

## In Ovo Progression of Borderline Ovarian Tumors

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### Abstract

**Objective:** Borderline ovarian tumors (BOTs) are generally considered to be non-invasive tumors with low malignancy potential. However, studies show that BOTs have the ability to progress to low-grade carcinomas and gain the potential to invade surrounding tissue and to metastasize. Here, we investigate whether a patient derived serous BOT (SBOT) could progress in low-grade serous carcinoma (LGSC) through *in vitro* and *in ovo* characterization.

**Methods:** Ovarian cancer cell line from a SBOT, named OCAM, were isolated and characterized by PCR, migration assay and chick chorioallantoic membrane (CAM) assay.

**Results:** OCAM cells express carcinoma characteristics and form a tumor in CAM. Moreover, OCAM cells get into membrane of chicken suggesting invasive properties.

**Conclusion:** SBOTs could be a precursor of LGSC involving consequences in adaptation of treatment provided by clinician.

**Keywords:** Borderline tumor; Ovarian; Low grade; Progression

### Introduction

Borderline ovarian tumors (BOTs), also named atypical proliferative ovarian tumours (APOTs), were first recognized in 2003 [1]. BOTs are viewed as an intermediate state between benign cystadenomas and adenocarcinomas. BOTs represent 15-20% of epithelial ovarian carcinomas (EOC), and 80% of them are diagnosed during the early stages of development [2,3]. Most women are diagnosed around 40 years of age but this can vary depending on ethnicity and geography [4]. Histological characteristics of BOTs are well-formed papillae with epithelial stratification and mild to moderate cellular atypia [5-7].

Patients diagnosed with stage I BOTs have excellent 5-year survival rates of between 90% and 100%, but this rate decreases when discovered in advanced stages [4]. In most cases, BOTs are asymptomatic and diagnosed during routine examination. The standard treatment for BOTs in advanced stages requires surgically removing the macroscopically visible tumor. Adjuvant therapy for advanced stages treatment remains controversial [8]. Risk factors such as age, histology, and type of surgery (for example fertility sparing surgery); appear to be associated with a higher risk for recurrence. Histologic parameters such as peritoneal implants, microinvasion, and micropapillary pattern do not seem to be associated with a higher recurrence rate [9,10]. Tumor markers, such as CA-125 and CA 19-9 are increased in BOTs, with a higher increase of CA-125 in serous BOT (SBOT) and CA 19-9 in MBOT [11].

Recent studies show that SBOTs have the potential to become a low-grade serous carcinoma (LGSC) and are associated with molecular modifications or microsatellite instability [12-14]. SBOTs and LGSCs share common mutations in KRAF, BRAF, ERBB2 supporting the hypothesis that SBOT might potentially progress to LGSC [14-16]. The hypothesis suggesting that BOTs are a precursor state for LGSC is now accepted, although clinical and molecular studies are still necessary to understand the mechanism and risk factors.

In this report, we isolated a clonal cell line established from a SBOT, termed OCAM, belonging to a 21 year old patient. OCAM cells were characterized and its ability to migrate was evaluated. Moreover,

the capacity of OCAM to grow and to invade was investigated using chick chorioallantoic membrane (CAM) assay.

### Materials and Methods

#### Patient and clinical background

A 21 year old asymptomatic white female consulted a gynecologist for contraception prescription. She has no particular medical history. She smoked 10 packs per year. Her physical examination was significant for a distended abdomen, with a palpable abdominal mass. Abdominal and pelvic computed tomographic (CT) scans and MRI confirmed the clinical evaluation of abnormal pelvic mass. Bilateral ovarian masses infiltrating the left paracolic gutter as well as a moderate quantity of ascites were noticed. Tumor markers were as follows: CA 125: 516 kU/l, Alpha-feto-protein: 3.7 uJ/l, LDH 146 U/l.

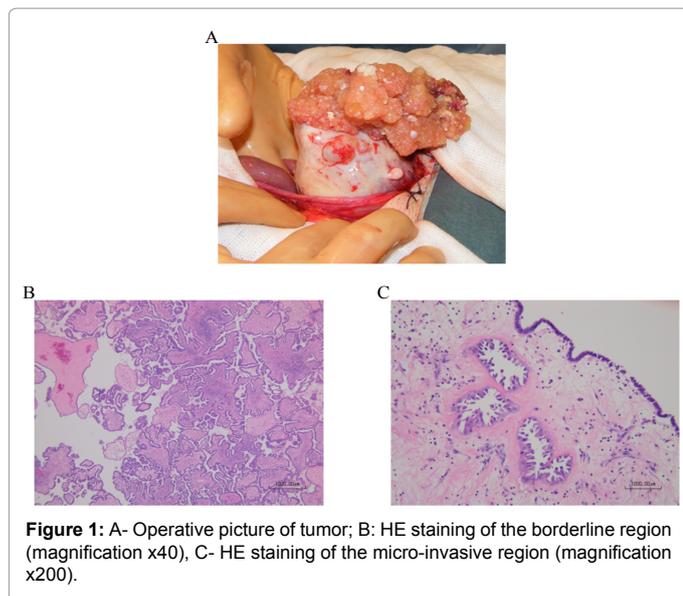
An exploratory laparoscopy converted into laparotomy followed by fertility-sparing surgery was performed. It included a right salpingo-oophorectomy, left ovarian cystectomy, omentectomy and appendectomy. The patient post-operative period was uneventful and she was discharged one-week post-op. Final pathological analysis showed serous borderline ovarian tumor with micro-invasion focus, FIGO stage of IIIB. Close follow-up was performed and 18 months later, a right ovarian mass with normal CA 125 was identified. The patient opted for left salpingo-oophorectomy, and pathology confirmed recurrence of a serous borderline ovarian tumour (SBOT) (Figure 1). Three years after the second surgery, no sign was observed.

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### Establishment of the ovarian cancer cell line: OCAM

Ovarian tissue was digested with 4mg/ml dispase (Gibco, Invitrogen, Basel, Switzerland) in HBSS-HEPES (filtered on 22 µm) containing 1 µg/ml DNase (Roche, Diagnostics GmbH, USA) for 30 min at 37°C. Ovarian tissue and supernatant were placed into 10 cm dishes and the tissue was scrubbed with a scalpel. The supernatant was collected, neutralized with 5% FBS, filtered through a 100 µm mesh (BD Biosciences, San Jose, USA) and centrifuged at 800 g for 8 min. The resulting pellet was resuspended in DMEM containing 10% FBS and 0.05 mg/ml gentamicin and cells were seeded in a 3 cm dish. The medium was replaced every two days. Cells were detached by using 0.25% trypsin-EDTA and were transferred into a 10 cm dish. OCAM cells were obtained by limiting dilution in a 96-well plate at passage 2. The cells were sub cultured every 5 days at a dilution 1:8.

### Cell culture

OCAM cell line was cultured at 37°C under 5% CO<sub>2</sub> in Roswell Park Memorial Institute medium 1640 (RPMI 1640, Gibco, Invitrogen,

Basel, Switzerland) containing 10% fetal bovine serum and 0.05 mg/ml gentamicin.

### Reverse transcription polymerase chain reaction (RT-PCR)

Expression of CD90, PAX8, KILK6, KRT7, KRT8, KRT19, Cytovillin, ESR1, ESR2, CLEC11A, HTRA1, COL12A1, COLT1, SLPI and HE4 mRNA were investigated in OCAM cells. Reverse transcription was performed with 1 µg of total RNA in a final volume of 20 µl using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA). The detection of the PCR product was performed using REDTaq<sup>™</sup> ReadyMix<sup>™</sup> PCR Reaction Mix from Sigma Aldrich (St Louis, MO, USA), with the Thermal cycler from Bioer (BioConcept, Switzerland). The experiment was performed in duplicate.

Oligonucleotide primers for PCR are listed in Table 1.

### Wound healing assay

1x10<sup>5</sup> OCAM cells were seeded in a 12-well plate. Twelve hours after, cells were treated with 2.5 µg/ml of mitomycin for 2 h. Then, mitomycin solution was removed, a straight scratch was performed with pipette tip stimulating a wound and medium was added. At different time points (0, 2, 4, 6, 8, 10, 24 h), a picture was assessed using xlicap software. The experiment was performed in triplicate.

### Immunocytochemistry

OCAM cells were seeded into Lab-tek chamber slide (2 × 10<sup>4</sup> cells /well) and cultured in RPMI medium. After 24 h, cells were washed in Phosphate Buffer Saline (PBS), fixed in 4% Paraformaldehyde (PFA) for 15 min and washed 3 times in PBS. Cells were permeabilised with 0.2% Triton X-100 in PBS for 10 min. Afterwards, non-specific sites were blocked with RPMI medium for 30 min and then incubated overnight at 4°C with mouse monoclonal antibodies against cytokeratin 18 (dilution 1:50, clone DC10, Dako, Baar, Switzerland), mouse monoclonal antibodies against cytokeratin 7 (dilution 1:50, clone OV-TL 12/30, Dako, Baar, Switzerland), mouse monoclonal antibodies against cytokeratin 19 (dilution 1:50, BA17, Santa Cruz Biotechnology, San Diego, CA, USA), mouse monoclonal antibodies against p21 (dilution 1:50, clone SX118, Dako, Baar, Switzerland), mouse monoclonal antibodies against p53 (dilution 1:50, Ab-3, Calbiochem, Merk Millipore, Switzerland) or mouse IgG (dilution 1:50, sc-2025, Santa Cruz Biotechnology, San Diego, CA, USA). Cells were

	Forward (5'-3')	Reverse (5'-3')
CD90	ATCGCTCTCCTGCTAACAGT	ACTTGACCAGTTTGTCTCTGA
CLEC11A	ACCTCTTCGAAAACGGCCAG	GCAGACGTAGTAGAGACGCC
COL12A1	CCTACAACGGATGGGCCTAC	ACTGAGCGTGGTTGTCTGAG
COLT1	TCCCTACTAAGGCCAGACC	CCTTCTCACCACCGAGCAAT
Cytovillin	AGGCGGTGGATCAGATAAAGA	CTCCTGGACATGGTAGCTCAC
ESR1	CCTTTGGCCAAGCCCGCTCA	TGGAGCGCCAGACGAGACCA
ESR2	GGTCGCGACACCACAAGCCC	TAACAGGGCTGGCGCAACGG
HE4	CGGCTTCACCCTAGTCTCAG	CCTCCTTATCATTGGGCAGA
HTRA1	CCAGACGTGATCTCAGGAGC	ACGGGAGAGTCTTTGAGGGA
KLK6	AGCCAAACTCTCTGAATCAT	AGTGATGCAAGGATGGAGCT
KRT19	ACTACAGCCACTACTACAGACCAT	GTCTCAAACCTTGGTTCGGAAGTC
KRT7	TTGCCTCTTCATCGACAAGGTGC	TCATACTGCGCCTTGACCTCAGCG
KRT8	AACAACCTTAGGCGGAGCT	GCCTGAGGAAGTTGATCTCG
PAX8	ACCTTCTCCATGTGGGACAC	GGATGCTGTGCTCAAAGTGA
SLPI	CCTGGATCCTGTTGACACCC	ACAGGGGAAACCGCAGGATTT
CYCLOPHILIN A	ATGGTCAACCCACCGTG	TGCAATCCAGCTAGGCATG

Table 1: Oligonucleotide sequences.

then washed 3 times in PBS and incubated for 1 hour with goat anti-mouse IgG-HRP (dilution 1:500 Santa Cruz Biotechnology, San Diego, CA, USA). After washing, cells were stained with diaminobenzidine (DAB) chromogen system (Dako, Baar, Switzerland) following by a hemalun counterstaining.

To perform this, Mayer's Hemalun solution (Merk Millipore, Switzerland) was diluted to 1/4 in water. The solution was then applied to the sample for 1 min and immersed in warm water. The slide was then dried, Aquatex (Merk Millipore, Switzerland) was added and a glass coverslip was put onto the slide.

### Tumor development on chick chorioallantoic membrane (CAM)

On Embryo Development Day 1 (EDD1), fertilized eggs (animal facility of the University of Geneva, Geneva, Switzerland) were incubated at 38°C with 80% relative humidity and periodic rotation.

On EDD4, rotation was stopped and eggs were drilled at their narrow apex. The hole was closed with adhesive tape. Incubation was carried out until use.

On EDD8, after gentle scratching of the membrane with a needle tip, a silicon O-ring (Apple Rubber products Inc., Lancaster, USA) was placed onto a blood vessel crossing. OCAM cells were resuspended at  $2.5 \times 10^6$  cells in 30  $\mu$ l of RPMI-1640 (containing 10% fetal bovine serum and 0.05 mg/ml gentamicin). The suspension was then placed into the silicon O-ring. The hole was hermetically covered with Parafilm<sup>®</sup> and eggs were returned to the incubator.

On EDD12, tumor growth was monitored using a Wild Heerbrugg M3Z microscope at 10x magnification with a Lumenera INFINITY2-1 CDD camera with Infinity Capture Software.

The ovarian tumor was rapidly washed with 0.1 M phosphate buffered saline (PBS) at pH 7.4 and fixed in 4% buffered formalin at 4°C for 24 h. The specimens were then dehydrated in ethanol and embedded in paraffin wax.

### Hematoxylin and eosin staining (HE)

The section of tissue was deparaffinised and rehydrated through graded ethanol before being immersed in the filtered Harris Hematoxylin solution modified (Sigma Aldrich, St Louis, MO, USA) for 1 min and rinsed with tap water. The section was immersed in eosin for 1 min, rinsed with tap water and was then dehydrated with a series of ethanol solutions: 50%, 70%, 80%, two baths with 95% and two baths with 100%. To finish, one bath of xylene for 5 min was performed. Then, Aquatex was added and a glass coverslip was put on slide.

### Immunohistochemistry

Serial sections of tissue were deparaffinised and rehydrated through graded ethanol as described previously. Antigen retrieval was performed by microwave pre-treatment in 10mmol/l citrate buffer (pH 6.0) for 5 min four times, followed by cooling in a cold water bath. Non-specific binding was blocked with 3% (v/v) bovine serum albumin (BSA) in PBS for 30 min at room temperature. The sections were incubated with mouse monoclonal antibodies against p21 (dilution 1:50), mouse monoclonal antibodies against p53 (dilution 1:50), mouse monoclonal antibodies against Ki-67 (dilution 1:50, BD Sciences, Allschwil, Switzerland) or mouse IgG (dilution 1:50). Sections were then washed with PBS and incubated with goat anti-mouse IgG-HRP (dilution 1/500 in 3% BSA-PBS) for 1 h. After washing, sections were stained with diaminobenzidine (DAB) chromogen system following

by a hemalun counterstaining. Aquatex (Merk Millipore, Switzerland) was added and a glass coverslip was put on slide.

## Results

### OCAM characterization through 15 genes

To characterize OCAM cell line, we investigated the expression of different genes known to participate in cancer cell invasion (keratin, KLK6, SLPI), growth (PAX8), adhesion (cytovillin, CD90), proliferation (HE4, oestrogen receptor, CLEC11A, COLT1, COL12A1) and chemoresistance (HTRA1). Most of these genes are currently studied for the characterization of ovarian cancer cell line, with the exception of CLEC11, HTRA1, COL12A1, COLT1 and SLPI, which were previously identified in our lab to be differentially expressed in borderline ovarian cancer cells compared to serous ovarian cancer cell.

OCAM cell line was positive for the following genes: PAX8, KRT8, KRT19, Cytovillin, KRT7, CLEC11A, HTRA1, COL12A1, SLPI and HE4 (Table 2). On the other hand, OCAM cells were negative for CD90, KLK6, ESR1, ESR2 and COLT1 genes.

### Tumour markers

Immunocytochemistry was performed to assess the expression levels of known oncoproteins p21, p53 as well as proliferative markers cytokeratin 7, cytokeratin 18 and cytokeratin 19 in OCAM cells. As observed in, OCAM cells were negative for p21. OCAM cells were also negative for CK19 while they expressed KRT19 mRNA. We also observed a heterogenous nuclear staining for p53 with a variable intensity an intense cytoplasmic staining for CK7 and a slight cytoplasmic staining for CK18 (Figure 2).

### OCAM migration

To examine the ability of OCAM cells to migrate, a wound-healing assay was performed. OCAM cells covered the half of the wound within 24 h thus showing their ability to migrate with 50% closure after 24 h (Figure 3).

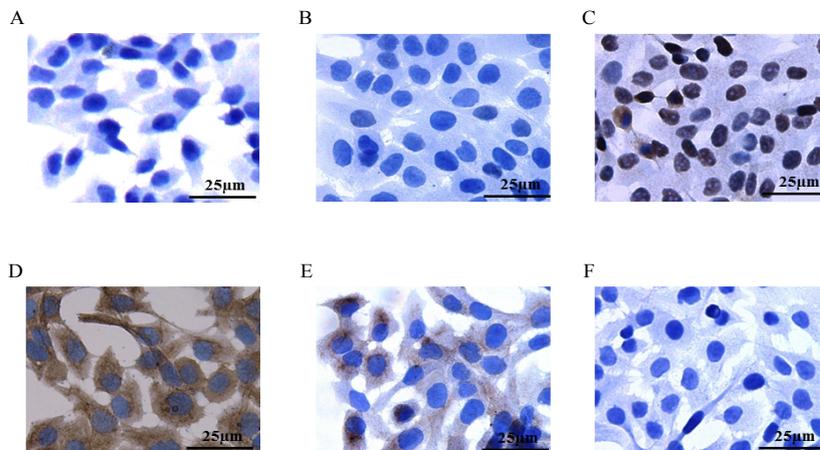
### OCAM tumor development

To further investigate the ability of OCAM cells to migrate and to invade into surrounding tissues in ovo, OCAM cells were inoculated on CAM. Seven days after the inoculation, the formation of a tumor was

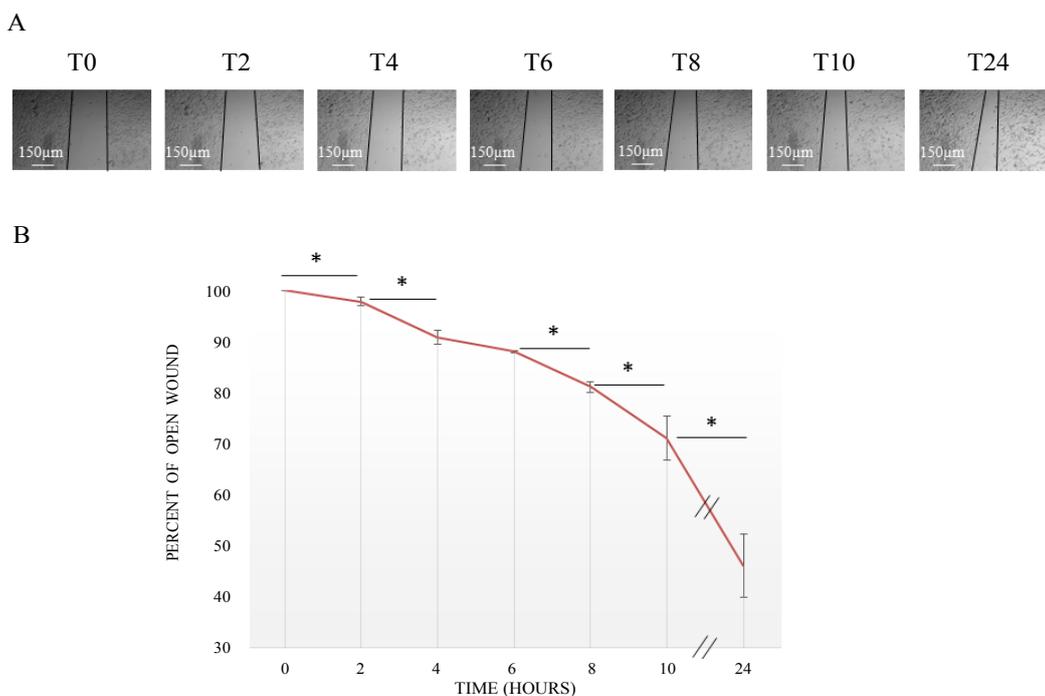
	OCAM
CD90	-
CLEC11A	+
COL12A1	+
COLT1	-
Cytovillin	+
ESR1	-
ESR2	-
HE4	+
HTRA1	+
KLK6	-
KRT19	+
KRT7	+
KRT8	+
PAX8	+
SLPI	+

+: expressed, -: not expressed

**Table 2:** Characterization of OCAM cell line mRNA by RT-PCR.



**Figure 2:** Tumor markers immunostaining of OCAM cells for mouse IgG as control (A), p21 (B), p53 (C), CK7 (D), CK18 (E) and CK19 (F). The magnification used is x400.



**Figure 3:** OCAM migration capacity OCAM cells were seeded in 12-well plate. The next day, cells were treated with mitomycin for 2 h to block cell proliferation and then a straight scratch was performed. To evaluate cell migration, a picture was assessed at different incubation time (0, 2, 4, 6, 8, 10, 24 h) (A). Figure B represents the quantification of open wound after the scratching (\* $p < 0.05$ , Student t-test).

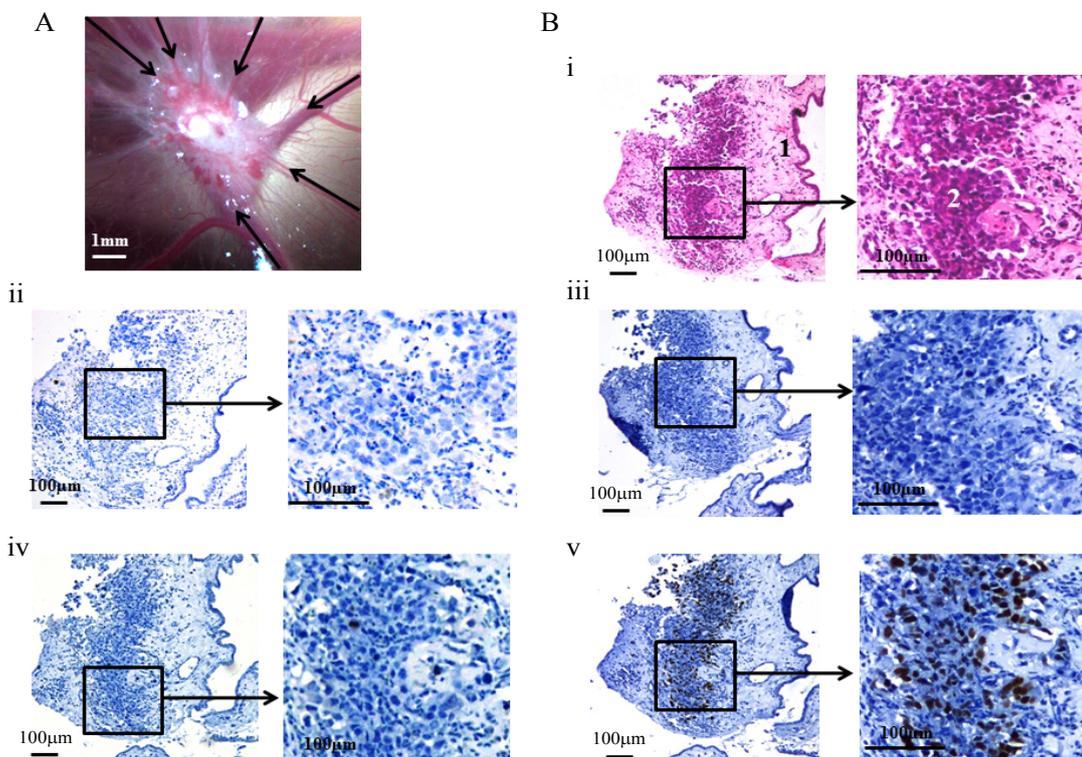
observed with several vessels converging to the tumor, thus showing the presence of a vascular network able to supply blood to the tumor.

At EDD 12, the tumor was removed embedded in paraffin and HE staining was performed. OCAM cells have formed a tumor (designated by number 2) next to the membrane of CAM (designated by number 1). The tumor is formed of small papillae with epithelial stratification and small regular nucleus, which is the characteristic of a low-grade tumor. Moreover, these cells seem to have infiltrated the membrane of the chicken. Immunohistochemistry further showed negative staining for p21 and very low Ki67, proliferation marker and a heterogeneous positive staining for wild type p53 (Figure 4).

## Discussion

Borderline ovarian tumors were long considered to be of benign evolution. It was proved this is not true, with overall survival (OS) rates lower than the general population in patients with advanced stage disease [17]. It is not clear if this decrease in OS is due to the borderline tumor per se, or to those tumors, that with time, mutate to low grade ovarian tumors, which are known to have very low response rates to systemic therapies. Unfortunately, to this day there is no way of identifying which patients will recur and if the recurrence will be as borderline tumor or low grade ovarian tumors.

This study report a cell line evaluation from a patient with a



**Figure 4:** OCAM tumour development A-On EDD8, after gently scratching of CAM, OCAM cells suspension was placed into the silicon O-ring. On EDD12, OCAM tumor was observed and a picture of tumor was taken. Arrows indicate the vascular network around the tumor. Bi- HE staining of OCAM tumor: 1. membrane of CAM; 2. OCAM tumor. B- Immunostaining of OCAM tumor for mouse IgG (ii), p21 (iii), Ki67 (iv) and p53 (v). The magnification used is x200. Each square represents an enlargement of the same location.

SBT named OCAM, and investigated its characteristics in vitro and in ovo. From a genetic point of view, we found that OCAM cell line is positive for PAX8, KRT8, KRT19, Cytovillin, KRT7, CLEC11A, HTRA1, COL12A1, SLPI and HE4 genes. These genes are known to participate in cell invasion, growth, cell adhesion, proliferation and chemoresistance, demonstrating the tumoral potential of this cell line. Moreover, we also observed that OCAM cells are highly proliferative in vitro (data not show). p53 expression was then investigated in these cells by immunocytochemistry. p53 is a well-known tumor suppressor which is inactivated in most cancer cells. Most of BOTs and LGSC were very rarely positive for p53 mutations and were almost wild type for p53 staining [18-21]. In contrast, most high grade serous ovarian cancers present a high expression of mutated p53 and a strong expression of p53 staining [18]. In our study, a heterogeneous nuclear expression of p53 was observed in OCAM cells which discriminates the high grade serous ovarian cancer. However, the heterogeneous character of p53 staining in vitro and in ovo is characteristic of BOTs and LGC. At the moment, the difference between SBOTs and LGSC is based on the invasive character of the tumor [7]. In ovo, OCAM cells have the capacity to grow and to form a tumor on the membrane and to get into the membrane of chicken showing thus a potential of invasion (Figure 4). These different characteristics could suggest the potential ability of SBOT to evolve in LGSC.

Risk factors associated with SBOTs progressing to LGSC may have important implications for the clinician, who may have to evaluate the potential benefit of adjuvant therapy, follow-up and treatment of recurrences. At the moment, most investigators do not prescribe adjuvant therapy for patients with BOTs due to its low response rate.

However, the presence of vessel proliferation around OCAM tumor may potentially change the practice as the use of new anti-angiogenesis target-based therapies as adjuvant settings. Sprouting angiogenesis is the most important mechanism for tumour vascularisation and thus tumour proliferation. The presence of a specific pathway that may be targeted, such as VEGF or its receptors, could open the way for directed therapies. The ability for angiogenesis in this line of BOT would explain why some patients with BOTs and LGSC treated with anti-angiogenic therapy (bevacizumab), with or without chemotherapy, have a survival improvement as compared to chemotherapy alone [22]. Another important issue is the confirmation of the migration of these cells. This capacity puts patients at risk who are considered R0 after surgery, and may have an impact not only in adjuvant therapy, as discussed above, but also in follow-up. To date, no definite follow-up strategies have been established, CA-125 and loco-regional ultrasound being the most used, but this capacity of migration by the tumoral cells may open the need for wider imaging [23].

## Conclusion

Molecular studies have already shed light on the relationship between BOTs and low grade ovarian tumors. Further molecular and cellular studies on human tissue are now needed to establish which patients will evolve from BOTs to low grade tumors, thus better orienting treatment and follow-up.

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