



## SV40 early genes induce neoplastic properties in serous borderline ovarian tumor cells

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### Abstract

**Objective.** Serous borderline ovarian tumors (SBOT) are slow growing, noninvasive ovarian epithelial neoplasms, which tend to recur as low-grade invasive carcinomas (LGC) with a much worse prognosis. We investigated the molecular basis of this progression.

**Methods.** We established cultures of three SBOTs and one LGC from tumor biopsies, and inactivated p53, Rb and PP2A in the cells with SV40 large T (LT) and small T (ST) antigen. They were examined for cadherins by immunofluorescence and immunoblotting, invasiveness in Boyden chambers, motility by scratch-wound healing assay, anchorage independence by growth in agarose, and protease activity by gelatin zymography, immunoassay and colorimetry. Cells were overexpressed with N-cadherin using an adenovirus.

**Results.** Inactivation of p53, Rb and PP2A by SV40 LT/ST antigen resulted in greatly enhanced growth potential, invasiveness, motility and anchorage independence, and in epithelio-mesenchymal transition, as indicated by morphology and substitution of N-cadherin for E-cadherin. Overexpressed N-cadherin did not induce invasiveness of SBOT cells and there was no consistent change in protease activities, suggesting that these were not primary effectors of the enhanced neoplastic characteristics. Low passage LGC cells were more invasive than SBOT cells, but this difference disappeared with the introduction of LT/ST into the two cell types.

**Conclusion.** Downregulation or inactivation of p53, Rb and/or PP2A plays a role in the progression from SBOT to invasive ovarian carcinomas.

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### Introduction

In the Western world, serous tumors account for about 80% of all epithelial ovarian tumors with 10 to 15% of these categorized as serous borderline ovarian tumors (SBOT) or of low malignant potential (LMP) [1]. Patients with SBOT have a significantly more favorable prognosis than those with invasive carcinomas, as reflected in their 95% five-year survival rate.

However, a recent retrospective study showed that 44% of patients with SBOT had recurrences and that three-quarters of these were low-grade serous adenocarcinomas (LGC) which are associated with a significantly worse prognosis, as 74% of these patients died of the disease [2].

SBOTs display nuclear atypia, mitotic activity and cellular stratification, but grow slowly and lack the destructive, infiltrative stromal invasion characteristic of high-grade serous adenocarcinomas. The etiology and progression of SBOT in the development of invasive epithelial ovarian cancer remain puzzling. In support of a progression model, case reports have documented the development of high-grade serous adenocarcinomas in patients previously diagnosed with SBOT [3]. However, there is growing evidence sug-

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gesting that high-grade serous adenocarcinomas are an entity distinct from SBOT, while LGC are closely related to borderline tumors [4,5]. Most convincingly, mutations in KRAS and BRAF occur in over 60% of LGC and SBOT while p53 mutations are rare. In contrast, high-grade serous adenocarcinomas infrequently harbor BRAF and KRAS mutations while p53 mutations occur in most of the tumors [4]. Importantly though, p53 appears to play a unique role in the gene regulation of SBOT and their transition to LGC: compared to normal ovarian surface epithelium as the control, the expression and signaling activity of p53 and its downstream targets are significantly increased in SBOT, resulting in repression of cell proliferation, promotion of senescence and stabilization of *CDKN1A*. With the progression of SBOT to LGC, this enhanced signaling activity of p53 is reversed [4].

In an effort to understand the nature of SBOT and their transformation to invasive carcinoma, we recently established an *in vitro* model of human SBOT [6]. These cultures resembled invasive ovarian carcinomas by CA125 secretion, telomerase activation, E-cadherin expression and morphologic resemblance to metaplastic OSE/serous differentiated adenocarcinoma cultures. They secreted proteinases but, in contrast to ovarian cancer cell lines, cultured SBOT cells were slow growing, minimally invasive and poorly motile, characteristics resembling their behavior *in vivo*. Here we report on the consequences on transfection of SBOT cultures with the simian virus 40 early genes, large T (LT) and small t (ST) antigens, which were initially carried out to extend the cells' life span in culture. Unexpectedly, the transfected SBOT cells acquired characteristics associated with neoplastic progression, the most important of which was the capacity to invade. Since LT inactivates p53, and in view of the unique role of p53 in the transition of SBOT to LGC, we decided to follow up on this observation.

LT binds to and inactivates the tumor suppressor p53, which is mutated or downregulated in most, if not all human tumors [7]. It is well known that p53 responds to cellular stresses and DNA damage by inducing either apoptosis, senescence or DNA repair. Recently, additional functions of p53 have emerged which include considerable evidence for p53 as a suppressor of cell migration. This effect is achieved through the inhibition of polarization, cells spreading and protrusion formation, largely via regulation of Rho signaling (reviewed in [8]). LT also inactivates pRb, thereby dysregulating control of the cell cycle. Among other effects of p53/pRb, the introduction of LT renders noninvasive mouse bladder carcinomas invasive [9] and p53 directly inhibits the transcription of focal adhesion kinase (FAK), a critical kinase in tumor cell invasion and survival [10]. ST promotes cell transformation primarily through negative regulation of the protein phosphatase 2A (PP2A) family of serine-threonine phosphatases [11]. Interference with the tumor-suppressive functions of PP2A complexes has transformation-promoting effects, including promotion of cell cycle progression, inhibition of apoptosis, activation of the MAPK and PI3K pathways, effects on the cytoskeleton, integrins and cell–cell

adhesion, and perturbation of p53 activation. Our findings suggest that perturbation of these key pathways may have a causative role in the induction of invasiveness in SBOTs.

## Methods

### *Tissue culture and transfection*

Institutional approval for experimentation with human tissues, including informed consent from each subject, was obtained prior to this study. Tumor specimens from patients with SBOTs and one LGC were collected and cultured in medium 199/MCDB105/10% FBS and gave rise to lines SBOT-1, SBOT-3, SBOT-4 and LGC-2. SBOT and LGC cells were separated from stromal cells by differential trypsinization [6]. The SV40 LT/ST antigens were introduced by transfection with FuGENE 6 (Roche Diagnostics) [11], generating lines ISBOT-1.5, ISBOT-3.3, and ILGC-2.2. The ovarian cancer lines SKOV3 and OVCA429 served as controls.

### *Adenoviral infection*

The human N-cadherin cDNA clone (Origene, Rockville, Maryland) was subcloned into the recombinant adenovirus pShuttle vector, followed by ligation to the adenoviral vector as per manufacturer's procedure (Clontech Laboratories Inc., Palo Alto, CA). The recombinant N-cadherin adenoviral vector was packaged into infectious adenovirus by transfecting HEK-293 cells. For infection, cells were trypsinized, infected with recombinant adenoviruses (either EGFP-virus or N-cadherin virus) for 1 h in suspension, replated and cultured for 24–72 h. Infection efficiency was nearly 100% as determined by immunofluorescence staining for N-cadherin. Invasion assays were carried out 72 h post-infection.

### *Immunostaining*

Immunofluorescent staining for keratin, SV40 LT, E-cadherin, and N-cadherin was as previously described [6]. Primary antibody against keratin (wide-spectrum screening, 1:1000) was from DakoCytomation (Glostrup, Denmark), SV40 LT (Ab-1, 1 µg/mL) from Oncogene Science (Hornby, ON), and E-cadherin (clone 36, 0.5 µg/mL) and N-cadherin (clone 32, 0.83 µg/mL) from BD Transduction Laboratories (San Jose, CA). Alexa 594-labeled goat anti-mouse (1:800) or Alexa 488-labeled goat anti-rabbit IgG (1:400) (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) were secondary antibodies. Cells were counterstained with Hoechst 33258 (0.5 µg/mL; Sigma, Oakville, Ontario).

### *Western blotting*

Lysates were separated on an 8% SDS-polyacrylamide gel and transferred onto nitrocellulose membranes that were blocked with 5% skim milk, 0.05% Tween-20 TBS, and incubated with anti-E-cadherin antibody (0.25 µg/mL) or anti-

N-cadherin antibody (0.1 µg/mL). Immunoreactive bands were visualized with peroxidase-conjugated anti-rabbit immunoglobulin (1:3000) (Bio-Rad Lab.), followed by enhanced chemiluminescence (Pierce, Nepean, ON).

#### *Anchorage independence*

Growth in soft agar was performed as previously described [6]. Triplicate cultures for each cell type were maintained for 3 weeks and colonies were then sized and counted.

#### *Tumorigenicity*

Assays were performed as previously described [6]. Four mice were injected with SBOT-3.1 or ISBOT-3.3 cells. Experiments were terminated after 6 months and the animals examined for evidence of tumors or ascites.

#### *Matrigel™ invasion assay*

The invasion assay was performed in Boyden chambers with minor modifications [12].

#### *Migration assay*

Confluent monolayers were serum starved overnight and scratched using a micropipette tip. Cell migration into the scratch was measured at 0 h, 6 h and 24 h by capturing images using a monochromatic digital CCD camera (Retiga 1300; Qimaging, Surrey, BC), and the covered area was analyzed with the Northern Eclipse 6.0 software. Results represent the mean area covered by migrating cells in one field ± SD of at least five fields.

#### *Protease assays*

Gelatin zymography for determining MMP-2 and MMP-9 secretion was performed as described [13]. Total MMP-2 was quantified by the Quantikine human/mouse MMP-2 (total) immunoassay kit (R&D Systems, Minneapolis, MN) [13]. Each sample was analyzed in duplicate. Analysis of uPA activity was quantified using a coupled assay to monitor plasminogen activation and the resulting plasmin hydrolysis of a colorimetric substrate (VLKpNA) [14]. Each sample was analyzed in triplicate in two separate assays.

#### *Measurement of internuclear distance*

Cells were stained with 0.5 µg/mL Hoechst 33258 and the internuclear distances [15] between adjacent cells were measured in arbitrary units (pixels) using the Northern Eclipse 6.0 software. Results are presented as internuclear distances from at least 75 cells per assay.

#### *Statistics*

Unless stated otherwise, all experiments were repeated at least 3 times.

## Results

### *Patient characteristics*

Fresh tissue samples of three SBOTs (SBOT-1, SBOT-3, SBOT-4) and one SBOT-derived LGC (LGC-2) were obtained from four women aged 38 to 46 years (Table 1).

### *SV40 LT/ST alter the lifespan of SBOT cells*

The lifespan of the SBOT and LGC cells in culture was limited, with the exception of a subline of SBOT-3, SBOT-3.1, which spontaneously developed into a permanent line [6]. The remaining cultures ceased growth and senesced after 3–4 passages. Since part of these passages was required to eliminate stromal cells, the amount of cells available for experimentation was quite limited. Therefore, we attempted to extend the lifespan of two SBOT and one LGC case by introducing the SV40 LT/ST antigens, generating lines ISBOT-1.5 and -3.3, and ILGC-2.2, respectively, the prefix “I” indicating “immortalization”. LT inactivates the cell cycle- and apoptosis regulators p53 and pRb, and thus delays senescence-related growth suppression. ISBOT-1.5, ISBOT-3.3, and ILGC-2.2 underwent 94, 80, and 56 population doublings, respectively, before undergoing senescence. These lines were used to compare the phenotypic and functional characteristics of the SBOT lines and the ISBOT lines. As the number of low passage cells was limited, not all experiments were performed on all of the cases, and not all experiments could be repeated 3 times.

### *SV40 LT/ST genes promote epithelio-mesenchymal transition (EMT)*

Typically, cultured low passage SBOT cells display an epithelial phenotype with long extensions, whorls and some overlap, resembling cultures of metaplastic surface epithelium and low-grade ovarian carcinomas (Fig. 1A) [6]. With the introduction of SV40 LT/ST, this phenotype was replaced by a more atypical and scattered morphology, suggestive of EMT (Fig. 1B). Such EMT is thought to contribute to the dissemination

Table 1  
Summary of patient characteristics

Patient no.	Age	Diagnosis	Cell line
1	46	SBOT <sup>a</sup>	SBOT-1
2	37	SBOT	
	45	LGC <sup>a</sup>	LGC-2
3	29	SBOT	
	32	SBOT	
	35	SBOT	
	38	SBOT <sup>a</sup>	SBOT-3
	40	LGC	
4	41	SBOT	
	43	SBOT <sup>a</sup>	SBOT-4

<sup>a</sup> Specimen obtained for cell culture; SBOT, serous borderline ovarian tumor; LGC, low-grade carcinoma; multiple ages for patients 2–4 indicate recurrences.

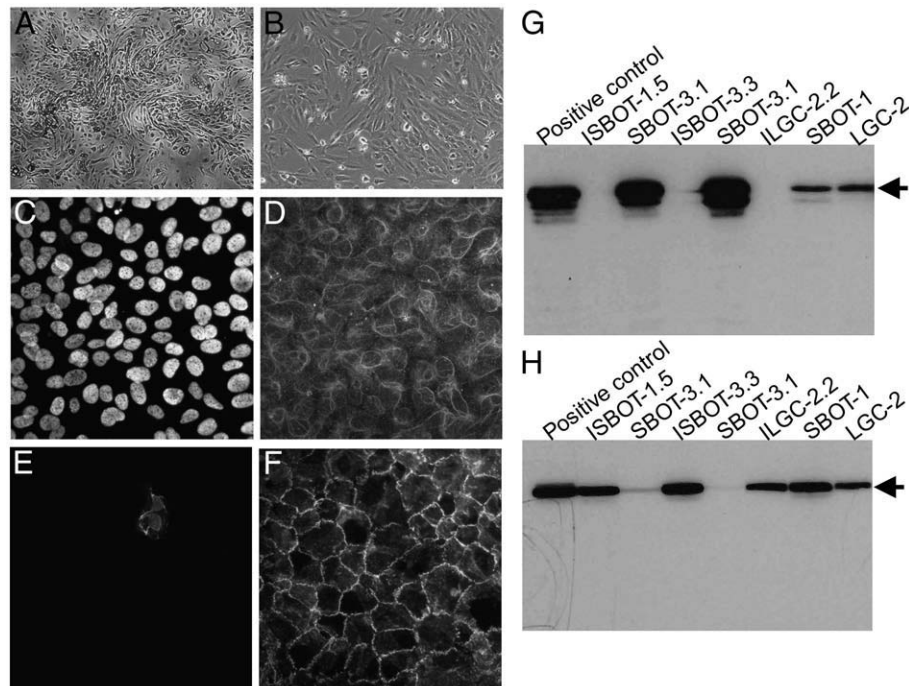


Fig. 1. SV40 LT and ST antigens promote EMT in SBOT cells. (A, B) Morphology of cells in culture. (A) SBOT cells display a whorly, epithelial growth pattern in primary culture while (B) cells transfected with SV40 LT/ST assume a more fibroblast-like morphology. ISBOTs are (C) LT-positive, (D) express keratin, (D) lose E-cadherin but (F) gain N-cadherin (immunofluorescence microscopy). Immunoblot analysis of (G) E-cadherin and (H) N-cadherin protein expression (bands shown by arrow). E-cadherin positive control, OVCAR-3. N-cadherin positive control, IOSE-80pc. Note that the lower expression of E-cadherin and the positive expression of N-cadherin in SBOT-1 and LGC-2 cells are due to contamination by fibroblast cells as keratin-positive cells, which is an indicator of epithelial differentiation, are E-cadherin positive and N-cadherin negative (data not shown).

of carcinoma cells [16], and therefore may play an important role in the progression from borderline to invasive cancer. Consequently, we examined molecular alterations associated with EMT. Immunostaining showed that all the cells were LT-positive (Fig. 1C) and continued to express keratin (Fig. 1D), indicating their epithelial origin, but lost the epithelial differentiation marker, E-cadherin (Fig. 1E). Complete loss of E-cadherin protein was also shown by immunoblotting (Fig. 1G). In addition, while the SBOT cultures secrete significant levels of CA125 [6], none was detected in the conditioned medium from the SV40 LT/ST-transfected cell lines. In contrast, expression of the mesenchymal marker, N-cadherin, which correlates positively with EMT, was strongly induced (Figs. 1F,H).

#### SV40 LT/ST increase invasiveness and cell motility

The clinical characteristic of SBOT cells, their inability to invade, was retained in culture [5]. As shown in Fig. 2A, all three SV40 LT/ST-transfected lines displayed significant increases in the number of invasive cells compared to SBOT cells. In contrast, ILGC-2.2, derived from an invasive carcinoma, was invasive prior to transfection, and the introduction of SV40 LT/ST did not enhance invasiveness. In parallel, SV40 LT/ST caused an increase in cell motility (Fig. 2B). Thus, SBOT cells can become invasive, and the signaling pathways altered by LT and/or ST can contribute to this phenotype.

#### SV40 LT/ST are not associated with tumorigenicity but induce anchorage independence

2/2 SBOT cell lines tested formed no colonies in soft agar, whereas all ISBOT and ILGC lines acquired a significant degree of anchorage independence (Fig. 2C). SBOT-3.1 and ISBOT-3.3 cells were injected intraperitoneally into SCID mice, but neither the permanent SBOT-3.1 line nor the ISBOT-3.3 cells developed tumors over 6 months.

#### Invasion and migration by ISBOT cells are not associated with changes in protease secretion

Since transfection with SV40 early genes resulted in increased motility and invasiveness, the conditioned media from the three ISBOT lines were analyzed for changes in three proteinases commonly studied in ovarian cancer, namely MMP2, MMP9, and uPA. ISBOT and ILGC expressed different levels of proMMP2 (Fig. 2D, bottom left panel). ILGC-2.2 cells expressed the most total MMP2 followed by ISBOT-1.5 and ISBOT-3.3 cells, respectively. Similar trends were observed in the levels of uPA activity (Fig. 2D, bottom right panel). Urokinase PA activity in the ILGC-2.2 cells was similar to those of SBOT-4 cells while ISBOT-1.5 and ISBOT-3.3 cells expressed little uPA, like SBOT-3 cells. Weak or no expression of proMMP9 was detected in the cell lines (Fig. 2D, top panel). Both SBOT-3 and SBOT-4 expressed proMMP2 and proMMP9 [6]. Thus,

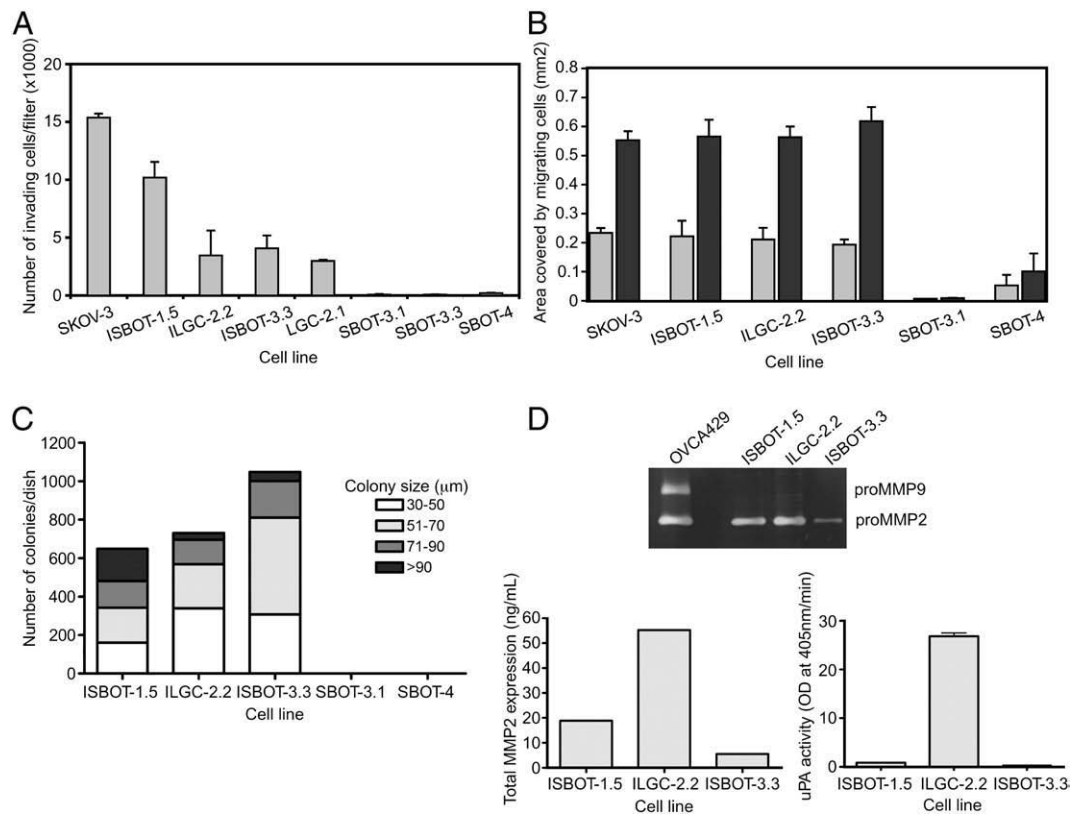


Fig. 2. Functional changes in SBOT cells following inactivation of p53, Rb and PP2A. (A) Matrigel™-invasion assay. SBOT cells are essentially noninvasive compared to ISBOT cells which invade Matrigel™-coated Boyden chambers. The invasiveness of LGC cells is comparable to ISBOT cells, and is not altered by the introduction of LT/ST. SKOV-3 cells serve as highly invasive controls. (B) Scratch-wound healing assay. The migratory activity (6 h, light grey bar; 24 h, dark grey bar) of ISBOT and ILGC cells is significantly increased over that of SBOT cells. (C) Anchorage-independent growth. ISBOT and ILGC lines but neither the SBOT-3.1 nor SBOT-4 cells form colonies in soft agar. (D) Protease expression. Conditioned media from an equal number of ISBOT-1.5, ILGC-2.2, and ISBOT-3.3 cells were analyzed by gelatin zymography for proMMP2 and proMMP9 (top panel). In contrast to the ovarian carcinoma cell line OVCA429, ISBOT cells show weak expression of proMMP9. Conditioned media were analyzed by ELISA to detect total MMP2 expression (bottom left panel) and for uPA activity using a coupled colorimetric plasminogen activation assay based on plasmin hydrolysis of VLKpNA (bottom right panel).

in spite of the differences in levels of protease activity among the ISBOT and ILGC lines and their similarities to SBOT-3 and SBOT-4 cells, there does not seem to be a clear relationship between the three proteinases examined and invasive capability.

#### *Manipulation of the cadherin profile of SBOT cells enhances cell spreading*

As the introduction of SV40 LT and ST antigens caused a switch from E- to N-cadherin, in association with the increased invasion and motility, we investigated whether manipulation of their cadherin profile would alter their invasive potential, as has been reported for other epithelial cell types [17,18]. N-cadherin was transiently overexpressed in SBOT cells (Fig. 3A). E-cadherin staining remained unchanged (Fig. 3B), and there was no significant increase in invasiveness, indicating that increased N-cadherin expression is insufficient to trigger invasiveness in the SBOT cells (Fig. 3C). However, the cells exhibited more filopodia and were more spread and flattened after N-cadherin overexpression (Figs. 3A, B), as determined by increased internuclear distances in N-cadherin overexpressing cells compared to controls (Fig. 3D).

#### **Discussion**

Relatively little is known about SBOTs and their relationship to invasive carcinomas, because few experimental systems for studies of this problem have been available [20,21]. We recently established and partially characterized cultures from several SBOT cases [6]. Here, we further characterized the SBOT cells and transfected them with SV40 LT/ST to extend their life span in culture. We found, unexpectedly, that the introduction of SV40 LT/ST into SBOT cells resulted in their acquisition of characteristics associated with neoplastic progression, including anchorage independence, increased motility and invasiveness. Thus, transformation of SBOT cells to a more aggressive phenotype may, in part, be mediated by changes in the expression and/or activity of p53, pRb and/or PP2A, the targets of the SV40 LT/ST. LT antigen inactivates the p53 and the retinoblastoma protein (pRB) tumor suppressor proteins [22], which control cell cycle arrest and apoptosis. Importantly, p53 also suppresses cell motility, a crucial step in invasion and metastasis [8,22]. This regulation is mediated by Rho GTPases, thereby controlling cytoskeletal organization and EMT [23], and also by negative regulation of FAK [10]. Interestingly, our results show that the introduction of LT/ST

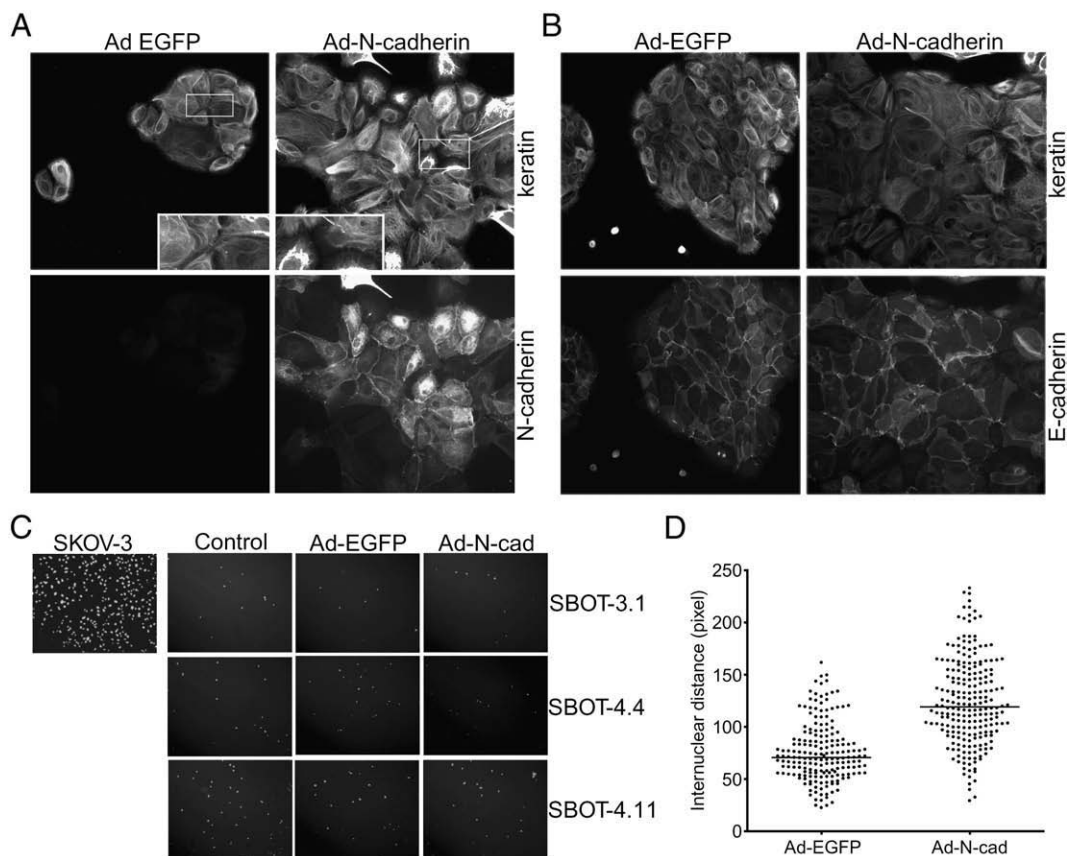


Fig. 3. Overexpression of N-cadherin does not alter E-cadherin or keratin expression, or the invasive capacity of SBOT cells, but increases cell spreading. Immunofluorescent staining, (A) N-cadherin and (B) E-cadherin in SBOT cells infected with EGFP control adenovirus (left panels) or N-cadherin adenovirus (right panels). No change in E-cadherin or keratin was observed with overexpression of N-cadherin. Note the increase in filopodia expressed in N-cadherin overexpressing cells compared to the control cells (inset box). (C) Overexpression of N-cadherin does not promote invasion of SBOT cells compared to controls (no adenovirus or EGFP adenovirus), while SKOV3 cells under comparable conditions are highly invasive. (D) Overexpression of N-cadherin caused increased cell spreading and flattening as determined by measuring the internuclear distances.

significantly increased invasiveness in SBOT lines but not in the LGC cultures. In view of the increased expression and activity of p53 in SBOT compared to LGC [4], in conjunction with the migration-inhibitory activity of p53 [8], it seems possible that in the LGC cells the p53 levels were low enough to permit migration, while migration/invasiveness of SBOT cells required downregulation of p53 by LT. In addition, PP2A, which is targeted by ST, influences motility [11]. PP2A is required for the proper localization of E-cadherin and  $\beta$ -catenin to the plasma membrane [24], and E-cadherin expression was greatly diminished in the ISBOT cells. Finally, both inactivation of p53 and of PP2A lead to apoptosis resistance, which may account for the anchorage independence acquired by the ISBOT cells.

Progression of SBOT cells to the invasive phenotype was accompanied by a transition from E- to N-cadherin expression. N-cadherin promotes motility and invasion in other carcinomas [18,19]. In ISBOT cells, overexpressed N-cadherin induced spreading of the cells and cell separation, as colony morphology was less compact, as well as an increase of filopodia, but the cells remained noninvasive. Cell spreading and the formation of filopodia are among the motility-related functions which are suppressed by p53 [8];

thus N-cadherin seems to have facilitated cell migration, but not sufficiently to induce invasiveness. Perhaps, downregulation of E-cadherin, in conjunction with N-cadherin expression is necessary for enhanced motility and invasiveness of this cell type [19].

A key factor enabling cells to invade is the ability to degrade extracellular matrix, but in the ISBOT lines, we found no association between invasive characteristics and the activities of the three proteinases, and although total MMP2 was increased in the ILGC line this did not affect its invasiveness. As shown previously [6], untransfected SBOT cells also expressed proMMP2 and proMMP9, which were both activated following treatment with the MMP activator APMA, as well as variable levels of active uPA. Thus, proteinase expression is not likely the limiting factor in the inability of SBOT cells to invade.

At present, the precise roles of p53, pRb and PP2A in the progression of SBOT cells to an invasive form are still undefined, but experiments are underway to discriminate among these influences. Further pursuit of the mechanisms underlying invasion in ovarian cancer may help to uncover better markers for predicting tumors that progress to a more aggressive and lethal invasive ovarian cancer.

**Conflict of interest statement**

The authors declare that there are no conflicts of interest.

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