

# EMT-associated factors promote invasive properties of uveal melanoma cells

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**Purpose:** Transcription factors regulating the epithelial-to-mesenchymal transition (EMT) program contribute to carcinogenesis and metastasis in many tumors, including cutaneous melanoma. However, little is known about the role of EMT factors in the growth and metastatic dissemination of uveal melanoma cells. Here, we analyzed the expression and functions of the EMT factors ZEB1, Twist1, and Snail1 in uveal melanoma cell lines and primary tumors.

**Methods:** ZEB1, Twist1, and Snail1 mRNA levels were measured using qPCR in five uveal melanoma cell lines and in 30 primary tumors. Gene expression was used to determine class 1 and class 2 signatures in the primary tumors. Short hairpin RNA was used to downregulate the expressions of the EMT factors; then, growth and transwell invasion assays were performed.

**Results:** ZEB1, Twist1, and Snail1 were expressed in all five uveal melanoma lines, with ZEB1 having the highest protein levels. ZEB1 mRNA was significantly elevated in highly metastatic class 2 primary tumors for which survival data were not available, whereas a high gene expression of Twist1 was associated with a worse prognosis in a separate tumor cohort analyzed by expression profiling. The genetic downregulation of ZEB1 in OCM1, OMM1, and 92.1 resulted in a more than 50% reduction in invasion, but only suppressed growth in OMM1 cells. Suppression of Twist1 in Mel290 and OMM1 reduced growth and invasion by more than 50%. The downregulation of Snail1 in the 92.1 cell line reduced invasion by 50%, but did not interfere with growth.

**Conclusions:** The downregulation of ZEB1, Twist1, and Snail1 reduces the invasive properties of uveal melanoma cells, and the elevated mRNA levels of ZEB1 and Twist1 are associated with a more aggressive clinical phenotype in uveal melanoma samples. Therefore, these factors could represent new therapeutic targets in patients with ocular melanoma.

Uveal melanoma is the most frequent intraocular tumor in adults, with an incidence of 5.1 per million in the United States. The five-year survival rate of approximately 80% has remained stable over the last 30 years [1]. While the primary tumor is generally treatable, metastatic dissemination to the liver or lungs occurs in nearly half of cases and is almost always fatal [2]. Therefore, understanding the molecular pathways that promote the ability of the uveal melanoma cells to disseminate outside the eye represents an important goal.

Epithelial-to-mesenchymal transition (EMT) is a reversible embryonic program that transforms polarized epithelial cells into motile cells. During this process, the intercellular interactions and the cellular adhesions with the basement membrane and extracellular matrix (ECM) are disrupted, releasing epithelial cells into the surrounding tissue. The

cytoskeleton is also reorganized to confer to the cells the ability to move through a three-dimensional ECM. A new transcriptional program is induced to promote this mesenchymal phenotype, characterized by morphological changes including a spindled shape and cytoplasmic protrusions that facilitate cellular movement [3].

This EMT mechanism is physiologically present during early embryonic morphogenesis, where it plays a critical role in mesodermal formation and the delamination of the neural crest [3,4]. However, the inappropriate re-expression of the EMT program during adult life can become harmful, promoting the intravasation of tumor cells into the blood stream or lymphatic vessels with the subsequent formation of distant metastases [5]. The EMT process also induces resistance to apoptosis, chemo-, and immunotherapy [6]. It has been shown that an EMT-like process also occurs in cutaneous melanoma and predisposes melanoma cells to gain independence from surrounding keratinocytes and to progress toward metastasis [7-10]. However, thus far, little is known about the expression and biologic functions of the

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EMT factors in the melanoma cells arising in the uveal tract of the eye, which are genetically and biologically distinct from those in the skin [11,12].

The EMT program is regulated by several zinc-finger transcription factors, including Snail [13], Slug [14,15], zinc finger E-box binding homeobox 1 (ZEB1) [16], and SIP1/ZEB2 [17], as well as by helix–loop–helix transcriptional regulators, such as Twist [18]. The expression of ZEB1 is induced by TGF- $\beta$  [19], estrogen [20], NF- $\kappa$ B [21], and HIF-1 [22], and it is negatively regulated by the miRNA-200 family and miRNA-205 [23]. The ZEB1 transcription is also promoted by Snail and Slug [24,25]. Twist1 induces EMT in mammary epithelial cells [18]. Its overexpression was also observed in metastatic cutaneous melanoma, where it correlates with a poor outcome [9], and in the most aggressive subtype of neuroblastoma [26].

Interestingly, both melanoma and neuroblastoma derive from the neuroepithelial cells of the neural crest, whose development requires the expression of Twist [27]. Here, we show that ZEB1 is highly expressed in uveal melanoma cell lines, while two other EMT factors, Twist1 and Snail1, were also expressed, but to a lesser degree. The genetic downregulation of these three EMT regulators reduced the invasiveness of uveal melanoma cells in vitro, and ZEB1 and Twist1 mRNA levels significantly increased in primary tumors with high metastatic risk.

## METHODS

**Cell culture and plasmids:** Our cellular models included four human uveal melanoma cell lines derived from primary tumors: OCM1, Mel285, Mel290, and 92.1 [28-31], and one originally derived from a subcutaneous metastasis, OMM1 [32], kindly provided by Dr. J. Niederhorn (UT Southwestern Medical Center, Dallas, TX). Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, L-glutamine (2 mM), HEPES buffer (10 mM), sodium pyruvate (1 mM), MEM essential vitamins mixture (1% solution), MEM non-essential amino acid (1% solution), 50 IU/ml penicillin, and 50  $\mu$ g/ml streptomycin at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere. Their identities were authenticated at the Johns Hopkins Molecular Core Laboratory through short tandem repeat (STR) analysis. Lentiviral particles containing the PLKO.1 transfer vector with short hairpin RNA (shRNA) targeting respectively ZEB1, Twist1, and Snail1 mRNA (target sequences are shown in Appendix 1) were purchased from Thermo Fisher Scientific (Waltham, MA) and prepared using HEK293T cells, as previously described [33]. Puro-mycin (5  $\mu$ g/ml) was used to select cells expressing the

transfer vector. Scrambled shRNA was used as the control in the loss-of-function experiments.

**RNA analysis of cell lines and primary tumors:** RNA extraction from cell lines was performed with the RNeasy Mini Kit (Qiagen, Germantown, MD). To avoid contamination by genomic DNA, on-column DNA digestion was performed during RNA extraction using the RNase-free DNase kit (Qiagen). Quantitative real-time PCR (qPCR) for ZEB1, Twist1, and Snail1 was performed as previously described [33]. The primer sequences are shown in Appendix 2. All reactions were performed in triplicate, and each triplicate experiment was repeated three times in a iQ5 Multicolor real-time PCR detection system (Bio-Rad, Hercules, CA) using SYBR Green (Applied Biosystems, Foster City, CA) as fluorescent dye and normalized to  $\beta$ -actin mRNA levels.

Excess tumor tissues not required for diagnosis from 30 primary uveal melanoma tumors removed by enucleation at the Wilmer Eye Institute (Baltimore, MD) from 2004 to 2006 and snap frozen were also analyzed, after approval of the local Institutional Review Board (IRB) and with written informed consent. The mRNA was extracted from these tissues using the RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol for human tissues. The mRNA levels of ZEB1, Twist1, and Snail1 were determined in these primary tumor tissues by qPCR. These specimens were stratified into risk categories and divided in class 1 (low metastatic risk, n = 20) or class 2 (high metastatic risk, n = 10) according to their gene expression profiles using a PCR-based platform that measures the expression of 12 selected genes [34,35]. Survival analysis was not possible in this anonymized cohort.

An additional gene expression analysis was performed using a different tumor cohort for which survival data were available. Fresh tumor material was obtained from the uveal melanoma of 64 patients who underwent enucleation between 1999 and 2008 at the Leiden University Medical Center (Leiden, The Netherlands). Patient's charts were scrutinized for clinical and histopathological features, including age at enucleation, largest basal diameter (in millimeters), prominence (in millimeters), scleral ingrowth, ciliary body involvement, cell type, mitotic count, and extracellular matrix patterns. Data on survival were obtained from patient's charts and the Dutch National Registry. A Twist1 gene expression analysis was performed in two runs with the Illumina HT-12v4 chip (Illumina, San Diego, CA). The probe ILMN\_1672908 was used to determine the gene expression level for Twist1. The Snail1 and ZEB1 gene expressions were not evaluable in this tumor cohort. Chromosome aberrations were determined by Single Nucleotide Polymorphism (SNP) analysis. The Affymetrix 250K\_NSP chip was used

to analyze 28 tumor specimens. The remaining 36 tumor specimens were analyzed with the Affymetrix Cytoscan HD chip [36]. Tumors were classified as disomic when a normal chromosome 3 was detected and classified as monosomic when the loss of one chromosome 3 arm occurred. An additional chromosome 8q analysis was performed with digital PCR (dPCR), as described [36]. In short, probes for PTK2 and TUSC3 were used to determine the copy number of chromosome 8q and 8p, respectively. The DNA of each tumor specimen was obtained and mixed in a reaction volume, which consisted of 2X droplet PCR supermix (Bio-Rad Laboratories, Inc.), 20X target probe (FAM), and 20X reference probe (HEX). Subsequently, this mixture was converted into an emulsion of 20,000 droplets. These emulsified samples were analyzed by PCR with the end point 95 °C, 10 min; (94 °C, 30 s; 60 °C, 1 min) 40X; 98 °C, 10 min; 4 °C, till end. A droplet reading was performed with the QX100 droplet reader (Bio-Rad Laboratories, Inc., Hercules, CA) and the dPCR software was used to analyze and visualize the data. TERT located on chromosome 5 was used as a reference.

**Protein analysis:** The protein levels of ZEB1, Twist1, and Snail1 were determined by western blot in uveal melanoma cells transduced with shRNAs targeting the mRNAs encoding these three EMT proteins. Cells were maintained under puromycin selection for one week and then lysed using TNE lysis buffer, as previously described [37]. The protein lysates were sonicated for 20 s. Protein concentration was measured using a BCA kit (Pierce, Rockford, IL). Equal amounts of proteins were analyzed by 4%–12% SDS–PAGE (Invitrogen, Carlsbad, CA), transferred on a nitrocellulose membrane (Invitrogen), and incubated for 1 h in a blocking solution containing 5% dried milk in TBS with 0.1% Tween-20 (TBS-T). The following primary antibodies were used during overnight incubation in a blocking solution at 4 °C: ZEB1 (in rabbit, 1:2000, Sigma-Aldrich, # HPA027524, St. Louis, MO), Snail1 (in mouse, 1:750, Cell Signaling Technology, #3895, Danvers, MA), Twist1 (in rabbit, 1:750, Santa Cruz Biotechnologies, #sc-15393, Santa Cruz, CA), and  $\beta$ -Actin (in mouse, 1:500, Sigma-Aldrich, #sc-47778, St. Louis, MO). Secondary antibodies bound to peroxidase and raised in mouse or in rabbit (KPL, Gaithersburg, MD) were used to visualize protein bands. Enhanced chemiluminescence (ECL) was used as a detection reagent (PerkinElmer, Waltham, MA).

#### Cell growth and Transwell invasion assays:

**Cell growth assay**—Cell growth was determined by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium) colorimetric assay

performed in 96-well plates [38]. Cells were seeded at  $5 \times 10^3$  per well and resuspended in 200  $\mu$ l of 5% FBS medium per each well. Cell viability was determined at the day of seeding ( $t_0$ ) and after 3, 5, and 7 days of incubation at 37 °C by adding 20  $\mu$ l of an MTS solution to each well and measuring the absorbance at 490 nm in a microplate reader (BioTek, Winooski, VT) after 1 h of incubation at 37 °C. The growth rate has been determined as follows:  $(\text{Abs } t - \text{Abs } t_0) / n$ . days in culture. Each experimental condition has been repeated in triplicate and data are presented as mean  $\pm$  standard deviation (SD).

**Transwell invasion assay**—Cellular invasion was determined using 6.5-mm-diameter cell culture filters (8- $\mu$ m pore size; Becton Dickinson, Franklin Lakes, NJ), which were precoated for 1 h with Matrigel (Becton Dickinson) and diluted at 1:100 in 10% FBS medium in 24-well plates, as previously described [37]. As well,  $1.5 \times 10^5$  cells resuspended in a 500- $\mu$ l serum-free medium were plated in each filter insert. A gradient of FBS was established by adding 800  $\mu$ l of a medium containing 10% FBS to the lower chamber. After 18 h of incubation, cells remaining on the upper surface of the filter were removed with a cotton swab. Cells migrated to the lower surface were fixed with 70% ethanol, stained for 10 min with hematoxylin, destained, photographed, and quantified by cell counting. For the quantification, cells present on the lower part of the filter were photographed in six random high-power fields (HPFs) in each of three independent experiments and manually counted. The data represent the mean  $\pm$  SD of the number of cells counted in six random HPFs in each of three independent experiments. The percentage of reduction in invasion has been calculated as follows:  $\left\{ \left[ \frac{\text{N. invaded cells}_{(\text{control: sh-Scramble})} - \text{N. invaded cells}_{(\text{condition: shRNA for gene of interest})}}{\text{N. invaded cells}_{(\text{control: sh-Scramble})}} \right] \times 100 \right\}$ .

To extract RNA from cells with different invasion rates, we used 25-mm-diameter transwell cell culture inserts (Falcon inserts, 8- $\mu$ m pore size; Becton Dickinson) precoated with Matrigel and diluted at 1:100 in 10% FBS medium. As well,  $5 \times 10^5$  cells were plated in 2 ml of a serum-free medium, whereas 2.7 mL of a 10% FBS medium were added to the well. After overnight incubation, cells that had migrated to the lower surface of the microporous membrane were lysed and RNA extracted using the RNeasy Mini Kit (Qiagen). The non-invading cells on the top of the filter were removed with a cotton swab. The opposite procedure was used in an adjacent well containing the same initial number of cells to collect RNA from non-invading cells located on the upper surface of the microporous membrane. The experiment was performed in triplicate, using three wells for the invading and three for the non-invading cells.

**Statistical analysis:** Levels of significance were determined by the two-sided Student *t* test. P values lower or equal to 0.05 were considered statistically significant. Experiments were performed three times, and data are presented as the mean  $\pm$  SD. Statistical evaluations were performed using GraphPad Prism4 software. An analysis of Twist1 gene levels was performed with SPSS 20.0.0 (IBM SPSS Statistics, IBM Corporation, Armonk, New York, NY). The Twist1 gene expression was dichotomized at the median to create two groups representing low and high Twist1 gene expressions in uveal melanoma. The following tests were used to compare the two groups: the  $\chi^2$ -test was applied for categorical prognostic parameters and the non-parametric Mann–Whitney U test was used for continuous prognostic parameters. The log-rank test of Kaplan–Meier survival curves was used to examine clinical outcomes associated with low and high Twist1 gene expressions. Death due to metastasis was considered an event. Loss to follow-up, death due to another cause, and death with an unknown cause were censored. To determine which clinical, histological, and genetic features influence survival in patients with uveal melanoma, a univariate cox regression analysis was performed. Parameters with a p value  $\leq$ 0.1 were further analyzed in a multivariate cox regression analysis to determine which significant parameters are independent prognostic factors; 95% confidence intervals were calculated for hazard ratios. For all the comparisons, a p value of  $\leq$ 0.05 was considered significant.

## RESULTS

**ZEB1, Twist1, and Snail1 are expressed in uveal melanoma cells and primary tumors:** To investigate the role of the EMT-like program in uveal melanoma, we first examined the expressions of ZEB1, Twist1, and Snail1 proteins in uveal melanoma cell lines. We observed a strong expression of ZEB1 in all the cell lines that we analyzed, as determined by western blot. Twist1 and Snail1 were also expressed, but less abundantly than ZEB1 (Figure 1A). These lines include one with a GNA11 mutation (OMM1), one with a GNAQ mutation (92.1), and one with a BRAF mutation (OCM1), which is uncommon in primary uveal melanoma [39].

An analysis of the mRNA expression of ZEB1, Twist1, and Snail1 was performed in 30 primary tumors with no clinical follow up available, but they were previously classified according to their metastatic risk (Appendix 3). We found that ZEB1 transcript levels were significantly higher in class 2 tumors, which have a high propensity to metastasize, compared to class 1 tumors with low metastatic risk ( $p = 0.019$ ), as found by the Fisher Exact probability test (Figure

1B). No significant correlation with class 1 or 2 was observed for Twist1 and Snail1 in that tumor group.

The Twist1 gene expression was also analyzed by microarray in a different tumor cohort with clinical follow-up data, which included 64 primary uveal melanomas collected at the Leiden University Medical Center (Leiden, The Netherlands). The Twist1 gene expression was dichotomized at the median level (6.50) into two groups (low and high gene expressions) to analyze the differences in the clinical, histological, and genetic parameters and survival. We compared the 32 uveal melanomas with the lowest and the 32 uveal melanomas with the highest Twist1 gene expressions. A high Twist1 gene expression coincided with the monosomy of chromosome 3 ( $p = 0.001$ ). A low-level gain of chromosome 8q (between 2.2 and 3.1 copies) occurred more frequently in uveal melanoma with a low Twist1 gene expression, while the amplification (more than 3.1 copies) of chromosome 8q was associated with a high Twist1 gene expression ( $p = 0.021$ ).

With regard to clinical and histologic features, the two groups were similar. However, metastases developed significantly more frequently in tumors with a high Twist1 gene expression (Appendix 4). According to the log-rank test of Kaplan–Meier curves, a high gene expression of Twist1 was associated with a worse survival than a low gene expression ( $p < 0.0001$ ; Figure 1C). The univariate Cox regression analysis showed that the age at enucleation ( $p = 0.036$ ), the largest basal diameter ( $p < 0.0001$ ), the presence of ciliary body involvement ( $p = 0.006$ ), a mixed or epithelioid cell type ( $p = 0.031$ ), the monosomy of chromosome 3 ( $p < 0.0001$ ), the gain or amplification of chromosome 8q ( $p = 0.025$  and  $p = 0.002$ , respectively), and a high Twist1 gene expression ( $p < 0.0001$ ) were associated with an increased risk of death due to metastasis (Appendix 5). A multivariate Cox regression was also performed to determine which of these associated parameters were independent prognostic factors in uveal melanoma. Patients with a gain or amplification of chromosome 8q were more likely to develop metastases than patients without aberrations in chromosome 8q were ( $p = 0.046$ ;  $p = 0.019$  respectively). In addition, the monosomy of chromosome 3, the largest basal diameter, and a high Twist1 gene expression were significant independent prognostic factors. The hazard ratios (HR) and corresponding p values of the univariate and multivariate analyses are listed in Appendix 5.

**Increased ZEB1, Twist1, and Snail1 mRNA levels in invasive uveal melanoma cells:** To determine whether ZEB1, Twist1, and Snail1 increase migration and promote the metastatic spread of uveal melanoma, we determined the mRNA levels of these three transcription factors in OMM1 cells that had moved overnight through a microporous membrane precoated



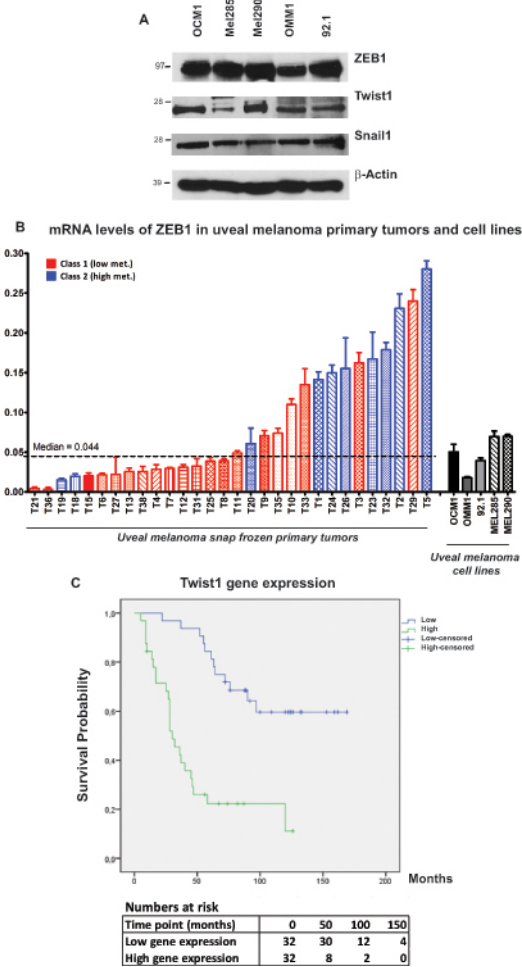


Figure 1. Protein expression of the EMT-associated factors in uveal melanoma cells lines and primary tumors. **A:** ZEB1, Twist1, and Snail1 protein levels were determined by western blot in OCM1, Mel285, Mel290, OMM1, and 92.1 uveal melanoma lines;  $\beta$ -actin was used as a loading control. The time of film exposure during the chemiluminescence reaction was 10 s for ZEB1 and  $\beta$ -actin and 2 min for Twist1 and Snail1. **B:** The ZEB1 mRNA expression was measured in 30 uveal melanoma snap-frozen primary tumors and in five cell lines. ZEB1 mRNA levels were significantly higher in class 2 tumors, compared to class 1, as determined by the two-tailed Fisher Exact probability test using the median value (0.044) as a cut-off ( $p = 0.0186$ ). **C:** Kaplan–Meier survival curve according to the gene expression levels of Twist1 (compared to the log-rank test); low and high expressions are respectively lower or higher than the median value, which is equal to 6.50.

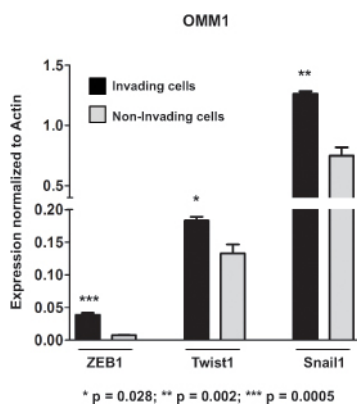


Figure 2. Increased ZEB1, Twist1, and Snail1 mRNA levels in invasive uveal melanoma cells. The mRNA levels of the EMT-factors ZEB1, Twist1, and Snail1 were significantly increased, as measured by qPCR, in invading OMM1 cells, which moved through a Matrigel-coated filter, as compared to non-invading OMM1 cells. Data shown are mean  $\pm$  standard deviation (SD); \* $p = 0.028$ ; \*\* $p = 0.002$ ; \*\*\* $p = 0.0005$  versus non-invading cells.

with Matrigel, which mimics the composition and the properties of the ECM (Figure 2). We observed that all these three factors were significantly increased at the transcriptional level in the invasive cells harvested from the lower surface of the Matrigel-coated membrane, as compared to those that

did not invade and were still located in the upper part of the filter after overnight incubation.

*Downregulation of ZEB1 suppresses invasion in uveal melanoma cells:* To determine the functional role of ZEB1 in

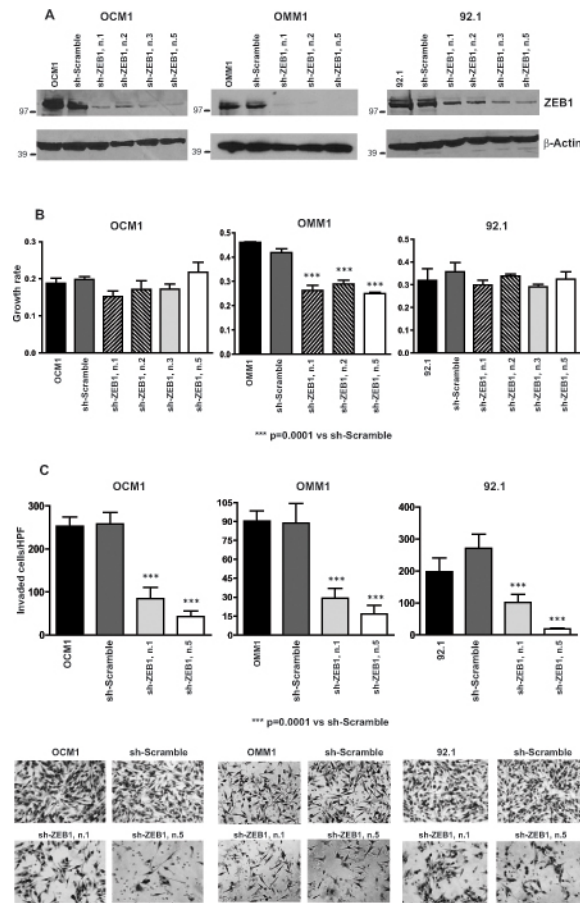


Figure 3. The downregulation of ZEB1 reduces Transwell invasion. **A:** ZEB1 protein levels were determined by western blot in OCM1, OMM1, and 92.1 cells, where the ZEB1 expression was reduced by shRNA. **B:** An MTS growth assay shows reduction in cell growth only in OMM1 cells, as opposed to OCM1 and 92.1, where growth inhibition was not observed. **C:** A Transwell invasion assay shows that ZEB1 downregulation significantly suppressed overnight invasion through Matrigel in all three cell lines; scrambled shRNA was used as a control. The microphotographs in the lower panel show the invading cells after staining with hematoxylin. Data shown are mean  $\pm$  SD; \*\*\* $p = 0.0001$  versus sh-Scramble.

our tumor cell lines, we performed loss-of-function studies using shRNA that specifically targets this key EMT factor. Four separate constructs, which are specific for different target sequences of ZEB1 mRNA (Appendix 1), were used to transduce OCM1, OMM1, and 92.1 cells. All four sequences successfully suppressed the ZEB1 protein expression in OCM1 and 92.1 cells, while only three inhibited ZEB1 translation in OMM1 cells, as determined by western blot (Figure 3A). Scrambled shRNA was used as a negative control. The growth rate was not inhibited by suppressing ZEB1 in OCM1 and 92.1 cells, and it was slightly reduced in OMM1 cells, as found by MTS assay (Figure 3B). Interestingly, we observed a profound inhibition of the ability of the cells to invade Matrigel by transwell assay in all three cell lines. ZEB1 shRNAs reduced the number of invasive cells by approximately 60% to 80% in OCM1 and OMM1 lines

and by 60% to 90% in 92.1 cells, as compared to scramble shRNA (Figure 3C).

*Downregulation of Twist1 affects both growth and invasion in uveal melanoma cells:* To assess the effects of Twist1 in uveal melanoma, we suppressed its expression using two separate shRNA constructs targeting Twist1 mRNA, both of which reduced the mRNA levels by 60%–70% in Mel290 and OMM1 cells, as compared to scramble shRNA (Figure 4A). This reduction correlated with a significant inhibition of growth in both cell lines (Figure 4B), as found by MTS assay. However, a transwell invasion assay showed that Twist1 inhibition produced a stronger effect on cellular invasion, which was inhibited by approximately 80%–90% in Mel290 and 60%–75% in OMM1 cells (Figure 4C).

*Downregulation of Snail1 reduces invasion in uveal melanoma cells:* The suppression of Snail1 was achieved in 92.1

cells using two separate shRNAs constructs, which reduced expression by approximately 40%–60% at the mRNA and protein levels, as compared to scramble controls (Figure 5A,B). We attempted to suppress the Snail1 expression in OCM1 cells as well, but we were not successful. The reduction of Snail1 in 92.1 cultures did not interfere with cell growth, as found by MTS assay performed for 3, 5, and 7 days (Figure 5C). Nevertheless, we observed that Snail1 shRNAs reduced by approximately 50% the number of 92.1 cells that moved through a Matrigel-coated filter after 24 h of incubation (Figure 5D), indicating that Snail1 can also promote invasion in uveal melanoma cells. Our working model for the activity of the EMT factors in the regulation of invasion in uveal melanoma cells is depicted in Figure 6.

## DISCUSSION

Uveal melanoma has a high propensity to metastasize hematogenously in visceral organs, particularly the liver, and thus far, no pharmacological treatment has succeeded in preventing or treating this dissemination. The EMT process is considered a crucial event during the metastatic progression of many solid tumors, because it increases their capacity to move through the ECM into the blood stream, promoting the formation of distant metastatic deposits. In addition, the induction of an EMT process in immortalized human mammary epithelial cells and other tumors leads to the expression of stem-cell markers [40]. Therefore, cells that undergo EMT, like cancer stem cells, are considered more resistant to apoptosis induced by chemo- and radiotherapy, as compared to better differentiated cells [6,41].

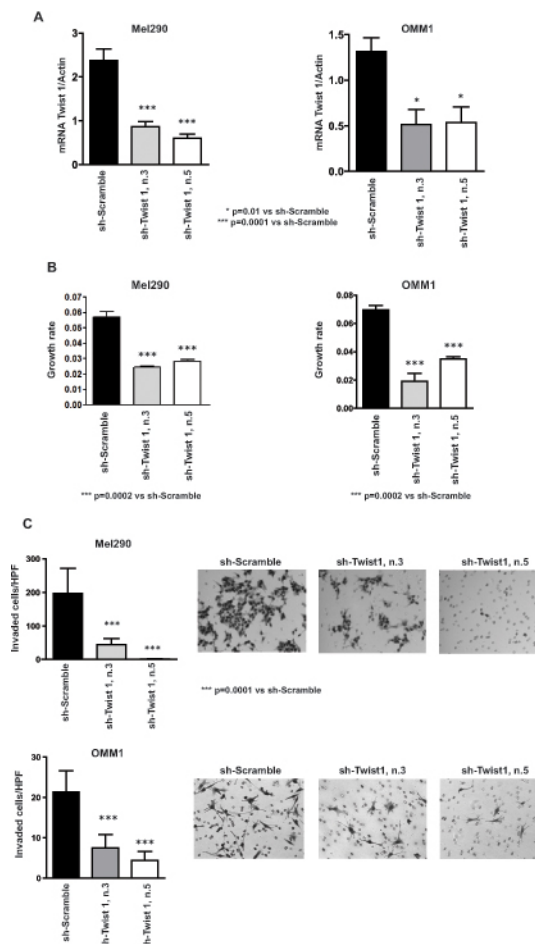


Figure 4. Reduction in Twist1 decreases both growth and invasion in uveal melanoma cells. **A:** Twist1 mRNA levels were determined by qPCR in Mel290 and OMM1 cells transduced with Twist1 shRNA. Data shown are mean  $\pm$  SD; \* $p = 0.01$ ; \*\*\* $p = 0.0001$  versus sh-Scramble. **B:** An MTS assay shows a reduction in cell growth when the Twist1 expression was reduced in both cell lines, as compared to scramble shRNA; \*\*\* $p = 0.0002$  versus sh-Scramble. **C:** A transwell invasion assay reveals a significant reduction in the invasive capacity of Mel290 and OMM1 cells upon the downregulation of Twist1, compared to vector control. Microphotographs in the right panels show the invading cells on the lower surface of the Matrigel-coated filter after overnight incubation; \*\*\* $p = 0.0001$  versus sh-Scramble.

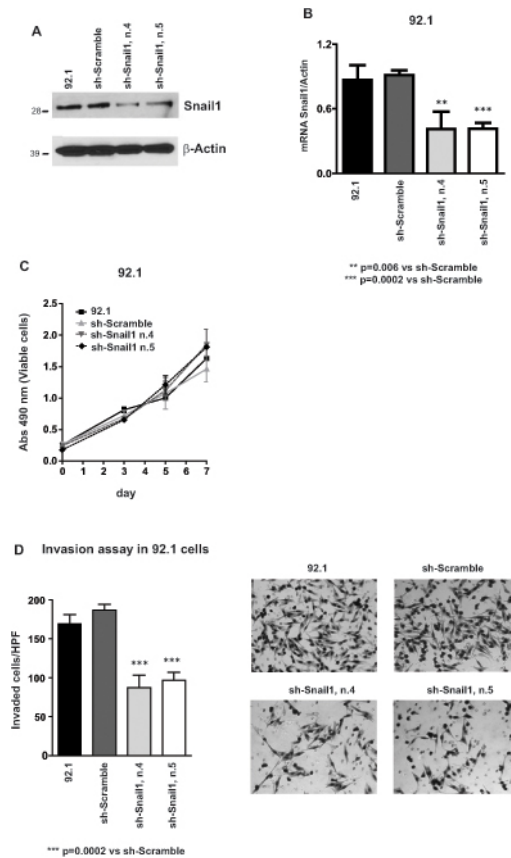


Figure 5. Snail1 downregulation reduces invasion in uveal melanoma cells. **A-B**: Protein and mRNA levels of Snail1 were determined respectively by western blot (**A**) and qPCR (**B**) in 92.1 cells transduced with Snail1 shRNA. Scrambled shRNA was used as a control. Data shown are mean  $\pm$  SD; \*\*p = 0.006; \*\*\*p = 0.0002 versus sh-Scramble. **C**: An MTS assay was performed in 92.1 cells transduced with Snail1 shRNA as compared to scrambled shRNA. **D**: An invasion assay in 92.1 cells shows a reduction in the ability of the cells to invade Matrigel following the downregulation of Snail1. Microphotographs in the right panels show the invading cells on the lower side of a Matrigel-coated membrane; \*\*\*p = 0.0002 versus sh-Scramble.

Accumulating evidence indicates that an EMT-like program contributes to neoplastic transformation, metastatic progression, and drug resistance in cutaneous melanoma,

but thus far, this program has not been studied in uveal melanoma. Recently, it has been found that during melanogenesis, the EMT regulators Snail2 and SIP1/ZEB2 behave

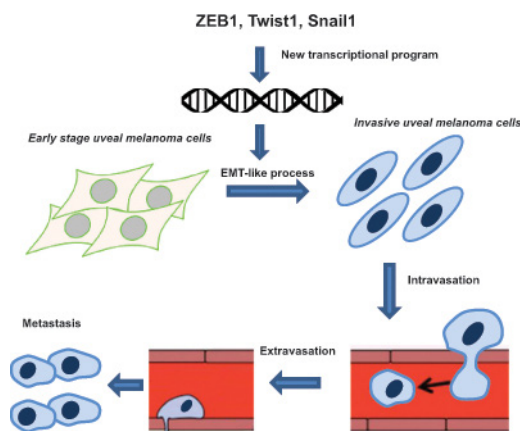


Figure 6. Working model for the activity of the EMT factors in the regulation of invasion in uveal melanoma cells.



as oncosuppressor proteins, activating an MITF-regulated differentiation program, while ZEB1 and Twist1 repress differentiation and cooperate with BRAF in promoting melanoma progression [42]. Therefore, the switch from ZEB2/Snail2 to ZEB1/Snail1 is indicative of a poor outcome in malignant melanoma. In addition, a comparison of gene expression profiles between metastatic and nonmetastatic cutaneous melanoma primary tumors showed that the EMT-regulator genes were significantly upregulated in the metastatic cohort and contributed to the promotion of a metastatic phenotype [43].

These concepts prompted us to study the potential role of the EMT-regulators ZEB1, Snail1, and Twist1 in inducing the metastatic progression of uveal melanoma. We found that the transcription factor ZEB1 was highly expressed in all the cell lines analyzed, while Snail1 and Twist1 were also expressed, but to a lesser degree. Interestingly, we found that ZEB1 mRNA levels were significantly elevated in class 2 uveal melanoma surgical samples with high metastatic risk, supporting the concept that this EMT factor might play an important role in the dissemination of the primary tumor. In a different tumor cohort including a larger number of cases and more detailed clinical follow up, we found that the high expression of Twist1 was associated with worse survival, suggesting a role for an additional EMT factor in promoting metastatic behavior in uveal melanoma.

We further observed that the downregulation of these three EMT-related genes using shRNA inhibited by more than 50% the ability of uveal melanoma cells to invade a Matrigel-coated filter. The most prominent effect was observed when ZEB1 was downregulated. These findings imply that these transcription factors could play an important role in promoting invasion in uveal melanoma cells. Interestingly, the effects of EMT factor reduction on tumor culture growth were not as pronounced. Only the reduction of Twist1 significantly suppressed cell growth in both the cell lines that we analyzed, while shRNAs targeting Snail1 did not have any effect in reducing growth. The suppression of ZEB1 slightly impaired the growth rate only in OMM1 cells and did not have any inhibitory effect on growth in OCM1 and 92.1 cells. These data are consistent with previous findings obtained in cutaneous melanoma cells, where the downregulation of ZEB1 did not modify cell proliferation, but it potently reduced intercellular adhesion and cell migration [25].

OMM1 cells carry GNA11 mutation, which is more commonly found in metastatic uveal melanoma cells, as compared to GNAQ mutation [39]. The presence of this mutation in OMM1 cells might be linked to a greater growth inhibition after the downregulation of ZEB1, as the other two

lines, OCM1 and 92.1, contain BRAF<sup>V600E</sup> and GNAQ mutations, respectively. Indeed, an important caveat to consider is that our functional studies used long-term cell cultures, which may not fully model the human tumors from which they are derived. In particular, no human uveal melanoma cell lines in common use contain mutations in BAP1, which are thought to play a key role in tumor spread. In addition, the mutation profile of OCM1 challenges the assumption that this cell line is derived from a uveal melanoma; therefore, multiple cell lines, including those carrying GNAQ/GNA11 mutations, were used in this study.

Nevertheless, our combined in vitro and primary human tumor data suggest that an EMT-like process may exist in uveal melanoma cells and may potentially be responsible for activating a metastatic phenotype. Therefore, we propose that the EMT regulators should be considered as potential therapeutic targets for clinical intervention to prevent the metastatic spread of uveal melanoma cells.

#### APPENDIX 1.

Target sequences for the short hairpin RNA (shRNA) specific for ZEB1, Twist1, and Snail1 mRNA. To access the data, click or select the words “[Appendix 1.](#)”

#### APPENDIX 2.

Primer sequences for ZEB1, Twist1, Snail1, and  $\beta$ -Actin. To access the data, click or select the words “[Appendix 2.](#)”

#### APPENDIX 3

The primary tumors used for the analysis of ZEB1 mRNA levels were classified by gene expression profile in class 1 tumors with low metastatic risk (red) and class 2 tumors with high metastatic risk (blue). To access the data, click or select the words “[Appendix 3.](#)”

#### APPENDIX 4.

Comparison of clinical and histopathological features in low and high Twist1 gene expression in 64 cases of uveal melanoma. n: number of patients; mm: millimeters. Percentages are rounded and may not be equal to 100. To access the data, click or select the words “[Appendix 4.](#)”

#### APPENDIX 5.

Univariate and Multivariate Cox regression of 64 uveal melanoma patients with death due to metastasis as end point. HR: hazard ratio; CI: confidence interval; HR for continuous variables is for each unit (mm, years). \*Reference group; NS:

Not significant. To access the data, click or select the words “Appendix 5.”

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