

## Effect of Ovarian Cancer Ascites on Cell Migration and Gene Expression in an Epithelial Ovarian Cancer *In Vitro* Model<sup>1</sup>

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### Abstract

A third of patients with epithelial ovarian cancer (EOC) present ascites. The cellular fraction of ascites often consists of EOC cells, lymphocytes, and mesothelial cells, whereas the acellular fraction contains cytokines and angiogenic factors. Clinically, the presence of ascites correlates with intraperitoneal and retroperitoneal tumor spread. We have used OV-90, a tumorigenic EOC cell line derived from the malignant ascites of a chemonaive ovarian cancer patient, as a model to assess the effect of ascites on migration potential using an *in vitro* wound-healing assay. A recent report of an invasion assay described the effect of ascites on the invasion potential of the OV-90 cell line. Ascites sampled from 31 ovarian cancer patients were tested and compared with either 5% fetal bovine serum or no serum for their nonstimulatory or stimulatory effect on the migration potential of the OV-90 cell line. A supervised analysis of data generated by the Affymetrix HG-U133A GeneChip identified differentially expressed genes from OV-90 cells exposed to ascites that had either a nonstimulatory or a stimulatory effect on migration. Ten genes (*IRS2*, *CTSD*, *NRAS*, *MLXIP*, *HMGCR*, *LAMP1*, *ETS2*, *NID1*, *SMARCD1*, and *CD44*) were upregulated in OV-90 cells exposed to ascites, allowing a nonstimulatory effect on cell migration. These findings were validated by quantitative polymerase chain reaction. In addition, the gene expression of *IRS2* and *MLXIP* each correlated with prognosis when their expression was assessed in an independent set of primary cultures established from ovarian ascites. This study revealed novel candidates that may play a role in ovarian cancer cell migration.

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## Introduction

Ovarian cancer is the fifth cause of cancer-related deaths in woman and the most lethal of all gynecological cancers. Largely asymptomatic, more than 70% of patients with ovarian cancer have already reached an advanced stage of disease at initial diagnosis [1,2], and the overall 5-year survival rate for these patients is less than 30% [3]. Intraperitoneal dissemination is common, and malignant cells can implant anywhere in the peritoneal cavity but are more likely to implant in sites of stasis along the peritoneal fluid circulation [4]. Ascites, a voluminous exudative fluid with a cellular fraction consisting of ovarian cancer cells, lymphocytes, and mesothelial cells, is present in more than one-third of ovarian cancer patients. The acellular fraction is known to harbor cytokines and angiogenic factors [5–9]. Clinically, the presence of ascites correlates with intraperitoneal and retroperitoneal tumor spread, suggesting that it may facilitate metastasis [10].

Previously, we used an *in vitro* invasion assay to monitor the effect of ascites on the potential of chemonaive epithelial ovarian cancer (EOC) cell lines to degrade and migrate across a Matrigel-based barrier [11]. We used the OV-90 cell line to perform our assays. This cell line is derived from an ovarian malignant ascites, is able to form tumors in a xenograft model, and has been extensively characterized at both the cellular and the molecular levels [11–13]. Ascites from more than 50 patients were tested and compared with either 5% fetal bovine serum (FBS) or no serum in an invasion assay. The ascites were classified into one of two categories for their effect on invasion on the basis of comparison to FBS-treated cells: stimulatory ( $\geq 5\%$  FBS activity) or nonstimulatory ( $< 20\%$  of FBS activity). We focused on gene expression profiles generated from the OV-90 cell line [12] treated with ascites possessing either stimulatory or nonstimulatory invasive potential. A supervised analysis of gene expression microarray data sets identified differentially expressed genes, which were validated by quantitative polymerase chain reaction (Q-PCR) assays on the basis of OV-90 cells exposed to a large number of ascites from different patients. In a previous study [11], the proliferation rates and the capacity to form three-dimensional spheroids in hanging drop cultures of the OV-90 cell line treated with different ascites were also described. The results from this previous study strongly supported the notion that ascites affect the cellular and molecular behaviors of ovarian cancer cells.

In the present study, we have further assessed the effect of the same ascites samples on the migration potential of OV-90 cells using an *in vitro* wound-healing assay and extended our analysis to include a larger number of ascites samples derived from ovarian cancer patients. We also assessed differential gene expression using OV-90 cells treated with no serum, with 5% of ascites, or with 5% FBS that correlated with the cellular migration potential of this cell line. Some candidate genes identified in our analysis were further validated using Q-PCR, and their association with survival was tested in an independent set of primary cultures derived from ovarian cancer patients with ascites.

## Materials and Methods

### Cell Culture Conditions, Material, and Patients

OV-90 cells were maintained in ovarian surface epithelium (OSE) complete medium (cat. no. 316-030-CL; Wisent, Quebec, Canada) supplemented with 10% FBS, 2.5  $\mu\text{g}/\text{ml}$  of amphotericin B (Wisent), and 50  $\mu\text{g}/\text{ml}$  of gentamicin (Invitrogen, Ontario, Canada) at 37°C [14]. Ascites were collected at the time of clinical intervention from ovarian cancer patients at the Centre hospitalier de l'Université de

Montréal (Montreal, Quebec, Canada). Informed consent for this study was obtained from all of the patients. After centrifuging at 1250g for 5 minutes, the acellular fractions of ascites were stored at  $-20^{\circ}\text{C}$  and tested within 6 months of collection. Histopathological diagnosis, grade, and stage of ovarian tumor samples were assigned according to the criteria of the International Federation of Gynecology and Obstetrics. Of the 31 ascites samples, one third were from patients diagnosed with papillary serous adenocarcinoma and most presented as stage IIIC grade 3 tumor diseases (Table 1). A small proportion (4/31) of patients had already received chemotherapy before ascites collection. The cohort of patients with ovarian cancer and the accompanying ascites used for this research and included in the survival analysis have been described previously [11].

### In Vitro Migration Assays

Cellular migration was assayed by determining the ability of cells to migrate in a culture plate using a wound-healing assay. To evaluate migration assays, OV-90 cells were plated in 12-well dishes and were grown at 37°C until confluent. Cell monolayers were scraped using a sterile 200- $\mu\text{l}$  yellow plastic tip to produce small wounds of similar size. Wounded monolayers were then washed with phosphate-buffered saline to remove cell debris, and OSE medium was added with no serum or with either 5% FBS or 5% ascites. For inactivation assays, ascites were heated for 10 minutes at 100°C to denature the proteins. Cells were incubated at 37°C under 5%  $\text{CO}_2$  for different lengths of time to evaluate their migration (0, 6, 24, 30, 48, and 54 hours after scratch formation). At the different time points, cells were methanol-fixed and treated with Giemsa stain (Sigma-Aldrich, Inc, St Louis, MO). Digital images were obtained at each time point of the experiment. Images were analyzed, and wound closures were quantified using Image Pro Plus software (Version 5.1; MediaCybernetics, Bethesda, MD) and Microsoft Excel. All experiments were performed twice using triplicate samples and were normalized to FBS-treated cultures.

### RNA Preparation

Total RNA was extracted using TRIzol reagent (Gibco/BRL, Life Technologies, Inc, Grand Island, NY) according to the manufacturer's protocol. RNA was extracted from OV-90 cells grown to 80% confluence in 100-mm Petri dishes. The quality of RNA was assessed using a 2100 Bioanalyzer with the RNA 6000 Nano LabChip kit (Agilent Technologies, Mississauga, Ontario, Canada) according to the manufacturer's protocol.

Microarray hybridization experiments were performed at McGill University and the Genome Quebec Innovation Center (Montreal, Quebec, Canada) using the HG-U133A GeneChip arrays. This chip allows the analysis of approximately 18,400 transcripts and variants, including 14,500 well-characterized human genes, composed of more than 22,000 probe sets. Protocols are available at the Affymetrix Web site ([www.affymetrix.com](http://www.affymetrix.com); Affymetrix, Santa Clara, CA). Methods for labeling and hybridization of RNA were previously described [11].

### Gene Expression Statistical Analysis

Gene expression profiles were analyzed using R (version 2.4.0; [www.r-project.org](http://www.r-project.org)), a statistical programming language, Bioconductor [15], and an open-source software library for the analyses of genomic data, which is based on R. Background subtraction, normalization (quantile normalization), and expression value calculations were performed using the justGCRma function available as part of the Bioconductor's gcrma package. Bioconductor's gene filter package was used to

**Table 1.** Compiled Clinical Characteristics of Patients from Which Ascites Were Obtained.

Ascites	Age (years)	Histopathological Diagnosis	Grade	Stage	Neoplastic Cells in Ascites	Previous Chemotherapy	Ascites Collected
A3331	73	AC	N/S	IIIC	N/S	N/S	N/S
A3312	62	MCT	0	N/S	Yes	N/S	Primary surgery
A3294	38	PSA	3	IIIC	Yes	No	Primary surgery
A3258	58	MMT	3	IIIC	Yes	No	Primary surgery
A3203	77	PSA	3	IV	Yes	No	Primary surgery
A3133	52	PSA	3	IIIC	Yes	No	Secondary cytoreduction
A2965	70	MCA	3	IIIC	Yes	No	Primary surgery
A2912	53	PSA	3	IIIC	Yes	No	Primary surgery
A2910	65	OT	N/S	N/S	No	N/S	Primary surgery
A2891	50	MCA	3	IC	N/S	No	Primary surgery
A2839	54	SA	3	IV	N/S	No	Primary surgery
A2834	62	PSA	3	IIIC	Yes	No	Primary surgery
A2775	48	PSA	2	IIIC	Yes	No	Primary surgery
A2774	41	EA	3	IB	N/S	No	Primary surgery
A2685	61	PSC	N/S	N/S	No	N/S	Primary surgery
A2652	49	MCT	0	N/S	N/S	N/S	Primary surgery
A2647	68	SA	3	III	Yes	N/S	Primary surgery
A2635	50	MC	N/S	N/S	N/S	N/S	Primary surgery
A2473	71	MpA	2	IIIC	Yes	No	Primary surgery
A2433	50	MCT	0	IA	No	N/S	Primary surgery
A2427	70	AF	N/S	IB	N/S	N/S	Primary surgery
A2295	59	SA	3	IIIC	Yes	No	Primary surgery
A2295(2)*	59	SA	3	IIIC	Yes	Yes	Secondary cytoreduction
A2090 <sup>†</sup>	76	UA	N/S	IIIC	N/S	Yes	N/S
A2085 <sup>†</sup>	65	PSA	3	IIIC	N/S	Yes	Secondary cytoreduction
A1946 <sup>†</sup>	75	PSA	3	IIIC	Yes	No	Primary surgery
A1835 <sup>†</sup>	69	PSA	3	IIIC	Yes	No	Primary surgery
A1592 <sup>†</sup>	35	MCA	3	IIIC	N/S	No	Primary surgery
A1337 <sup>†</sup>	45	PSA	3	IIIC	Yes	No	Primary surgery
A1322 <sup>†</sup>	71	PSA	3	IIIC	Yes	No	Primary surgery
A1317 <sup>†</sup>	60	PSA	3	IV	No	Yes	Primary surgery

AC indicates adenocarcinoma; AF, adenofibroma; EA, endometrioid adenocarcinoma; MC, mucinous cystadenoma; MCA, mixed cell adenocarcinoma; MCT, mucinous cystic tumor in extreme cases of the malignity; MMT, mixed mullerian tumor; MpA, mucipare adenocarcinoma; MsA, mucinous adenocarcinoma; N/S, not specified; OT, ovarian torsion; PSA, papillary serous adenocarcinoma; PSC, papillary serous cystadenoma; SA, serous adenocarcinoma; SC, serous cystadenoma; UA, undifferentiated adenocarcinoma.

\*Denotes second ascites collected from patient with the same number.

<sup>†</sup>Results previously reported in Puiffe et al. [11].

filter genes with insufficient variation in expression across all samples tested. Expression values retained after this filtering process had intensities greater than 100 units in at least two samples and a log base 2 scale of at least 0.2 for the interquartile range across all tested samples. Differentially expressed genes were identified using Bioconductor's limma package that implements moderate *t* tests by fitting a linear model for each group of samples and using empirical Bayes method to moderate SEs of the estimated log-fold changes between the predefined groups.

Kaplan-Meier survival plots, univariate Cox proportional hazard regressions, as well as log-rank tests were performed to determine the significance of using gene expression levels to predict survival of EOC patients as described earlier [11]. The expression threshold cutoff was determined by survival tree using the recursive partitioning and regression tree (RPART) method [16]. The survival analysis and tree building were performed using R's survival and RPART packages, respectively. The Pearson correlation coefficient test (two-tailed) was used to calculate the correlation between gene expression and migration rate and was performed with SPSS software 11.0 (SPSS, Inc, Chicago, IL).

### Quantitative Reverse Transcription-PCR Validation

The complementary DNA synthesis was prepared using the QuantiTech Reverse Transcription for two-step reverse transcription-PCR (Qiagen, Inc, Mississauga, Ontario, Canada) according to the manufacturer's instruction. First-strand synthesis for reverse transcription-PCR was performed with 1  $\mu$ g of RNA and a mix of Oligo dT and random hexamers. Samples were diluted 1:10 in water before Q-PCR.

Q-PCR was performed using Rotor-gene 3000 (Corbett Research, Montreal Biotech, Inc, Montreal, Quebec, Canada). The Quantitect SYBR Green PCR (Qiagen, Inc) reaction mixture was used to label 5  $\mu$ l of sample complementary DNA and 10 pg of the different primers in a final volume of 25  $\mu$ l. Reactions were performed at least twice according to the manufacturer's instructions. Serial dilutions were performed to generate a standard curve for each gene tested to define the efficiency of the Q-PCR, and a melt curve was constructed to confirm reaction specificity. The analytical method of Pfaffl [17] was used to measure the relative quantity of gene expression. The first sample (with 5% FBS) served as the reference sample in each experiment. Gene expression was evaluated in the OV-90 cell line with no serum or with either 5% FBS or 5% ascites, under the same conditions used to evaluate the migration potential of the OV-90 cell line.  $\beta$ -Actin was used as reference gene on the basis of its stable expression in all samples by microarray analysis. For every marker, a Pearson correlation was calculated between the scored migration result (1 < 100% and 2  $\geq$  100% of migration) and the scored gene expression (1 < median and 2  $\geq$  median). All experiments were performed in duplicate.

## Results

### Effect of Ascites on OV-90 Migration Potential

Ascites from 31 EOC patients were studied to determine their effect on OV-90 cell migration in an *in vitro* scratch assay (Table 1 and Figure 1). Cells were grown in a monolayer until confluence in a

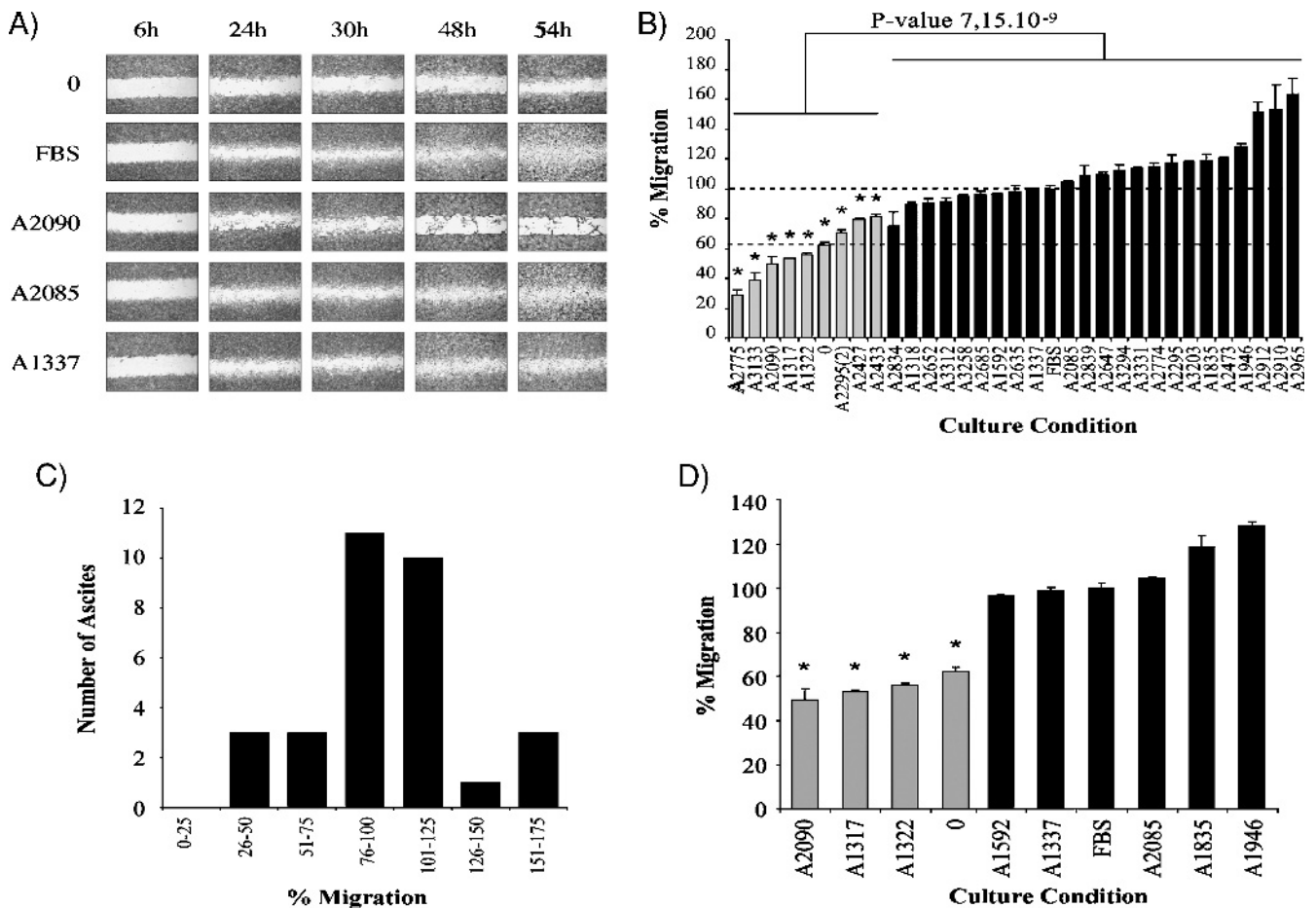
12-well plate. After scratching the monolayer with a pipet tip, cells were maintained in medium with or without 5% FBS. To test the effect of ascites, FBS was replaced by 5% ascites (acellular fraction) from EOC patients and sampled after 0, 6, 24, 30, 48, and 54 hours of incubation. OV-90 cells are able to seal the wound in 48 hours, similar to the average seen in comparable EOC cell lines [18]. The influence of each ascites sample on cell migration was scored in comparison to medium supplemented with 5% FBS (Figure 1). In OV-90 cells, a difference in cell migration was observed between cells in contact with medium alone and cells in medium supplemented with 5% FBS. When cells were incubated with the medium without FBS (Figure 1B), cell migration was reduced by 40% in comparison to 5% FBS. In contrast, a large number of ascites samples ( $n = 23$ ) stimulated OV-90 cell migration at levels similar to 5% FBS (Figure 1B). A smaller number of ascites samples ( $n = 8$ ) did not stimulate cell migration when compared with migration in the presence of FBS (Figure 1B).

To determine whether the migration effect of ascites is protein component-dependant, two stimulatory and two nonstimulatory ascites were selected and heated for 10 minutes at 100°C before adding to the OV-90 cell cultures (Figure 2). The results suggest that protein

inactivation abolished the stimulatory effect (FBS, A2647 and A2839). For the two nonstimulatory ascites, samples A2295(2) and A2090, we also observed a decreased effect on cell migration from ascites that had been heat-treated. The pH of FBS and the four ascites was not altered by heating (data not shown).

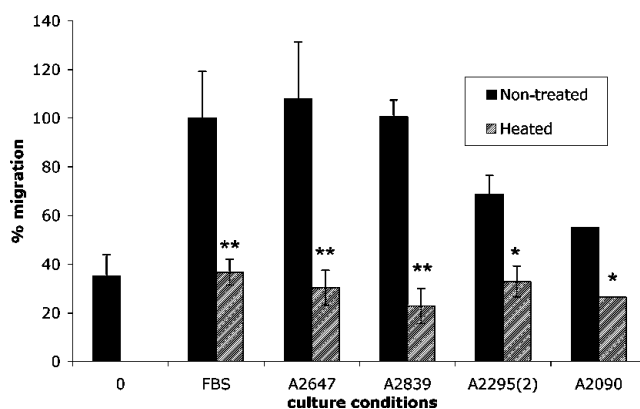
**Effect of Ascites on Gene Expression in OV-90 Cells**

To identify potential molecular players in migration regulated by ascites, gene profiling analysis was performed. Total RNA was extracted from OV-90 cells after a 24-hour exposure to no serum, 5% FBS, or 5% of one of eight ascites sampled from ovarian cancer patients as previously described (Table 1) [11]. The RNA samples were each hybridized on Affymetrix HG-U133A GeneChip arrays, and gene expression profiles were analyzed using 6489 probe sets (see Materials and Methods). A supervised analysis was performed using expression data sets representing the following groups. The GSTIMUL group contained the ascites (A1946, A1835, A2085, A1337, and A1592) that stimulated cell migration and included the 5% FBS control. The GnSTIMUL group contained ascites (A1322, A1317, and A2090) that demonstrates less cell migration and included the no-FBS sample. This supervised analysis identified 129 genes that



**Figure 1.** Effect of ascites on OV-90 cell migration. Migration was assessed by determining the ability of cells to migrate in a culture plate using a wound-healing assay in the presence of ascites compared with 5% FBS (% migration) after 54 hours of incubation. The symbol "0" indicates medium without ascites or FBS. (A) Effect of FBS and ascites on OV-90 cell migration. (B) Migration profile of OV-90 with OSE medium in the presence or absence of 5% FBS or with 5% of several ascites. (C) Effect of 31 ascites on OV-90 cell migration potential. (D) Migration profile of OV-90 with OSE medium in the presence or absence of 5% FBS or with 5% of ascites. \*Statistical significance,  $P < .05$ .





**Figure 2.** Effect of heat-treated ascites in OV-90 cell migration assays *in vitro*. Both stimulatory (A2647 and A2839) and nonstimulatory (A2295(2) and A2090) ascites were heated at 100°C for 10 minutes to inactivate the proteins before adding to OV-90 cell culture media. The effect on cell migration was subsequently evaluated. \* $P < .05$ , \*\* $P < .01$ .

were differentially expressed ( $P < .05$ ,  $t$  test) between the GSTIMUL and GnSTIMUL groups (Table 2).

#### Differential Expression Validation of Selected Candidates by Q-PCR

Forty gene candidates involved in the migration potential of OV-90 cells were selected for further validation by Q-PCR on the basis of their  $P$  values obtained in the previous microarray analysis and also on their gene functions. The candidates tested by Q-PCR are described in Table 2. Of the 40 selected genes, 21 were downregulated in the GnSTIMUL group (*HIST1H2AC*, *F1P1L1*, *RBM10*, *H2BS*, *BTG3*, *CNTNAP2*, *IGF1R*, *DKC1*, *HIST1H2BD*, *CDCA4*, *MDC1*, *SMARCD3*, *TSTA3*, *SMARCD2*, *PDPK1*, *ADAMTS1*, *ASH2L*, *SMARCA4/BRG1*, *CMKOR1*, *ANAPC5*, and *GPR125*) and 19 were upregulated in the GnSTIMUL group (*HSPA1B*, *CALM3*, *IRS2*, *CBFB*, *CEBPA*, *LIPG*, *CRLF1*, *MDK*, *CTSD*, *NRAS*, *MLXIP*, *HMGCR*, *CALM1*, *LAMP1*, *MKRN1*, *ETS2*, *NID1*, *SMARCD1*, and *CD44*). Q-PCR was performed on RNA derived from OV-90 cells exposed individually to the entire panel of 31 ascites (Table 1). The relative expression ratio of each candidate, based on the Pfaffl method, was quantified, and for each experiment, the median ratio was calculated, and the result was categorized as above or below the median expression. Pearson correlations were calculated for the migratory effect (stimulatory or nonstimulatory) of each ascites with a gene expression score, as shown in Table 3. Of the 40 candidates, 10 (*IRS2*, *CTSD*, *NRAS*, *MLXIP*, *HMGCR*, *LAMP1*, *ETS2*, *NID1*, *SMARCD1*, and *CD44*) tested by Q-PCR correlated significantly with the ascites migration effect. Three candidates (*MDC1*, *SMARCA4*, and *GPR125*) correlated significantly, but their downregulated expression pattern by Q-PCR was in the opposite direction expected from the microarray expression analysis.

#### Survival

In a previous study [11], we showed that the genes exhibiting a significant correlation with ascites invasion effect could be good prognosis predictors. The prognostic potential of the candidate genes that Q-PCR expression correlated significantly with the ascites migration effect was evaluated using microarray expression in 28 primary cultures derived from the cellular fraction of ascites of ovarian cancer patients.

A survival tree was used to determine the expression cutoff that could lead to the identification of prognostic groups among patients. In the case of *IRS2* and *MLXIP*, Kaplan-Meier curves coupled with a log-rank test identified a patient group with an overall good survival rate associated with a high expression (Figure 3). A trend toward significance was observed for the *HMGCR* candidate, although its association between gene expression and patient survival was not significant ( $P = .061$ ; Figure 3).

#### Discussion

EOC is the second most common gynecological cancer and accounts for more than half of the deaths associated with gynecological pelvic malignancies [19,20]. This gynecological malignancy is associated with vague symptoms, which results in a diagnosis at a late stage [2]. The need for reliable biomarkers in ovarian cancer detection is increasing because EOC mortality has not significantly decreased during the past years because of a poor understanding of the biology [4]. Although ascites are commonly found in patients with EOC, its association with a poor prognostic factor is not universally accepted, and mechanisms that lead to ascites formation are not well characterized in EOC [10,21,22]. The presence of ascites correlates with intraperitoneal and retroperitoneal tumor spread, which supports a role in metastasis [2,10]. It is also known that ascites contain factors that increase vascular permeability [5]. In this study, we assessed the effects of ascites on OV-90 cell migration and correlated this effect with the alteration of gene expression that occurred in the same cell line as a consequence of exposure to several heterologous ascites obtained in different clinical settings (Table 1) [12].

In control tests, the presence of FBS in culture medium stimulated the cellular migration of the OV-90 cell line. An analysis of the migration behavior of OV-90 with 31 different ascites showed that more than two-thirds of the ascites samples stimulated cell migration in a fashion similar to the serum control. In contrast, one-third of the ascites did not stimulate migration in a fashion similar to the non-FBS control. Of the ascites samples collected from the same patient (A2295 and A2295(2), respectively) but at different times during the course of her treatment, it is interesting that the prechemotherapy ascites stimulated cell migration, whereas the postchemotherapy ascites inhibited cell migration. This observation raises the intriguing possibility that chemotherapy treatment is associated with a diminution of tumor cell migratory potential but requires validation with a larger sample set.

In a previous study, we determined how ascites affected the invasive capacity of OV-90 cells [11]. Because several of the ascites tested in that study were also tested in the present study, we looked for correlations between the migration and invasion results. Although some ascites were able to increase both the migratory and the invasive properties of OV-90 cells, no correlation was observed between ascites stimulating migration and invasion (data not shown), suggesting that these two events are activated by different stimuli. Both the stimulatory and nonstimulatory effects of selected ascites and FBS were lost when the samples were heated. With this treatment, the results were similar to those observed with the medium lacking FBS, suggesting that the effects of FBS and both stimulatory and nonstimulatory ascites were due to the presence of a protein or a protein component rather than other soluble factors.

Gene expression was assessed to link molecular events with the migration effect of ascites. For this purpose, RNA from OV-90 cells exposed to five stimulatory ascites or 5% FBS (GSTIMUL) were compared with RNA from cells in the absence of serum or treated

**Table 2.** List of Differentially Expressed Genes among the GSTIMUL and GnSTIMUL Groups That Were Tested in Q-PCR.

P	Gene Expression Level in the GnSTIMUL*	Probe Set HG-U133A	UniGene	Description	Symbol	Molecular Function (Gene Oncology)	Cytoband
.004	Down	215071_s_at	Hs,484950	Histone 1, H2ac	<i>HIST1H2AC</i>	DNA binding	6p21,3
.004	Down	221007_s_at	Hs,518760	FIP1-like 1 ( <i>Saccharomyces cerevisiae</i> )	<i>FIP1L1</i>	RNA binding	4q12
.018	Down	208984_x_at	Hs,401509	RNA binding motif protein 10	<i>RBM10</i>	RNA/metal ion binding	Xp11,23
.020	Down	208579_x_at	Hs,473961	H2B histone family, member S	<i>H2BFS</i>	DNA binding	21q22,3
.027	Down	213134_x_at	Hs,473420	BTG family, member 3	<i>BTG3</i>	Not available	21q21,1-q21,2
.028	Down	219300_s_at	Hs,190621	Contactin-associated protein-like 2	<i>CNTNAP2</i>	Protein binding	7q35-q36
.029	Down	203628_at	Hs,592020	Insulin-like growth factor 1 receptor	<i>IGF1R</i>	Nucleotide/protein binding, receptor activity, ATP binding	15q26,3
.030	Down	201478_s_at	Hs,4747	Dyskeratosis congenita 1, dyskerin	<i>DKC1</i>	Telomerase activity/RNA binding	Xq28
.032	Down	209911_x_at	Hs,591797	Histone 1, H2bd	<i>HIST1H2BD</i>	DNA binding	6p21,3
.032	Down	218399_s_at	Hs,34045	Cell division cycle-associated 4	<i>CDCA4</i>	Not available	14q32,33
.035	Down	203062_s_at	Hs,632002	Mediator of DNA damage checkpoint 1	<i>MDC1</i>	Protein binding	6pter-p21,31
.036	Down	204099_at	Hs,438823	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily D, member 3	<i>SMARCD3</i>	Transcription activity, receptor binding	7q35-q36
.036	Down	36936_at	Hs,404119	Tissue-specific transplantation antigen P35B	<i>TSTA3</i>	Catalytic activity, isomerase activity	8q24,3
.040	Down	201827_at	Hs,250581	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily D, member 2	<i>SMARCD2</i>	Transcription coactivator activity, protein binding	17q23-q24
.040	Down	204524_at	Hs,459691	3-Phosphoinositide-dependent protein kinase-1	<i>PDPK1</i>	Nucleotide/Protein binding, protein kinase activity	16p13,3
.040	Down	222162_s_at	Hs,534115	ADAM metallopeptidase with thrombospondin type 1 motif, 1	<i>ADAMTS1</i>	Peptidase activity, integrin binding	21q21,2
.044	Down	209517_s_at	Hs,521530	ash2 (absent, small, or homeotic)-like ( <i>Drosophila</i> )	<i>ASH2L</i>	DNA/metal ion/promoter/protein binding, histone methyltransferase activity, transcription regular activity	8p11,2
.046	Down	212520_s_at	Hs,327527	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily A, member 4	<i>SMARCA4</i>	DNA/Protein binding, transcription factor activity	19p13,2
.047	Down	212977_at	Hs,471751	Chemokine orphan receptor 1	<i>CMKOR1</i>	Signal transducer activity, receptor activity	2q37,3
.048	Down	211036_x_at	Hs,7101	Anaphase-promoting complex subunit 5	<i>ANAPC5</i>	Ubiquitin-protein ligase activity, binding	12q24,31
.050	Down	210473_s_at	Hs,99195	G protein-coupled receptor 125	<i>GPRI25</i>	Actin/protein binding	4p15,31
.002	Up	202581_at	Hs,274402	Heat shock 70-kDa protein 1B	<i>HSPA1B</i>	Nucleotide binding, ATP binding	6p21,3
.019	Up	200622_x_at	Hs,515487	Calmodulin 3 (phosphorylase kinase, delta)	<i>CALM3</i>	Calcium ion binding, protein binding	19q13,2-q13,3
.019	Up	209185_s_at	Hs,442344	Insulin receptor substrate 2	<i>IRS2</i>	Signal transducer activity, receptor activity, protein binding, cell proliferation	13q34
.021	Up	206788_s_at	Hs,460988	Core-binding factor, beta subunit	<i>CBFB</i>	Transcription factor activity, protein binding	16q22,1
.026	Up	204039_at	Hs,643434	CCAAT/enhancer binding protein (C/EBP), alpha	<i>CEBPA</i>	DNA binding, transcription factor activity, RNA pol II transcription factor	19q13,1
.027	Up	219181_at	Hs,465102	Lipase, endothelial	<i>LIPG</i>	Catalytic activity, protein binding	18q21,1
.028	Up	202046_s_at	Hs,509447	Glucocorticoid receptor DNA binding factor 1	<i>GRLF1</i>	Nucleotide binding, DNA binding, receptor activity, GTP binding	19q13,3
.038	Up	209035_at	Hs,82045	Midkine (neurite growth-promoting factor 2)	<i>MDK</i>	Cytokine activity, growth factor binding	11p11,2
.039	Up	200766_at	Hs,121575	Cathepsin D (lysosomal aspartyl peptidase)	<i>CTSD</i>	Peptidase/hydrolase activity	11p15,5
.042	Up	202647_s_at	Hs,486502	Neuroblastoma RAS viral (v-ras) oncogene homolog	<i>NRAS</i>	Nucleotide/GTP binding, GTPase activity	1p13,2
.043	Up	202519_at	Hs,437153	MLX-interacting protein	<i>MLXIP</i>	DNA binding, transcription regulator activity	12q24,31
.045	Up	202540_s_a	Hs,643495	3-Hydroxy-3-methylglutaryl-coenzyme A reductase	<i>HMGCR</i>	Oxidoreductase activity, NADP binding	5q13,3-q14
.045	Up	211985_s_at	Hs,282410	Calmodulin 1 (phosphorylase kinase, delta)	<i>CALM1</i>	Calcium ion binding, protein binding	14q24-q31
.046	Up	201552_at	Hs,494419	Lysosomal-associated membrane protein 1	<i>LAMP1</i>	Not available	13q34
.046	Up	201285_at	Hs,490347	Makorin, ring finger protein, 1	<i>MKRN1</i>	Chromatin binding, ligase activity, metal ion binding, nucleic acid/protein binding	7q34
.048	Up	201329_s_at	Hs,517296	v-ets Erythroblastosis virus E26 oncogene homolog 2 (avian)	<i>ETS2</i>	DNA binding, transcription factor activity	21q22,3
.048	Up	202008_s_at	Hs,356624	Nidogen 1	<i>NID1</i>	Calcium ion binding, protein binding, extracellular matrix structural constituent	1q43
.048	Up	203183_s_at	Hs,79335	SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily D, member 1	<i>SMARCD1</i>	Transcription coactivator activity	12q13-q14
.049	Up	210916_s_at	Hs,502328	CD44 molecule (Indian blood group)	<i>CD44</i>	Receptor activity, protein binding	11p13

The GSTIMUL group consists of OV-90 samples with 5% FBS or with 5% of five ascites that stimulated cell migration (A1337, A1592, A1835, A1946, and A2085). The GnSTIMUL group consists of OV-90 samples with no FBS or with 5% of the three ascites that did not stimulate cell migration (A1317, A1322, and A2090).

\*All upward or downward changes observed in the gene expression levels were determined by microarray data using the Affymetrix HG-U133A GeneChip array and subsequently confirmed by Q-PCR. Up refers to the gene expression level being higher in the GnSTIMUL group than in the GSTIMUL. Down refers to the gene expression level being lower in the GnSTIMUL group than in the GSTIMUL.

with three nonstimulatory ascites (GnSTIMUL). Microarray analysis identified 129 genes differentially expressed between the group of ascites that stimulated cell migration and the group that did not stimulate cell migration. Among those genes, 40 were tested in Q-PCR. Pearson correlations were calculated for the migratory effect (stimulatory or non-stimulatory) of each ascites with a gene expression score, as shown in Table 3. Among these 40 genes, differential expression of 13 candidates was confirmed by Q-PCR in a larger set of 31 ascites samples (Table 3). Three candidates correlated significantly, but the expression pattern was opposite to that expected by microarray analysis. Of 27 additional candidates tested by Q-PCR, no significant correlation between gene expression and OV-90 cell migration could be established. The fact that the differential expression of these genes was identified by microarray analysis on a limited number of ascites samples (only eight), although the validation was tested on 31 different ascites, could explain this poor validation rate. These results also suggest that an extensive validation in a supervised analysis by Q-PCR of gene candidates is essential to uncover the richness of genes implicated in migration process.

Many genes differentially expressed in cells stimulated by the two groups of ascites are closely related to the mitogen-activated protein

kinase (MAPK) pathway and apoptosis. A similar association between MAPK-related genes and invasion was also observed in our recent study [11], although different gene candidates were identified. In the present study, the MAPK pathway-related genes include *NRAS*, *ETS2*, *Cathepsin D (CSTD)*, and *HMGCR*. Interestingly, recent studies suggest that *Cathepsin D* could be responsible for the positive regulation of proliferation, survival, motility, and invasion of fibroblasts by triggering activation of ras/MAPK/Rds [23]. The Ras proteins were some of the first proteins identified involving the regulation of cell growth [24]. Nearly 30% of human cancers are associated with mutations in the Ras genes [25]. In ovarian cancer research, Ahmed et al. [26] showed that ascites enhanced the activation of Ras by increasing Ras-GTP levels in the study of four ovarian cancer cell lines. This study also presented evidence that activation of Ras and downstream Erk pathway is involved in maintaining growth, adhesion, and invasiveness of cancer cells. It was also recently shown that a high expression of HMG-CoA reductase (HMGCR) induced acceleration of the cholesterol synthesis pathway in cancer cells, and this may have promoted Ras isoprenylation, a posttranslational modification activating Ras [27]. In addition, the Ras-MAP kinase signaling pathway leads to the phosphorylation of ETS transcription

**Table 3.** Correlation between Migration and Gene Expression.

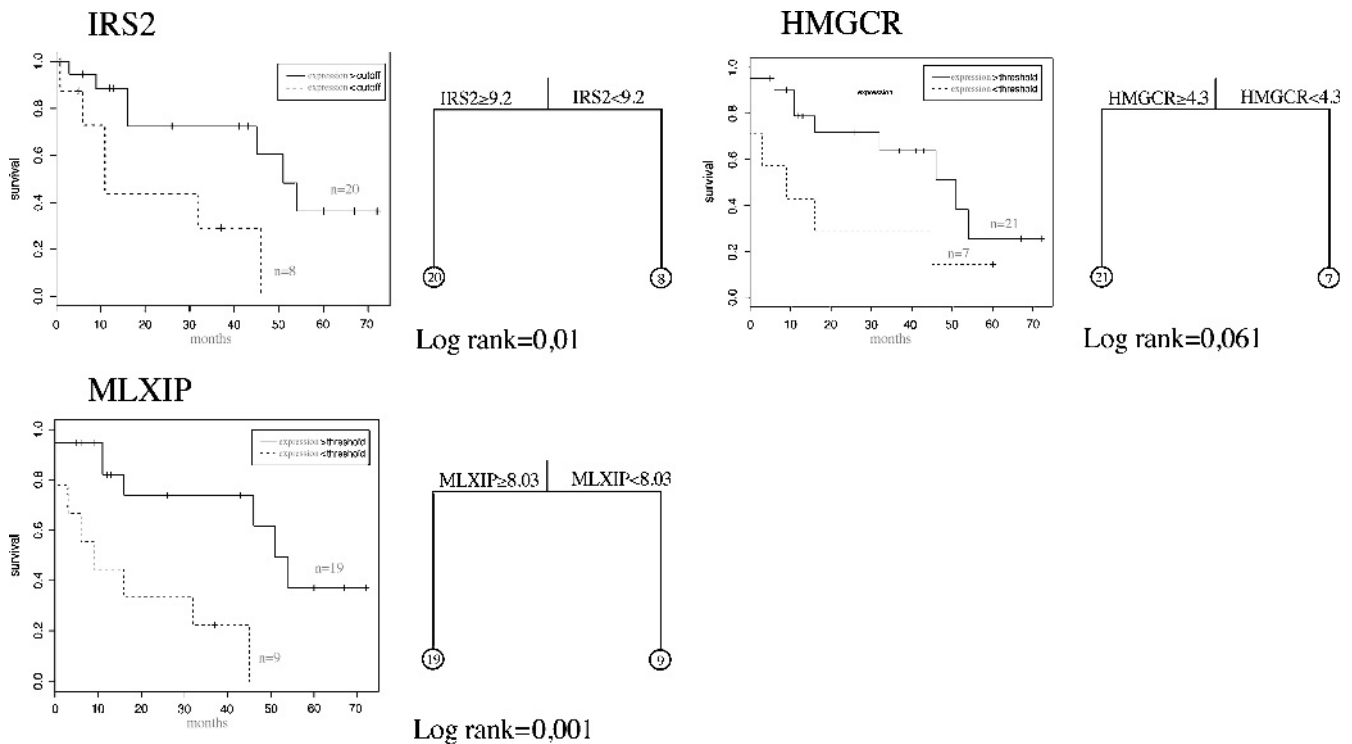
Genes	Median of Ratio in the GSTIMUL*	Median of Ratio in the GnSTIMUL*	Pearson Correlation	P
<i>HIST1H2Ac</i>	1.14	1.37	-0.160	.389
<i>FIP1L1</i>	0.36	0.54	-0.160	.389
<i>RBM10</i>	0.41	0.47	-0.160	.389
<i>H2BFS</i>	0.81	0.81	-0.022	.905
<i>BTG3</i>	0.80	0.71	-0.160	.389
<i>CNTNAP2</i>	0.85	1.01	-0.298	.103
<i>IGF1R</i>	0.52	0.67	-0.298	.103
<i>DKC1</i>	0.60	0.61	-0.298	.103
<i>hist1h2bd</i>	0.79	1.12	-0.298	.103
<i>CDCA4</i>	1.08	1.38	0.116	.535
<i>MDC1</i>	1.02	1.38	-0.437 <sup>†</sup>	.014
<i>SMARCD3</i>	0.68	0.86	-0.254	.168
<i>TSTA3</i>	0.66	0.55	0.116	.535
<i>SMARCD2</i>	0.98	1.14	-0.160	.389
<i>PDPK1</i>	0.41	0.61	-0.160	.389
<i>ADAMTS1</i>	0.36	0.48	-0.160	.389
<i>ASH2L</i>	0.89	1.01	-0.298	.103
<i>SMARCA4</i>	0.68	0.76	-0.437 <sup>†</sup>	.014
<i>CMKOR1</i>	0.41	0.52	-0.160	.389
<i>ANAPC5</i>	0.58	0.76	-0.160	.389
<i>GPR125</i>	1.00	0.80	-0.437 <sup>†</sup>	.014
<i>HSPA1B</i>	1.00	1.49	-0.160	.389
<i>CALM3</i>	1.14	1.53	-0.254	.168
<i>IRS2</i>	1.60	2.49	-0.437 <sup>†</sup>	.014
<i>CBFB</i>	0.74	0.83	-0.160	.389
<i>CEBPA</i>	0.58	0.58	0.254	.168
<i>LIPG</i>	0.37	0.66	0.116	.535
<i>GRLF1</i>	0.80	0.84	-0.160	.389
<i>MDK</i>	0.52	0.82	-0.298	.103
<i>CTSD</i>	1	1.64	-0.437 <sup>†</sup>	.014
<i>NRAS</i>	0.74	1.04	-0.437 <sup>†</sup>	.014
<i>MLXIP</i>	0.53	0.84	-0.437 <sup>†</sup>	.014
<i>HMGCR</i>	0.59	1.25	-0.392 <sup>‡</sup>	.029
<i>CALM1</i>	1.14	1.53	-0.298	.103
<i>LAMP1</i>	1.00	1.57	-0.392 <sup>‡</sup>	.029
<i>MKRN1</i>	1.00	1.16	-0.116	.535
<i>ETS2</i>	0.47	0.83	-0.575 <sup>‡</sup>	.001
<i>NID1</i>	0.56	0.72	-0.437 <sup>†</sup>	.014
<i>SMARCD1</i>	0.99	1.22	-0.575 <sup>‡</sup>	.001
<i>CD44</i>	0.39	0.58	-0.392 <sup>‡</sup>	.029

Pearson correlations were calculated between scored migration results (1 < 100% of migration and 2 ≥ 100% of migration) and scored genes expression (1 < median and 2 ≥ median) for the 40 candidates quantified by Q-PCR.

\*Gene expression ratio relative to OV-90 stimulated with FBS.

<sup>†</sup>Correlation is significant,  $P = .05$ .

<sup>‡</sup>Correlation is significant,  $P = .01$ .



**Figure 3.** Relationship between *IRS2* and *MLXIP* expression and cumulative survival of patients with ovarian cancer in the context of concomitant ascites. The cutoffs were determined by survival tree. Kaplan-Meier graphical representation of the survival curves illustrates the survival associated with candidate gene expression levels (log-rank).

factors, including Ets-2, which plays an important role in the regulation of growth and cell cycle-related genes [28] and protects cells from apoptosis [29].

The other candidate genes identified in this study have been linked to tumor growth and apoptosis. For example, *CD44*, a gene upregulated in the group of nonstimulatory ascites, mediates the interaction between ovarian carcinoma cells and the mesothelial cells lining abdominal organs [30]. Through strong affinity binding to the extracellular matrix in ovarian carcinoma cells, *CD44* has been shown to affect cell adhesion [30] and migration [31] as well as to increase tumor growth [32]. In addition, increased *CD44* expression is associated with an increased expression of Bcl-2, an antiapoptotic factor [33]. Mammary tumor cells that are deficient in *Irs-2*, another candidate, have been shown to be significantly more sensitive to apoptotic stimuli such as serum deprivation [29]. Conversely, *BRG1/SMARCA4*, a member of the SWI/SNF complex, which is down-regulated in OV-90 cells treated with stimulatory ascites, is required for the growth arrest induction and cell senescence induced by p21 [34,35]. These results show that gene candidates that decrease the migratory potential of OV-90 cells are also involved in growth promotion and apoptotic protection of tumor cells, whereas genes up-regulated by migratory ascites are involved in cell growth arrest. Taken together, this suggests that cell migration and growth may be sequential events during tumor progression where cells need to stop growth to be able to start migrating. Further experiments will be needed to confirm this hypothesis. In line with this is the fact that most gene candidates alone are not associated with survival of patients, which also means that the migratory potential of tumor cell is necessary but insufficient *per se* to affect the aggressive behavior of tumor cells.

In summary, this study provides evidence for some novel gene candidates and molecular pathways that may play an important role in ovarian cancer cell migration. Combined with our previous research [11], this work continues to define the importance of studying the effect of both ascites and the tumor environment on ovarian cancer cells. Our data also suggest that ascites may contain either positive or negative regulators of tumor behaviors and can play a role in future clinical outcomes. Future studies assessing the relative expression of these candidates in clinical specimens will no doubt help to better refine those that are important in ovarian cancer progression.

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