decrease in pRBP5780 levels plays a role in regulating senescence of human EOC cells. Expression of Wnt5a is altered in many types of cancers (45). For example, in melanoma, Wnt5a overexpression correlates with cancer progression and a higher tumor stage (16). However, in colorectal and esophageal squamous cell carcinomas, Wnt5a has been described to be a tumor suppressor and was frequently silenced by promoter hypermethylation (16, 46). Consistently, we also observed Wnt5a promoter hypermethylation in a number of human EOC cell lines in which Wnt5a is downregulated (Fig. 2; Supplementary Table S1). This result is consistent with the idea that Wnt5a promoter hypermethylation contributes to Wnt5a downregulation in human EOC cells.

Wnt5a function is highly dependent on cellular context (45). For example, the cellular Wnt receptor/coreceptor context dictates the downstream signaling pathways upon the binding of Wnt5a, which include activating noncanonical Wnt signaling or antagonizing canonical Wnt/β-catenin signaling (47). These reports illustrate that Wnt5a expression and its resulting activity are cell type and context dependent. The Wnt receptor/coreceptor profile in EOC cells is currently unknown, and our future studies will elucidate the mechanism by which Wnt5a

Figure 6. Wnt5a restoration inhibits tumor growth and promotes senescence of human EOC cells in vivo. A, OVCAR5 cells were transduced with luciferase-encoding hygromycin-resistant retrovirus together with a control or Wnt5a-encoding puromycin-resistant retrovirus. Drug-selected cells were unilaterally injected into the periovarian bursa sac of the female immunocompromised mice (n = 6 for each of the groups). The radiance of luciferase bioluminescence, an indicator of the rate for tumor growth, was measured every 5 days from day 10 until day 30 by using the IVIS imaging system. Shown are images taken at day 10 and day 30, respectively. B, quantitation of tumor growth from injected OVCAR5 cells expressing Wnt5a or control at indicated time points. *, P = 0.038 compared with controls. C, following tumor dissection, expression of Wnt5a in tumors formed by control or Wnt5a-expressing OVCAR5 EOC cells was determined by immunohistochemical staining against Wnt5a (magnification, 40×). Bar, 50 μm. D, same as (C), but examined for expression of Ki-67, a marker of cell proliferation (magnification, 40×). Bar, 50 μm. E, quantitation of (D). *, P = 0.008 compared with controls. F, expression of SA-β-gal activity was examined on sections of fresh-frozen tumors formed by OVCAR5 cells expressing control or Wnt5a (magnification, 40×). Bar, 100 μm. G, quantitation of (F). *, P = 0.003 compared with controls. Arrow points to an example of SA-β-gal positive cells.
antagonizes Wnt/β-catenin signaling in human EOC cells. Regardless, our data show that Wnt5a downregulation is an independent predictor for overall survival in EOC patients. In contrast, 2 other studies showed that higher Wnt5a expression predicts poor survival in EOC patients (48, 49). The basis for this discrepancy remains to be elucidated. An explanation may be that our study included more cases than the other 2 studies (130 EOC cases in our study vs. 38 cases in the study by Badiglian and colleagues or 63 cases in the study by Peng and colleagues). It may also be due to the difference in the composition of type I and type II cases in this study compared with the other 2 studies. The vast majority of EOC cases in this study are of type II high-grade serous subtypes. Consistently, our data showed that there is a difference in Wnt5a expression between type I and type II EOC (P = 0.005; Table 1). Furthermore, it has been shown in microarray analysis that Wnt5a is expressed at lower levels in laser capture and microdissected high-grade serous EOC compared with normal primary HOSE cells (50).

In summary, the data reported here show that Wnt5a is often expressed at lower levels in human EOCs compared with either normal human ovarian surface epithelium or fallopian tube epithelium. A lower level of Wnt5a expression correlates with tumor stage and predicts shorter overall survival in EOC patients. Reconstitution of Wnt5a signaling inhibits the growth of human EOC cells both in vitro and in vivo. In addition, Wnt5a reconstitution suppresses the proliferation-promoting canonical Wnt/β-catenin signaling in human EOC cells. Significantly, Wnt5a reconstitution drives cellular senescence in human EOC cells and this correlates with activation of the senescence-promoting HIRA/PML pathway. Together, our data imply that reconstitution of Wnt5a signaling to drive senescence of human EOC cells is a potential novel strategy for developing EOC therapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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