BORIS/CTCFL promotes a switch from a proliferative towards an invasive phenotype in melanoma cell lines

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Abstract

Melanoma is among the most aggressive cancers due to its tendency to metastasize early. Phenotype switching between a proliferative and an invasive state has been suggested as a critical process for metastasis. The mechanisms that regulate these transitions are poorly understood, but are associated with transcriptional changes. Brother of Regulator of Imprinted Sites (BORIS), also known as CCCTC binding factor-Like (CTCFL), is a transcriptional modulator that becomes aberrantly expressed in melanoma. Here, we provide the first evidence that BORIS is involved in phenotype switching in melanoma. Genetic modification of BORIS expression in melanoma cells combined with whole transcriptome analysis indicated that BORIS expression contributes to an invasion-associated transcriptome. In agreement with this finding, inducible BORIS overexpression in melanoma cells reduced proliferation and increased migration and invasion, demonstrating that the transcriptional switch is accompanied by a phenotypic switch. Overall, our study indicates a pro-invasive role for BORIS in melanoma via transcriptional reprogramming.

Introduction

Primary cutaneous melanoma is one of the most aggressive cancers due to its ability to rapidly disseminate and develop distant metastases. The progression of melanoma from the primary site to regional lymph nodes and distant sites requires cells to migrate and invade into tissue containing lymphovascular channels that allow the tumor cells to enter the circulation. During melanoma progression, cells undergo phenotype switching by transitioning from a proliferative to an invasive state, a process similar to epithelial-mesenchymal transition (EMT) [1,2]. Gene expression profiling of melanoma cell lines and tumor samples has revealed the different transcriptional states that underlie the proliferative and invasive phenotypes of melanoma cells [3-7]. While proliferative cells tend to express high levels of developmental and lineage specification genes such as *MITF*, *MLANA*, and *DCT* as well as *SOX10* and *PAX3* [1,4,6], cells with an invasive transcriptional state are marked by expression of amongst others, *ZEB1*, *SNAI1*, *AXL*, *NGFR*, *SPARC*, *WNT5A* and genes involved in TGF-β signaling [4,6-10]. Currently, various transcriptional regulators have been implicated as drivers of the transition towards an invasive phenotype, including BRN2, AP-1, TEAD, NFATC2, and NFIB [4,11-13]. These findings suggest complex transcriptional regulation that requires context-specific involvement of various transcriptional regulators to achieve the transition towards an invasive state. In line with this idea it was proposed that phenotype switching comprises a gradient of phenotypic states that is accompanied by different transcriptional states [14,15], similarly to what is reported for EMT in epithelial cancers [16,17]. To gain insight into the transcriptional landscape that underlies these different phenotypic spectra it is important to identify transcriptional regulators of the gene expression changes that confer migratory and invasive properties to melanoma cells.

Brother of Regulator of Imprinted Sites (BORIS) is a DNA binding protein with high similarity to CCCTC binding factor (CTCF) [18], a multifunctional transcription factor that plays an important role in genome organization [19]. Like CTCF, BORIS plays a role in transcriptional regulation [20-23]. The mechanisms through which BORIS can alter transcription rely on BORIS' ability to bind the DNA at specific binding motifs, which are often found near promoter regions [24-26]. At these binding motifs BORIS can compete with DNA-bound CTCF [26], form a BORIS-CTCF heterodimer, or occupy a so-called BORIS-only binding site [25]. Via these mechanisms BORIS can alter transcription by acting as a transcriptional activator [20,21,23,27], recruiting a transcriptional activator [28], altering DNA methylation [20,29,30] or histone

modifications [20,22,23,29], or recruiting chromatin modifying proteins [31,32]. In addition, it is believed that BORIS impacts the transcriptional landscape by interfering with CTCF-mediated chromatin loops [25,33]. The *BORIS* gene is located at 20q13 locus, within a chromosomal region that is often amplified in cancer [34]. Furthermore, hypomethylation of the *BORIS* promoter is frequently observed in cancer cells [35,36]. Besides regulation of expression via DNA methylation, increased BORIS levels have been observed upon CTCF knockdown [37]. *BORIS* is expressed in 60-90% of melanoma cell lines [30,38], in 16% (4 out of 25) of primary melanomas and in 34% (13 out of 38) of melanoma metastases [38]. The higher frequency of BORIS expression in metastatic tumors compared to primary tumors suggests that BORIS plays a role in melanoma progression [38]. Currently, little is known about the role of BORIS in melanoma.

In this study, we sought to determine if BORIS plays a role in melanoma progression through its role as transcriptional modulator. We found that BORIS expression in melanoma cells led to altered gene expression as determined by RNA-sequencing (RNA-seq). While the upregulated genes were enriched among invasion-related processes and gene signatures, the downregulated genes were enriched among proliferation-related processes and gene signatures, suggesting that BORIS plays a role in phenotype switching. Phenotypically, we found that BORIS overexpression reduced proliferation and increased migration and invasion of melanoma cell lines. Overall, we demonstrate that BORIS-mediated transcriptional reprogramming was accompanied by a switch towards an invasive phenotype.

Results and Discussion

BORIS expression in the MM057 melanoma cell line induces large-scale differential gene expression

First, we sought to confirm that BORIS is expressed in melanoma cell lines as reported previously [30,37,38]. Our panel of melanoma and non-malignant congenital nevi cell lines was screened for expression of BORIS mRNA and protein. This revealed *BORIS* mRNA in ~60% (11 out of 18, **Fig S1A**) and BORIS protein in ~33% (4 out of 12, **Fig S1B**) of melanoma cell lines. We did not detect *BORIS* expression in the congenital nevi cell lines (**Fig S1A**). In the tested melanoma cell lines *BORIS* mRNA expression was not proportional to BORIS protein level. High BORIS protein expression was observed in 3 cell lines with low to undetectable *BORIS* mRNA expression. This discordance between BORIS mRNA and protein has been reported previously [39] and is proposed to be due to a short half-life of the BORIS transcript and high protein stability [40]. We observed no correlation between the expression of BORIS and its ubiquitously expressed paralogue CTCF in the tested cell lines (**Fig. S1B and C**). Together, these data indicate that BORIS is expressed in melanoma cells and that this expression is independent of CTCF expression.

Next, we wanted to assess if BORIS, through its role as transcriptional modulator, can alter the transcriptome of melanoma cells. To this end we used the MM057 melanoma cell line, which harbors a proliferative gene signature as reported by Verfaillie *et al.* [4] and expresses no detectable BORIS protein and low BORIS mRNA (**Fig. S1A and B**). BORIS overexpression was established in this cell line by lentiviral transduction with the doxycycline (dox)-inducible BORIS expression vector. In parallel, MM057 cells with inducible empty vector (EV) expression were generated to control for potential dox treatment-related side effects (**Fig 1A**). Subsequently, the effect of BORIS expression on the transcriptome of MM057 cells was assessed by RNA-seq using three biological replicates. Since a strong correlation was observed between the biological replicates ($r \geq 0.97$, **Fig. S2A**), all of the data was included for downstream analyses. The unsupervised analysis of gene expression demonstrated clustering of the samples into two groups, one representing the controls [EV without dox (EVneg) and with dox (EVpos), and BORIS without dox (BORneg] and the other representing BORIS expressing cells [BORIS with dox (BORpos)] (**Fig S2B**). Differential gene expression analysis between BORneg and BORpos

cells identified 2248 differential expressed genes (DEGs), of which 1361 genes were upregulated and 887 downregulated following BORIS expression (**Fig. 1B and Table S2**). The large number of detected DEGs is not surprising because of BORIS' function as a transcriptional modulator [20,21,23,27] and regulator of various cellular processes [36,41]. Analysis of the log2 counts per million (CPM) for the top 100 up- and down-regulated genes demonstrated robust differential expression, as well as clustering of all control samples versus BORpos samples (**Fig. 1C**). To validate the RNA-seq dataset, BORIS expression was induced in the MM057 cells and qPCR was performed for genes distributed throughout the list of DEGs. A significant differential expression of upregulated and downregulated genes was observed following BORIS expression compared to the controls (**Fig. 1D**). Together, these data demonstrate that ectopic BORIS expression results in large-scale differential gene expression.

BORIS expression contributes to a switch from a proliferative to invasive transcriptional state

The dataset was further analyzed to investigate if BORIS-regulated genes are involved in processes related to melanoma progression. First, gene set enrichment analysis (GSEA) [42] was performed for biological processes on a pre-ranked gene list. The ranked gene list was established by including genes with a CPM>0.5 for all replicates in at least one condition and ranked according to the $\log_2$ fold-change ($\log_2$FC). A strong enrichment (false discovery rate (FDR)<0.05) for migration and invasion-related processes was observed, including EMT, cell motility and locomotion, while a strong negative enrichment was found for proliferation-related processes like cell cycle/division, telomere maintenance, and DNA replication/repair (**Fig. 2A**). This analysis provides the first line of evidence that BORIS could promote a pro-invasive transcriptome.

Next, we performed GSEA for the melanoma-specific Hoek [3] and Verfaillie [4] gene signatures that correspond to either the proliferative or invasive state of melanoma cells. For these gene sets, we identified a strong positive correlation with the Hoek (NES=2.21, FDR=0.001) and Verfaillie (NES=5.99, FDR<10^{-4}) invasive gene signatures (**Fig. 2B**) and a negative correlation with the Hoek (NES=-1.98, FDR=0.006) and Verfaillie (NES=-3.84, FDR<10^{-4}) proliferative gene signatures (**Fig. 2C**). The overlap with the Hoek gene signatures was less significant, likely due to the smaller number of genes within this gene set. These data

support the hypothesis that BORIS expression enables a transcriptional program that promotes a switch from a proliferative to invasive state. To validate the effect of BORIS expression on genes within the gene signatures we measured the expression of four invasion-associated and four proliferation-associated genes from the Hoek and Verfaillie studies in the MM057 cell line following BORIS expression. We observed a significant upregulation of the invasion-associated genes *DKK1, FBN1, INHBA* and *VEGFC* (**Fig. 2D**) and significant downregulation of the proliferation-associated genes *GYG2, LZTS1, MLANA* and *TFAP2A* (**Fig. 2E**) compared to the controls. Together, these results indicate that BORIS promotes a switch from a proliferative to an invasive gene expression signature in melanoma cells.

Transcriptional reprogramming and subsequent phenotype switching have been linked to differences in the expression of transcription factors that are associated with EMT [4,11,12]. In melanoma, reduced expression of the melanoma-specific transcription factor MITF has been reported in invasive cells compared to proliferative cells [1]. MITF is regulated by the switch in expression from ZEB2 in melanocytes to ZEB1 in melanoma cells, with ZEB1 functioning as a transcriptional repressor of E-cadherin (*CDH1*) [43,44]. In the MM057 cells BORIS did not significantly reduce *MITF* expression (**Fig. S3A**). Similarly, BORIS did not significantly alter *ZEB1*, *ZEB2* or *CDH1* expression. The latter is also not expressed in the control cells, indicating that the equilibrium between ZEB1/2 and CDH1 might already be in favor of an invasive phenotype in the MM057 cells. In contrast, expression of SPARC appears to be regulated by BORIS. SPARC is a matricellular protein involved in cell-matrix interactions that promotes EMT-like changes [45], is associated with the metastatic potential of melanoma [46,47] and induces expression of SNAIL (*SNAI1*) [48]. Transcription of both *SPARC* and *SNAI1* were increased in BORIS-expressing MM057 cells: $\log_2$FC 0.74, FDR 3.9E-04 and $\log_2$FC 1.5, FDR 0.056, respectively (**Fig. S3A**). Increased SNAIL expression in melanoma cells was shown to induce EMT via upregulation of invasive markers and reduced immunosuppression, which is in part driven by increased secretion of TGF-beta (*TGFB*) and thrombospondin (*THBS*) [49]. In agreement, we observed increased expression of *TGFB1* ($\log_2$FC 0.65, FDR 5.7E-03) and *THBS2* ($\log_2$FC 1.49, FDR 1.88E-09) upon BORIS overexpression (**Fig. S3A**). In addition to *TGFB1*, we observed upregulation of all TGF-beta family members that are expressed at a detectable level in the MM057 cell line (**Fig. S3B**). Based on our findings, we propose that BORIS expression promotes a transcriptional switch from a proliferative towards an invasive

state via the upregulation of *SPARC* that leads to *SNAI1* expression and secretion of THBS2 and TGF-beta family members.

BORIS expression in melanoma cells results in reduced proliferation

In line with the acquisition of an invasive transcriptional state, the GSEA on BORIS-induced DEGs revealed a negative enrichment for processes involved in proliferation. To investigate the effect of BORIS expression on proliferation we induced BORIS expression with increasing concentration of dox. Expression of BORIS at each time point was confirmed using immunoblot (**Fig. S4A**). We observed a dose-dependent reduction in proliferation over time compared to untreated cells, which was not observed in the control cells (**Fig. 3A**). This observation is in agreement with previous studies reporting reduced proliferation upon ectopic BORIS expression in both primary cells and various cancer cell lines [50,51]. Though the literature regarding proliferation in the context of BORIS is not uniform, as others have reported increased proliferation upon BORIS overexpression [20,52,53]. Our findings support the hypothesis that BORIS expression reduces proliferation and promotes a more invasive state in melanoma cells.

In addition to an effect of BORIS expression on proliferation, BORIS knockdown in breast cancer and colon cancer cell lines was shown to increased apoptosis [53,54]. To address whether the reduction in proliferation that we observed is due to increased apoptosis, we investigated the effect of BORIS expression on cell death. Compared to control cells, BORIS expression increased the expression of cleaved PARP, a marker for cell death (**Fig. 3B**). In addition, we observed an increase in the number of early apoptotic, apoptotic and necrotic cells following BORIS expression compared to controls (**Fig. 3C and D**), indicating that BORIS expression in melanoma cells leads to increased apoptosis. Importantly, while we observed an increase in apoptosis of ~12%, this cannot solely explain the 30% reduction in proliferation. Taken together, our results show that expression of BORIS in melanoma cells leads to increased apoptosis and decreased proliferation.

BORIS expression promotes an invasive phenotype in melanoma cells.

The contribution of BORIS expression to an invasive transcriptional state and a reduction in proliferation prompted us to ask if BORIS expression promotes an invasive phenotype. Currently, few studies have investigated BORIS in the context of EMT in cancer and results have

been contradictory [55,56]. First, we assessed the effect of BORIS overexpression on migration using a transwell assay in two melanoma cells lines (MM057 and MM074) that harbor a proliferative transcriptional state [4]. Inducible BORIS expression was confirmed by immunoblot (**Fig. S4B**). In both cell lines we observed a significant increase in the percentage of migrating cells compared to the controls (**Fig. 4A and B**). Furthermore, we observed a significant increase in the invasive capacity of both cell lines following BORIS expression in a Matrigel transwell invasion assay (**Fig. 4C and D**). Overall, these results demonstrate that BORIS expression leads to an increase in the migratory and invasive potential of the MM057 and MM074 melanoma cell lines.

Here we provide the first evidence that BORIS can rewire the gene expression program in favor of a more invasive phenotype of melanoma cells. We demonstrate that BORIS expression in melanoma cell lines resulted in large-scale transcriptional changes, decreased proliferation, and increased migration and invasion. Taken together, these data indicate that expression of BORIS promotes a switch from a proliferative to invasive state at both the transcriptional and phenotypic level in melanoma cell lines. BORIS may therefore be an important regulator of phenotype switching in melanoma. Other consequences of BORIS expression have not yet been investigated. Given the large-scale transcriptional changes, additional effects on cell biology are very likely. Furthermore, it remains elusive how BORIS mediates these transcriptional effects. For example, via competition with CTCF for DNA binding sites, direct promoter regulation, epigenetic modifications, or chromatin looping. Given the importance of BORIS as an invasion promoting transcriptional regulator, these questions warrant further studies to fully understand BORIS' role in cancer.

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Author contributions

SMJ, LCK and AS conceived the idea and designed the study; SJ, ME and AIP performed the tissue culture; ME generated the expression vectors; SJ carried out the qPCRs and immunoblots; SJ and ME conducted the *in vitro* experiments with help of MR and HW. Experiment were carried out under the supervision of AS; RM performed the bioinformatics analysis under the supervision of ER; the manuscript was written by SJ, LCK and AS with input from all other authors.

Conflict of interest

The authors declare no competing financial interests

Figure legends

Figure 1. BORIS expression in the MM057 melanoma cell line leads to large-scale differential gene expression.

BORIS expression was induced in the MM057 cell line using 50ng/ml dox for 5 days.

A: Expression of BORIS mRNA and protein as assessed by qPCR and immunoblot. Anti-Tubulin was used as a loading control for immunoblot.

B: MA plot representing the RNA-seq data of all BORneg versus BORpos samples. The blue horizontal lines mark $\log_2FC = 0.5$

C: Heat map representing the $\log_2CPM$ for the top 100 upregulated and downregulated genes across all RNA-seq samples.

D: qPCR for upregulated and downregulated genes.

For all qPCR experiments, a technical triplicate was performed for two biological replicates. Expression was normalized to *HPRT1* and *TBP*. Error bars represent the standard error of the mean.

R: replicate, dox: doxycycline, FDR: false discovery rate.

Figure 2. BORIS promotes a switch from a proliferative to invasive gene signature in the MM057 melanoma cell line.

A-C: GSEA on the RNA-seq data for: **A-** biological processes and the hallmark EMT gene set from the Molecular Signatures Database, **B-** the invasive, and **C-** proliferative gene signatures.

D, E: BORIS expression was induced in the MM057 cell line using 50ng/ml dox for 5 days followed by qPCRs for **D-** invasive and **E-** proliferative genes. For each sample, a technical triplicate was performed for two biological replicates. Expression was normalized to *HPRT1* and *TBP*. Error bars represent the standard error of mean.

GO: gene ontology (biological process), H: Hallmark gene set, NES: normalized enrichment score, FDR: false discovery rate.

Figure 3. BORIS expression in the MM057 melanoma cell lines results in reduced proliferation.

A: Cell proliferation upon expression of BORIS protein using an increasing dose of dox for 3, 5 or 7 days of cell culture.

B: Whole cell lysate from cells with or without 50ng/ml dox for 5 days was used for immunoblotting with anti-BORIS, anti-cleaved PARP (c-PARP) and anti-PARP antibodies. Anti-Tubulin is used as a loading control.

C: Bar graph representing the mean percentage of Annexin V and PI positive cells as determined by flow cytometry. BORIS expression was induced with 50ng/ml dox for 5 days.

D: Representative image of the percentage of apoptotic and necrotic cells as determined by flow cytometry analysis of Annexin V and PI staining.

* indicates significantly different from untreated (*$p<0.05$. **$p<0.01$, ***$p<0.001$).

Error bars represent the standard error of mean.

Figure 4. BORIS expression results in increased migration and invasion of melanoma cells with a proliferative gene signature.

A, B: *in vitro* transwell cell migration upon induction of BORIS expression with 100ng/ml dox for 5 days. Bar graphs display the average number of cells per field that migrated through the membrane and are accompanied by a representative image.

C, D: *in vitro* transwell cell invasion upon induction of BORIS expression with 100ng/ml dox for 5 days. Bar graphs display the average number of cells per field that invaded through the membrane coated with Matrigel and are accompanied by a representative image.

For each assay, images of six fields were captured and counted using the Cell Counter option in ImageJ. Three independent experiments were performed, each consisting of two technical replicates. * indicates significantly different from untreated (*$p<0.05$. **$p<0.01$, ***$p<0.001$).

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Figure 1

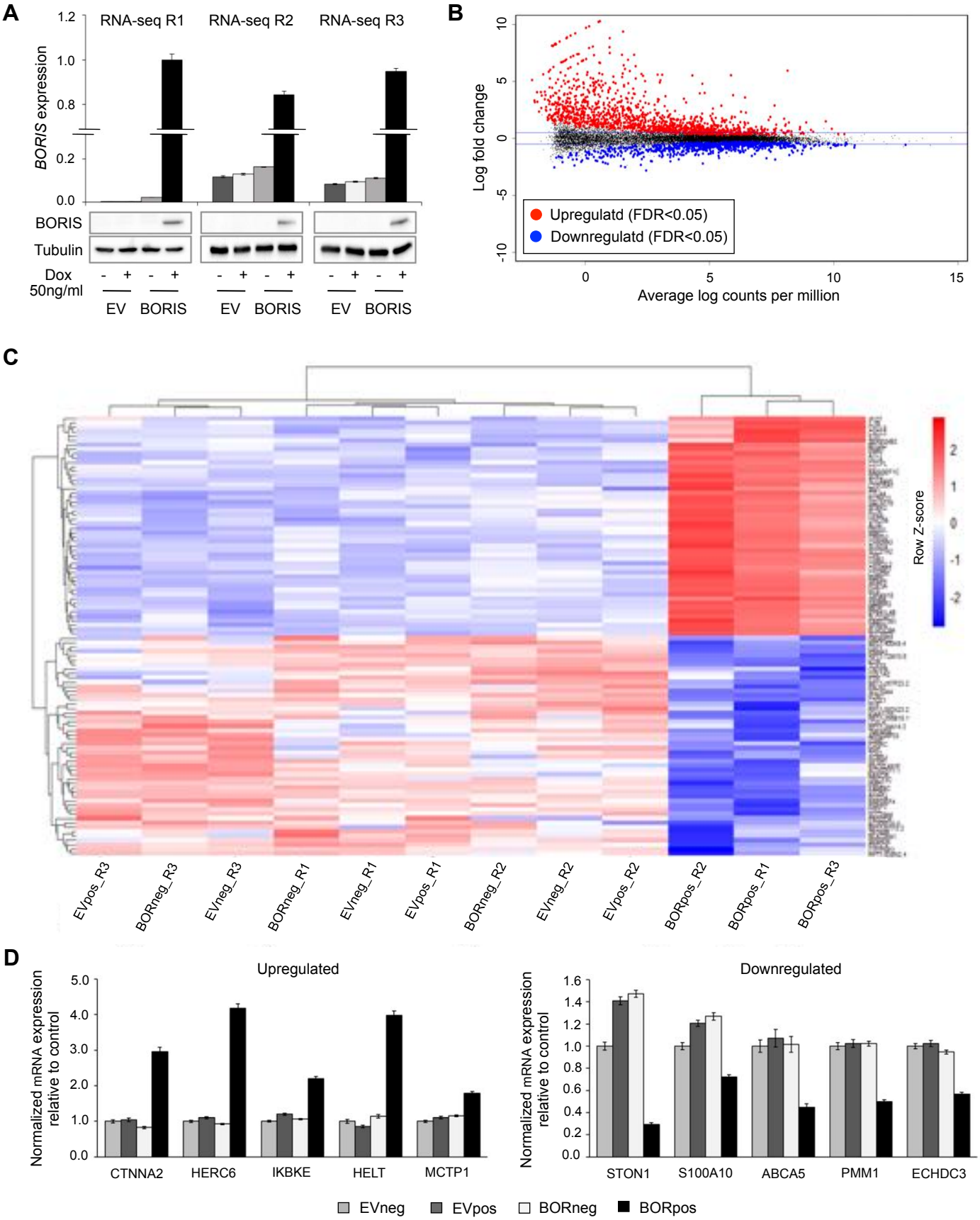
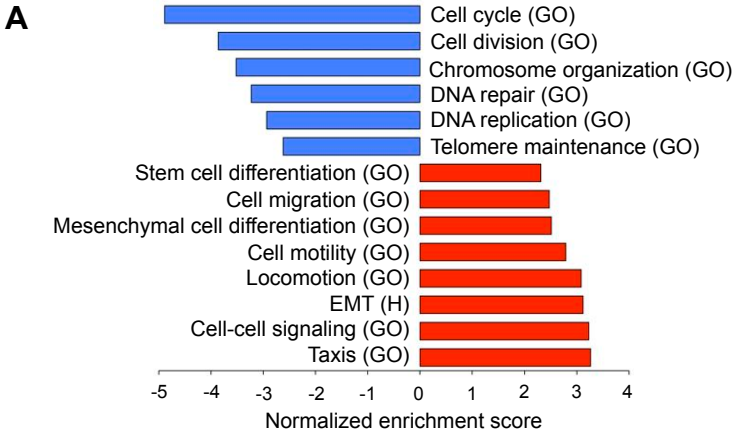
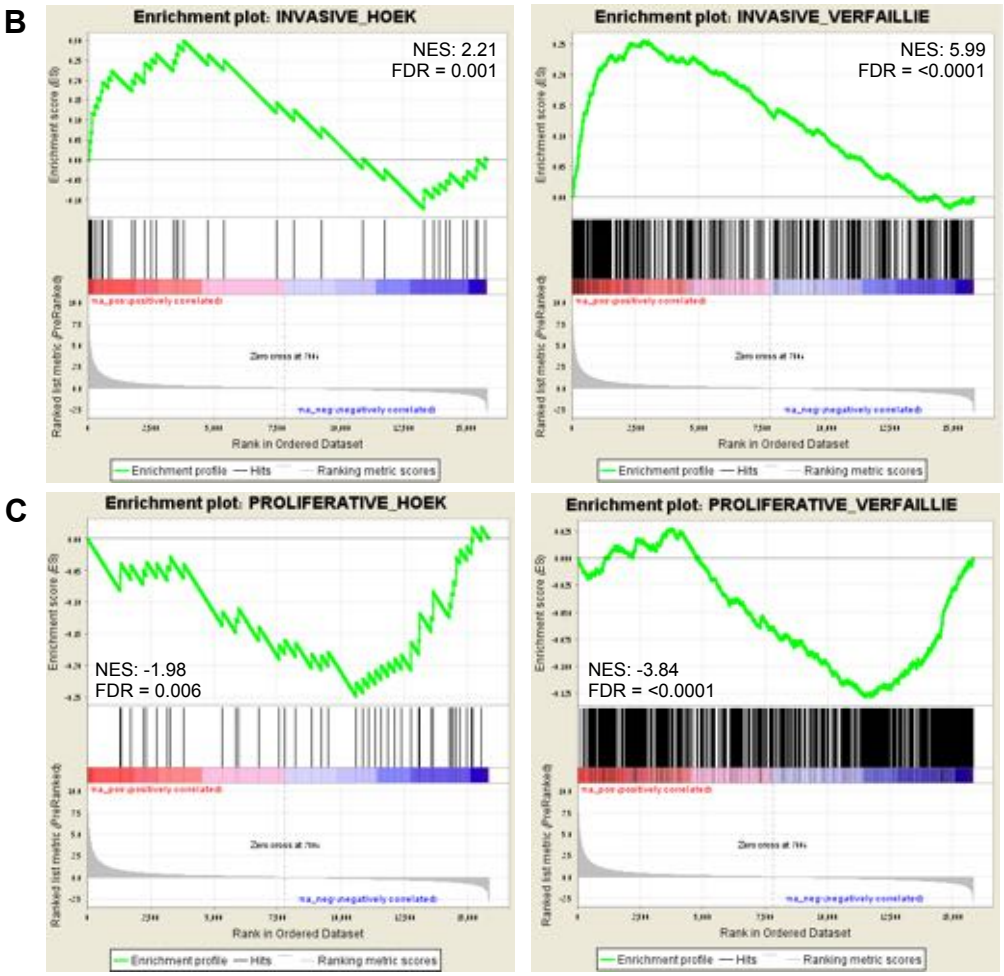
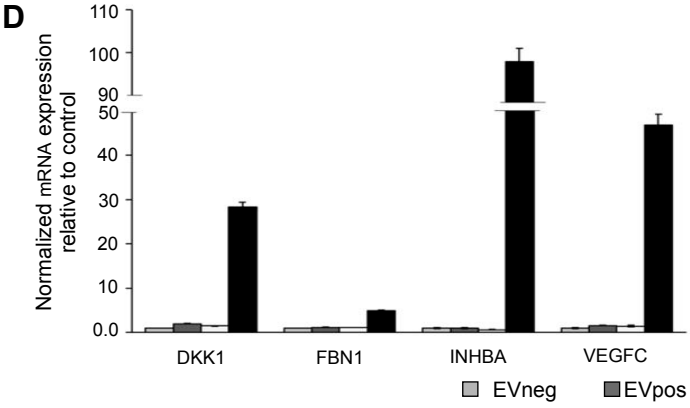

A
RNA-seq R1
RNA-seq R2
RNA-seq R3
BORIS expression
1.2
1.0
0.8
0.2
0.0
BORIS
Tubulin
Dox 50ng/ml
- + - +
EV BORIS
B
Log fold change
Average log counts per million
Upregulatd (FDR<0.05)
Downregulatd (FDR<0.05)
C
Row Z-score
EVpos_R3
BORneg_R3
EVneg_R3
BORneg_R1
EVneg_R1
EVpos_R1
BORneg_R2
EVneg_R2
EVpos_R2
BORpos_R2
BORpos_R1
BORpos_R3
D
Upregulated
Downregulated
Normalized mRNA expression relative to control
CTNNA2
HERC6
IKBKE
HELT
MCTP1
STON1
S100A10
ABCA5
PMM1
ECHDC3
EVneg
EVpos
BORneg
BORpos

Figure 2


A
Cell cycle (GO)
Cell division (GO)
Chromosome organization (GO)
DNA repair (GO)
DNA replication (GO)
Telomere maintenance (GO)
Stem cell differentiation (GO)
Cell migration (GO)
Mesenchymal cell differentiation (GO)
Cell motility (GO)
Locomotion (GO)
EMT (H)
Cell-cell signaling (GO)
Taxis (GO)
-5 -4 -3 -2 -1 0 1 2 3 4
Normalized enrichment score


B
Enrichment plot: INVASIVE_HOEK
NES: 2.21
FDR = 0.001
Enrichment plot: INVASIVE_VERFAILLIE
NES: 5.99
FDR = <0.0001
C
Enrichment plot: PROLIFERATIVE_HOEK
NES: -1.98
FDR = 0.006
Enrichment plot: PROLIFERATIVE_VERFAILLIE
NES: -3.84
FDR = <0.0001
Rank in Ordered Dataset
Enrichment profile
Hits
Ranking metric scores


D
Normalized mRNA expression relative to control
110
100
90
50
40
30
20
10
0.0
DKK1
FBN1
INHBA
VEGFC

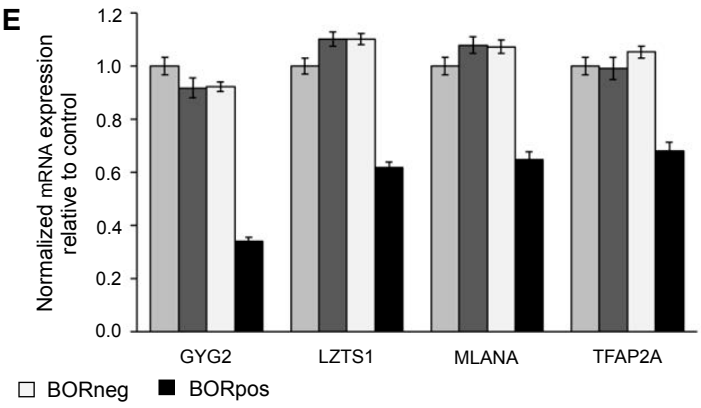
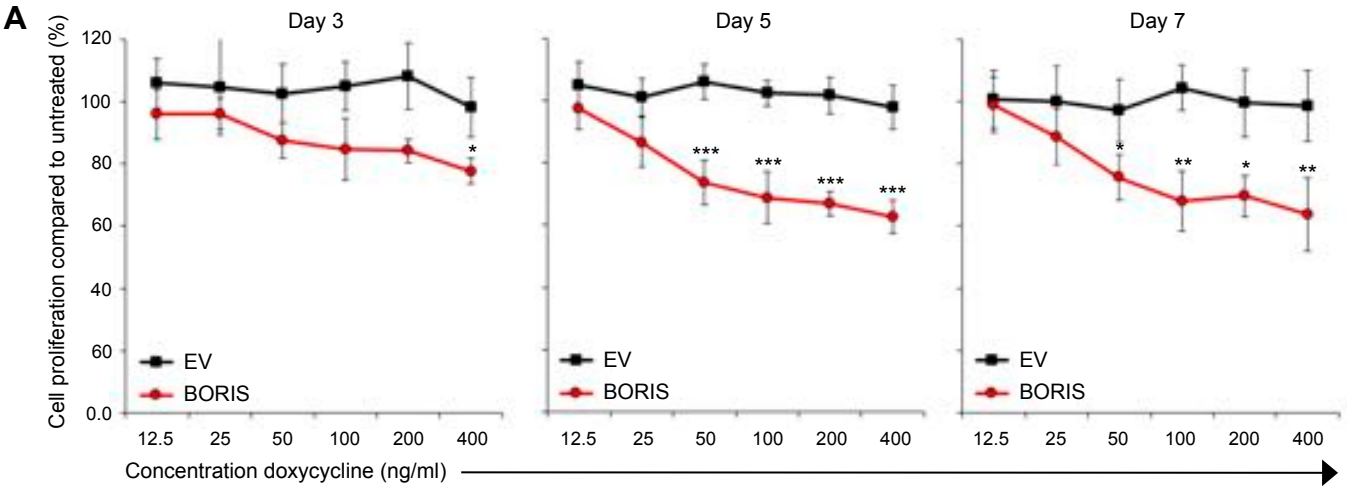
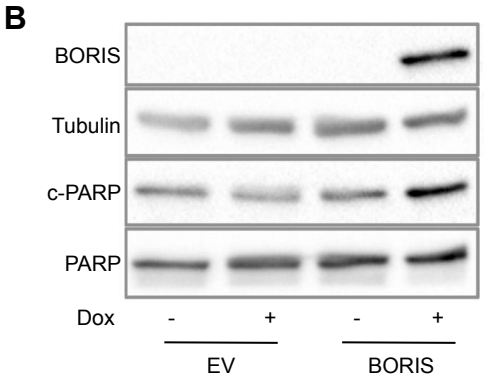
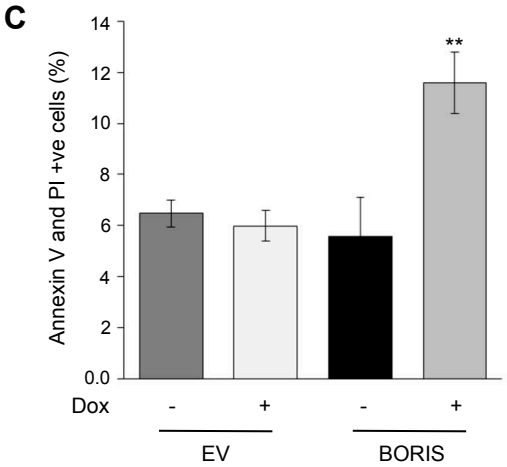

E
Normalized mRNA expression relative to control
1.2
1.0
0.8
0.6
0.4
0.2
0.0
GYG2
LZTS1
MLANA
TFAP2A
EVneg
EVpos
BORneg
BORpos

Figure 3


A
Day 3
Day 5
Day 7
Cell proliferation compared to untreated (%)
120
100
80
60
40
60
0.0
EV
BORIS
12.5
25
50
100
200
400
Concentration doxycycline (ng/ml)


B
BORIS
Tubulin
c-PARP
PARP
Dox
-
+
EV
BORIS


C
Annexin V and PI +ve cells (%)
14
12
10
8
6
4
2
0.0
**
Dox
-
+
EV
BORIS

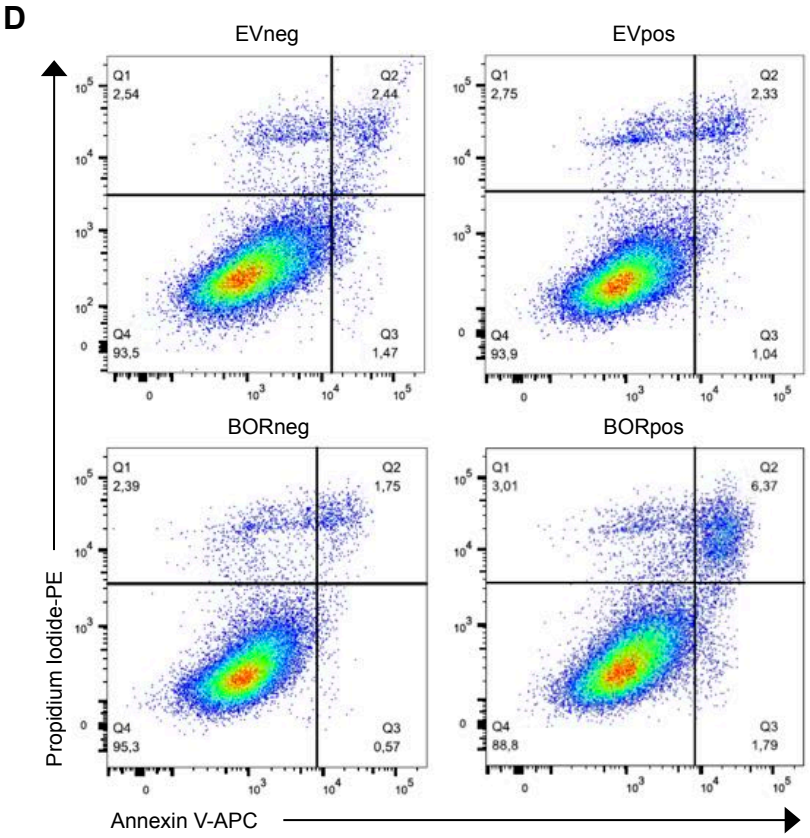
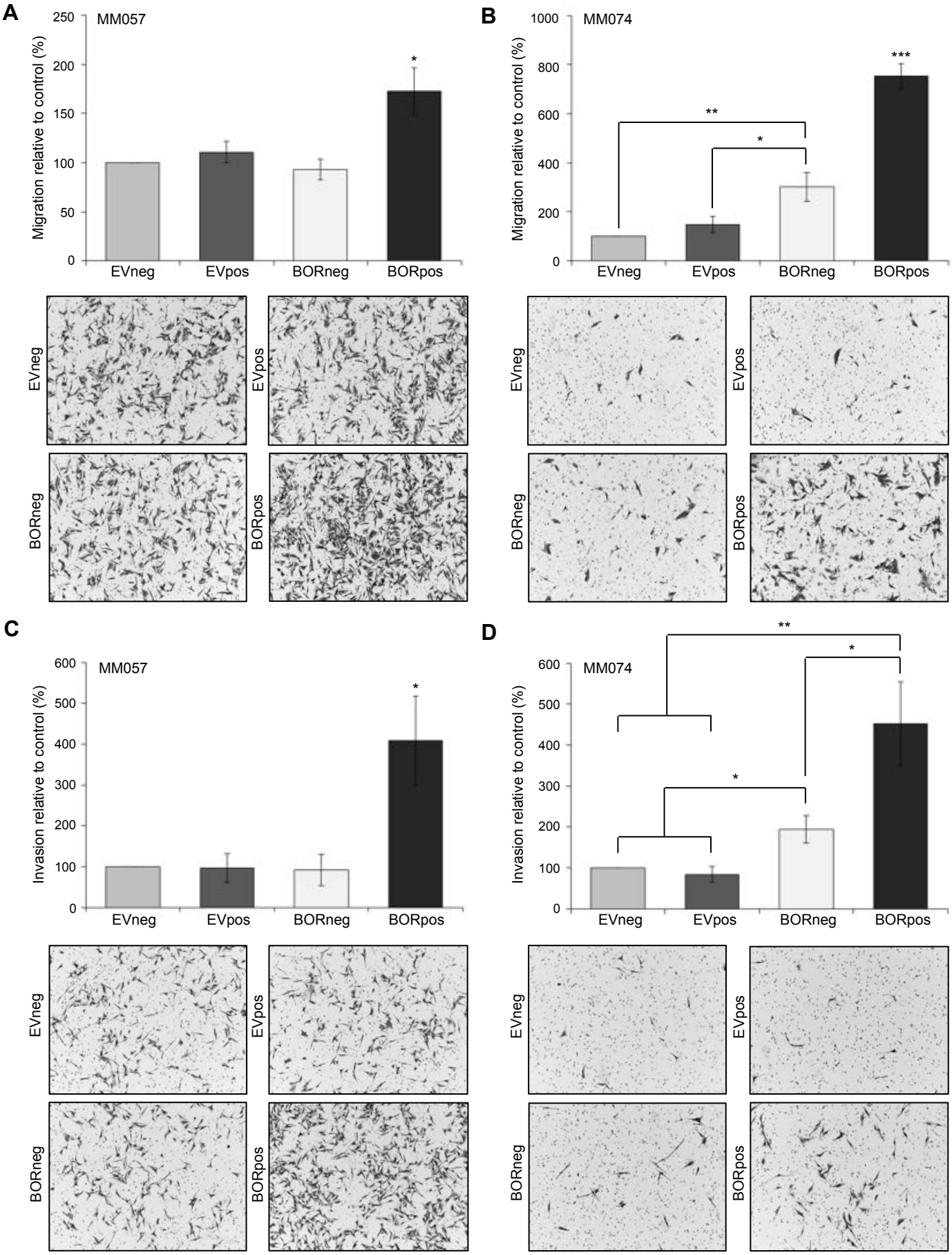

D
EVneg
EVpos
BORneg
BORpos
Q1 2,54
Q2 2,44
Q4 93,5
Q3 1,47
Q1 2,75
Q2 2,33
Q4 93,9
Q3 1,04
Q1 2,39
Q2 1,75
Q4 95,3
Q3 0,57
Q1 3,01
Q2 6,37
Q4 88,8
Q3 1,79
Propidium Iodide-PE
Annexin V-APC

Figure 4


A
MM057
Migration relative to control (%)
250
200
150
100
50
0
*
EVneg
EVpos
BORneg
BORpos
B
MM074
Migration relative to control (%)
1000
800
600
400
200
0
**
*
***
EVneg
EVpos
BORneg
BORpos
C
MM057
Invasion relative to control (%)
600
500
400
300
200
100
0
*
EVneg
EVpos
BORneg
BORpos
D
MM074
Invasion relative to control (%)
600
500
400
300
200
100
0
**
*
*
EVneg
EVpos
BORneg
BORpos