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ABCC4/MRP4 contributes to the aggressiveness of Myc-associated epithelial ovarian cancer

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Novelty & Impact Statements: Epithelial ovarian cancer (EOC) is a complex disease, however tailored therapies for the heterogeneous disease are yet to be developed. Here we demonstrated that the Myc-regulated Multidrug resistance protein 4 (MRP4) transporter has significant impact on patient outcome in distinct histological and molecular EOC subtypes in which Myc onco-proteins are highly expressed. Moreover, we found evidence for dual roles of MRP4 in EOC biology and drug efflux, suggesting MRP4 to be a potential therapeutic target within certain EOC tumors.

Abbreviations

ABC: The ATP-binding cassette family of transporters, AOCS: Australian Ovarian Cancer Study, EOC: Epithelial ovarian cancer, HG-SOC: High grade serous EOC, MRP: Multidrug resistance protein, PARP: Poly(ADP-ribose) polymerase,

Key words: ABCC4/MRP4, Epithelial ovarian cancer, Novel therapeutic targets, Myc-driven cancers

Abstract

Epithelial ovarian cancer (EOC) is a complex disease comprising discrete histological and molecular subtypes, for which survival rates remain unacceptably low. Tailored approaches for this deadly heterogeneous disease are urgently needed. Efflux pumps belonging to the ATP-binding cassette (ABC) family of transporters are known for roles in both drug resistance and cancer biology and are also highly targetable. Here we have investigated the association of *ABCC4*/MRP4 expression to clinical outcome and its biological function in endometrioid and serous tumors, common histological subtypes of EOC. We found high expression of *ABCC4*/MRP4, previously shown to be directly regulated by c-Myc/N-Myc, was associated with poor prognosis in endometrioid EOC (*P=0.001*) as well as in a subset of serous EOC with a "high-*MYCN*" profile (C5/Proliferative; *P=0.019*). Transient siRNA-mediated suppression of MRP4 in EOC cells led to reduced growth, migration and invasion, with the effects being most pronounced in endometrioid and C5-like serous cells compared to non-C5 serous EOC cells. Sustained knockdown of MRP4 also sensitized endometrioid cells to MRP4 substrate drugs. Furthermore, suppression of MRP4 decreased the growth of patient-derived EOC cells *in vivo*. Together, our findings provide the first evidence that MRP4 plays an important role in

the biology of Myc-associated ovarian tumors and highlight this transporter as a potential therapeutic target for EOC.

Introduction

Epithelial ovarian cancer (EOC) accounts for almost 90% of ovarian cancer and is one of the deadliest gynecological malignancies in women. Due to the non-specific symptoms of the disease and a paucity of biomarkers for early detection, EOC is usually diagnosed at an advanced disease stage with 5-year survival rates of 10-40% [1, 2]. EOC is a complex malignancy comprising of histologically, molecularly and clinically distinct tumors that vary widely in terms of genetic profile, response to chemotherapy and clinical behaviors [3-7].

Despite the heterogeneous nature of the disease, the current standard treatment for advanced EOC patients is similar for all patients, being cytoreductive surgery followed by platinum/taxane based chemotherapy, with the addition of an anti-angiogenic agent, bevacizumab, an anti-angiogenic agent in high-risk patients [8-11]. Unfortunately, even with the high initial response to treatment, most patients relapse and subsequently develop resistance to

chemotherapy. Salvage treatment for recurrent disease includes other chemotherapeutic agents, such as pegylated liposomal doxorubicin, docetaxel, topotecan, gemcitabine or vinorelbine, however objective response rates to the second line non-platinum chemotherapies have remained below 30% [12, 13]. Thus, a better understanding of the factors contributing to EOC biology and a refined approach to identify targets for this deadly complex disease are critically important to improve treatment outcome for individual patients. The introduction of poly(ADP-ribose) polymerase (PARP) inhibitors has shown substantial benefit in treating EOC patients with BRCA1 or BRCA2 mutations [14, 15], however tailored therapies for other subsets of EOC are yet to be developed.

Multidrug resistance protein (MRP) 4, encoded by the *ABCC4* gene, belongs to the C branch of the ATP-binding cassette (ABC) superfamily of transporters and is capable of mediating ATP-dependent efflux of endogenous and exogenous molecules [16]. MRP4/*ABCC4* has a particularly broad substrate specificity ranging from antiviral agents to anticancer drugs, such as methotrexate, 6-thioguanine, 6-mercaptopurine, topotecan and irinotecan [17, 18]. Consistent with functioning in drug efflux in EOC, MRP4 was recently reported to be enriched in recurrent tumors [19] and its expression was associated with chemo-resistance [20]. In addition, MRP4 transports a wide variety of endogenous signaling molecules, such as cAMP, ADP, cGMP, prostaglandins, leukotrienes and folic acid [17, 18], implying functional roles in cellular biology. Accordingly, a growing body of evidence implicates MRP4 in many human disease states. For instance, MRP4 has been found to promote platelet aggregation and smooth muscle cell proliferation in cardiovascular diseases [21, 22] and more recently the transporter protein was highlighted in tumor biology through studies demonstrating that MRP4 mediates cell

proliferation, migration and/or differentiation in a range of cancer types, including lymphoma [23], leukemia [24], non-small cell lung cancer [25] and neuroblastoma [26]. Of particular interest, high MRP4 expression was strongly associated with a poor patient outcome independent of drug efflux in neuroblastoma [26, 27].

Additional studies of MRP4 in neuroblastoma, which is commonly driven by aberrant activity of the N-Myc onco-protein, revealed that MRP4 is under direct transcriptional control of N-Myc [28]. The Myc proto-oncogene family members (*MYC*, *MYCN* and *MYCL1*) encode nuclear transcription factors that can globally regulate the expression of their downstream target genes involved in multiple cellular processes [29], and thus dysregulated Myc has been implicated in many human malignancies. A genome-wide profiling study of EOC revealed that amplification of the 8q24 chromosomal region that harbors *MYC* is one of the most common focal amplifications in high grade serous EOC (HG-SOC), although the major activated pathways identified across the cohort in that study did not include the Myc pathway [30]. However, additional gene expression profiling studies have identified molecular subtypes of EOC with signal activation pathways involving either c-Myc [6] or N-Myc [7] and a significant association between the level of overall Myc activity and clinical outcome in the high N-Myc subtype of HG-SOC [31], suggesting highly enriched Myc activity in subsets of EOC.

Despite c-Myc/N-Myc being attractive targets in a wide variety of cancers, they have currently proven to be 'undruggable' due largely to the lack of small molecule binding domains. However, the identification of specific downstream molecular targets within subsets of EOC tumors with hyperactive Myc presents the opportunity for more tailored treatment for this complex malignancy. It is of interest to note that MRP4 is a known direct transcription target

of c-Myc or N-Myc oncoprotein, depending on the predominant Myc family member in a given cancer type [26, 28], and is also highly targetable using pharmacological inhibitors that are currently available [26-28, 32-35].

In the present study we investigated *ABCC4*/MRP4 expression in tumor samples selected from large cohorts of women with endometrioid and serous EOC and utilized *in vitro* and *in vivo* models to study its biological roles in distinct histological and molecular subtypes of EOC. Our results suggest that MRP4 represents a valid molecular target in Myc-associated ovarian cancers with therapeutic potential for the treatment of this poor outcome tumor type.

Materials and Methods

Patient cohorts

Samples from 150 patients with serous EOC and 61 patients with endometrioid EOC were obtained from the Australian Ovarian Cancer Study (AOCS), a population-based case-controlled study undertaken between 2002 and 2006. An additional 19 endometrioid EOC samples were separately obtained from the Gynecological Oncology Biobank at Westmead Hospital (GynBiobank), Sydney, Australia. Fresh frozen tumor specimens were obtained at the time of surgical debulking, prior to chemotherapeutic exposure. Samples were verified as containing at least 70 % tumor tissue. Both serous and endometrioid EOC cohorts consist of tumors from all surgical stages (according to FIGO classification) with median clinical follow-up of 46.4 months. Clinicopathological characteristics for the serous and endometrioid EOCs are summarized in Table S1 and S2 respectively. Matched genomic DNA and RNA samples

were extracted using standard methods. The use of tissue samples and medical record data was approved for each patient cohort by the individual Institutional Review Boards. This project was approved by the University of New South Wales Human Research Ethics Committee (HC12551).

Cell lines

27/87 [36] and COV318 (RRID:CVCL_2419) cell lines were gifts from Terry Hurst (University of Queensland, Brisbane, Australia) and Deborah Marsh (Kolling Institute of Medical Research, Sydney, Australia) respectively. HEY (RRID:CVCL_0297) was a generous gift from Georgia Chenevix-Trench (QIMR Berghofer, Brisbane, Australia). Neuroblastoma cell lines, NBL-S (RRID:CVCL_2136) and SK-N-BE(2)-C (RRID:CVCL_0529), were gifts from Susan L. Cohn (Northwestern University, Chicago, IL, USA) and Barbara Spengler (Fordham University, New York, NY, USA) respectively. MCF7 (RRID:CVCL_0031) cell line was kindly provided by Rob Sutherland (Garvan Institute, Sydney, Australia). HG-SOC patient-derived xenograft cell line (WEHICS27) [37] was kindly provided by Clare L. Scott (The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia). All cells were cultured in either DMEM (COV318, NBL-S, SK-N-BE(2)C and MCF7) or RPMI1640 (HEY and 27/87) media supplemented with 10% FCS, except WEHICS27 cells that were cultured in DMEM/F12 media supplemented with GlutaMAX, epithelial growth factor (0.5mg/ml), insulin (1mg/ml), hydrocortisone (0.5mg/ml) and 10% FCS. Cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C and were mycoplasma free. All cell lines were authenticated using

short tandem repeat profiling at Cell Bank Australia and Kinghorn center for clinical genomics, Australia within the last three years.

Immunohistochemistry (IHC)

Tissue microarray sections containing tumor samples from 144 and 26 women diagnosed with serous and endometrioid EOC respectively were obtained from GynBiobank at Westmead Hospital. Cores of the formalin-fixed paraffin-embedded tumors were stained with hematoxylin and eosin (H&E) and with MRP4 (rat monoclonal anti-MRP4 M4I-10, Abcam at dilution 1:20), c-Myc (rabbit monoclonal anti-c-Myc Y69, Abcam, at 1:200 dilution) or N-Myc (mouse monoclonal anti-N-Myc NCM II 100, Abcam at dilution 1:200). Photos of the cores were taken with Olympus BX53 microscope with Olympus DP73 camera at 400x magnification. Cores with positive staining for MRP4, c-Myc, or N-Myc were then stratified according to the intensity of staining for c-Myc as follows: score 0: negative, ≥1 and <2: weak, ≥2 and <3: moderate, >3: strong membrane staining for MRP4 or nuclear staining for c-Myc and N-Myc.

Quantitative real-time PCR (qPCR)

qPCR was performed to determine gene expression levels of *MYC*, *MYCN*, or *ABCC4* with the 7900HT Fast Real-Time PCR system (Applied Biosystems). RNA was reverse-transcribed using MMLV reverse-transcriptase (Life Technologies, Sydney, Australia) as previously described [38]. mRNA expression levels of *MYC* (ID: Hs00905030_m1), *MYCN* (ID:

Hs00232074_m1), or *ABCC4* (ID: Hs00988708_m1) were detected using TaqMan® gene expression assay in 96-well plate format (20ng cDNA (mRNA equivalent) per well). Gene expression levels were quantified in relation to the expression of control genes (*HPRT* (ID: Hs99999909_m1) and *GUSB* (ID: Hs99999908_m1) using the ΔΔCt method and the average of the expression values was calculated.

Transfections

Transient siRNA transfection of ABCC4 in HEY, 27/87 and COV318 cells was carried out using Lipofectamine RNAiMAX reagent (Life Technologies, Mulgrave Australia) and 20 nM of each siRNA (Dharmacon, Lafayette, CO, USA) alongside a non-targeting control siRNA duplex (Ctrl; Dharmacon) according to manufacturer's protocol. Briefly, 20nM of siRNA per well was transiently transfected into 1.5 x 10^5 (27/87), 8 x 10^4 (HEY) or 2.5 x 10^5 (COV318) grown in 6-well plates. Suppression of MRP4 protein expression was examined at intervals up to 96 hours post-transfection. Unless stated otherwise, siRNA transfected cells were used for colony formation, migration and invasion assays following 48 hours transfection. Stable 27/87 and a patient derived cell line, WEHICS2737 expressing doxycycline-inducible MRP4 shRNA were generated by lentiviral transduction with the pFH1UTG vector39. Viral packaging was achieved using the psPAX2 and pMD2.G plasmids (a gift from Didier Trono, Lausanne, Switzerland). MRP4 knockdown was induced using 1 μg/mL doxycycline (72 hours treatment). Transient or stable suppression of MRP4 expression were confirmed by western blotting. Sequences of MRP4 siRNA/shRNA are listed in Table S4.

Protein extraction and Western blot analysis

Whole-cell extract was prepared with lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.2% Nonidet P40, 50 mM NaF, 5 mM EDTA, 0.1 mM orthovanadate, plus protease inhibitor cocktail (Sigma-Aldrich)). Protein (20 µg per well) was loaded onto a 4-15% SDS-PAGE gel (BIO-RAD), and were then transferred to Nitrocellulose membrane (BIO-RAD), followed by primary and secondary antibody incubation. The antibodies used were against MRP4 (rat monoclonal M4I-10; Enzo Life Sciences; 1:1000), C-Myc (mouse monoclonal 9E10; Sigma-Aldrich; 1:5000), N-Myc (mouse monoclonal B8.4.B; Santa Cruz; 1:2000), cleaved PARP (rabbit polyclonal Asp214, Cell signaling Technology, 1:2000), γH2AX (rabbit monoclonal 20E3, Cell signaling technology, 1:1000) and ß-actin (rabbit polyclonal A2066; Sigma-Aldrich; 1:5000). Signals were detected using Clarity™ ECL Western Blotting Substrate (BIO-RAD).

Colony formation assays

Following 24h siRNA transfection, HEY, 27/87 or COV318 cells were replated into 6-well plates at a density of 200 cells/well, 300 cells/well, or 250 cells/well respectively, and incubated for 6–12 days. For 27/87 cells with inducible MRP4 shRNA, cells were seeded into 6-well plates and allowed to adhere in the presence or absence of 1µg/mL doxycycline per well overnight. The cytotoxic drug, (SN-38 at 0.25 – 2 nM, topotecan at 1 – 25 nM or cisplatin at 0.25 – 2 µM), was then added into appropriate wells and cells were incubated for 72 h. Medium was replaced and cells were incubated a further 7-8 days. Colonies were then fixed and stained

with 0.5% w/v Crystal Violet in 50% methanol, counted, and expressed as % colony formation relative to untreated cells. Plates were scanned and colonies counted using ImageJ.

Transwell migration and invasion assays

Prior to performing migration and invasion assays, cells were siRNA transfected for 48 h. For transwell migration assays, 2 x 10^4 27/87 cells, 3 x 10^4 HEY cells, or 5 x 10^4 COV318 cells were suspended in serum free medium and seeded into 24-well Transwell migration chambers (8µm-pore PET membrane, BD Biosciences). For HEY cells, 5% FBS was added to the lower chamber of the 24-well companion plate (BD Bioscience) followed by incubation for 5 h, while 10% FBS was used for 27/87 and COV318 with incubation for 16 h. Transwell invasion assay was performed using the BioCoat Growth Factor Reduced Matrigel Invasion Chamber for 24 well plates with an 8µm pore PET membrane (BD Biosciences). 1 x 10^4 27/87 cells or 2 x 10^4 HEY cells were seeded into the top chamber with medium containing 1% and RPMI medium containing 10% FBS was added into the lower chamber. Cells were incubated 8 or 30 hr for HEY or 27/87 cells respectively. Cells were fixed in 100% methanol for 20 mins. After air-drying, non-migrated or non-invaded cells were stained with Light Green SF Yellowish dye (1.25% in Milli-Q water; MP Biomedicals) and Safranin O dye (0.2% in Milli-Q water; MP Biomedicals) was used to stain migrated or invasive cells. Five random fields were taken with Olympus CKX41 microscope (Olympus) with QImaging Micro Publisher 3.3RTV camera (QImaging), under 20x objective and cells were counted using ImageJ (NIH, Bethesda, MD, USA).

BrdU cell Proliferation Assay

Cell proliferation was measured using the Cell Proliferation ELISA BrdU colorimetric kit (Roche) according to the manufacturer's instructions. Cells were mixed with Lipofectamine RNAiMAX reagent (Life Technologies, Mulgrave Australia) and 20 nM of each siRNA (Dharmacon, Lafayette, CO, USA) or non-targeting control vector and seeded into 96 well plates in duplicate at 3000 cells per well for HEY and 27/87 and 5000 cells per well for COV318 with additional duplicate wells for assay controls. Following 48 hrs in culture, cells were labelled with BrdU for 6 hrs at 37ºC. Cells were fixed, denatured and BrdU probed using the solutions provided. The absorbance was measured at 370 nm (reference wavelength at 492 nm) using the Benchmark Plus Microplate Reader and MicroPlate Manager Software (Version 5.2.1) (Bio-Rad, Hercules, CA, USA).

Cell cycle analysis

The effect of MRP4 suppression on cell cycle phase distribution in EOC cells was analyzed. Cells were fixed at 48, 72 or 96 hrs post-transfection for HEY, 27/87 or COV318 cells respectively and incubated for 15 mins in 100 µl of PBS containing propidium iodide (50µg/ml; Sigma-Aldrich) and RNaseA (100µg/ml; Sigma-Aldrich) before analyzing on the FACScalibur flow cytometer (BD Biosciences, San Jose, CA, USA). The proportion of cells in G1, G2 and S phase was analyzed using FlowJo software.

Human ovarian cancer xenograft

For xenograft experiments, 5 x10^6 WEHICS27 cells stably transduced with non-silencing control, MRP4.6 or MRP4.7 inducible shRNAs were subcutaneously engrafted into one flank of NOD scid gamma (NSG) mice. Tumors were measured every second day using a vernier caliper. When tumors reached 75 – 100 mg, mice either remained on control diet or were switched to a diet supplemented with 600 mg/kg doxycycline (Specialty Feeds, Glen Forrest, WA, Australia). Mice were sacrificed when tumors reached 1000 mg, and tumors were taken for analysis. Tumors were examined histologically to determine tumor morphology and viability following H&E staining. Tumor sections were stained for MRP4 (rat monoclonal M4I-10; Enzo Life Sciences; 1:1000), which was scored by an investigator blinded as described previously [37]. Briefly, percent of strong (+++), moderate (++), low (+) and absent staining was documented ((+++% × 3) + (++% × 2) + (+% × 1) + (0% × 0), out of a possible total score of 300). All animal studies were approved by the University of New South Wales Animal Care and Ethics Committee and conducted according to the Animal Research Act, 1985 (New South Wales, Australia) and the Australian Code of Practice for Care and Use of Animals for Scientific Purposes (2013), approval number 16/93B.

Statistical Analysis

For MRP4 gene expression analysis in patient cohorts, the statistical methodology described in detail in London *et al.* [40] was used to determine the optimal cut-off points between low and high expression in each cohort in this study. Briefly, tumors were repeatedly divided into either

"high" or "low" groups with cut-off points at lower quartile, median or upper quartile (UQ) of Myc family gene expression and signature score. For each cut-off, a Cox regression model was used to produce a P-value and hazard ratio (HR) using SPSS software *v*22 (IBM). The one with the highest HR was selected as the optimal cut-off. Kaplan-Meier survival analysis was conducted at the optimal cut-off and compared by two-sided log-rank tests. A Cox proportional hazards model was used for univariate and multivariate comparisons to generate HR and 95% confidence intervals (95% CI). Comparisons of gene expression values between tumor groups were made using either Welch's t-test or one-way ANOVA as appropriate. Fisher's exact test was used to study the association between clinicopathological characteristics. Data visualization of the subgroup tests and correlation were performed with GraphPad Prism v6.01 for Windows (GraphPad software, Sydney, Australia). For all other *in vitro* experiments including colony formation, migration and invasion assays, when comparing more than two groups, One-Way ANOVA with Dunnett's multiple comparison test was performed using GraphPad Prism. For all in vitro experiments, a minimum of three independent, biological replicates were performed.

Results

High MRP4 mRNA expression is associated with poor outcome in "high-MYCN" subtypes of EOC

We examined the relationship between *ABCC4* gene expression and clinical outcome in the two common histological subtypes of EOC, endometrioid and serous tumors. Kaplan-Meier analysis revealed that high *ABCC4* expression was associated with a shorter progression-free

survival (PFS: *P*=0.001; HR=4.295 (95% CI:1.690-10.918)) or overall survival (OS: *P*=0.006; HR=3.869 (95% CI:1.357-11.029)) in endometrioid EOC (n=80; Figure 1A and B). In contrast, no association between *ABCC4* expression and clinical outcome was found in the overall serous cohort (PFS: *P*=0.399; HR=1.188 (95% CI:0.796-1.773); OS: *P*=0.950; HR=1.016 (95% CI:0.613-1.686) (Figure 1C, 1D). In light of the molecularly defined subtypes of serous EOC, including a "high-*MYCN*" C5 molecular subtype of HG-SOC [5], and the observation that *ABCC4* is a downstream target gene of c-Myc/N-Myc [28], the relationship between *ABCC4* and clinical outcome was further tested in this subset of patients. Of the 150 serous EOC tumors tested in this study, 14 cases were identified to be C5-like tumors and as expected found to have higher than average *MYCN* expression (Fig 2A). Within this subset high *ABCC4* gene expression was significantly associated with poor clinical outcome (PFS: *P*=0.019; HR=4.187 (95% CI:1.169-14.990) and OS: *P*=0.027; HR=5.202 (95% CI:1.031-26.258)) (Figure 1E, 1F), but not in any other molecular subtypes of HG-SOC, including C1, C2 and C4 (Data not shown).

Since an association of high *ABCC4* with poor outcome was observed in endometrioid tumors, we further examined *MYCN* gene expression levels using RT-qPCR in the endometrioid and serous EOC cohorts and found that like the C5 tumors, endometrioid tumors expressed higher *MYCN* compared to serous tumors (One-way ANOVA and Dunnett's post-hoc test *P=0.003*; Figure 2A). Furthermore, twenty-six endometrioid tumors, which included 17 cases that had been analyzed for *MYC* and *MYCN* mRNA levels, were characterized by immunohistochemical staining for c-Myc, N-Myc and MRP4 proteins. As expected, MRP4 staining is confined to the membrane while staining for both c-Myc and N-Myc was exclusively nuclear (Figure 2B). N-Myc staining score positively correlated with MRP4 staining intensity scores in these tumors

(Spearman's r=0.54, *P*=0.004) (Table S3). Additionally, the two cases with the highest MRP4 staining score exhibited positive staining for both N-Myc and c-Myc proteins (Figure 2B), suggesting that MRP4 may be highly activated in the context of Myc regulation in certain EOC subsets. Together, these data highlight the relationship between Myc transcription factors and MRP4 in subsets of EOC.

Suppression of MRP4 impairs growth, migration and invasion ability of EOC cells

We sought to investigate the biological relevance of MRP4 in the different EOC subtypes using representative ovarian cancer cell lines. MRP4 and Myc protein expression levels were determined in a panel of EOC cell lines (Figure S1). Cell lines representing endometrioid (27/87) and serous (HEY) EOC, that expressed appreciable levels of MRP4 and Myc proteins and were also suitable for phenotypic assays, were selected. Depletion of MRP4 was achieved using two distinct MRP4 siRNA duplexes in 27/87 endometrioid and HEY serous EOC cells. Each of two independent siRNA sequences markedly reduced MRP4 protein expression levels 48hr post transfection although MRP4.5 was less effective than MRP4.6 siRNA (Figure 3A). MRP4 knockdown was found to significantly attenuate colony forming ability of 27/87 cells (MRP4.6: *P*=0.022) and HEY cells (MRP4.5: *P*=0.03 and MRP4.6: *P*=0.036) (Figure 3B). In addition, the migratory and invasive potentials of 27/87 and HEY cells following MRP4 siRNA knockdown were determined by using Transwell migration or invasion assays. We observed that MRP4 depletion markedly impaired cell migration in 27/87 cells with the proportion of migrated cells reduced by more than 60% compared to the control group (one-way ANOVA

P<0.0001; Figure 3C). Similarly, the invasive ability of 27/87 cells was also significantly impaired by MRP4 depletion (Figure 3D). The impact of MRP4 suppression in HEY cells was less clear, whereby only the more effective MRP4.6 siRNA duplex reduced cell migration (Figure 3C) and there was no clear effect on HEY cell invasion (Figure 3D).

To study the effects of MRP4 suppression in a potentially more relevant model of serous EOC, we utilized the HG-SOC cell line (COV318) that was found to express N-Myc (Figure S1) and which, through molecular profiling, had been previously reported to resemble the 'proliferative' subtype defined by TCGA [30] that is equivalent to the C5 molecular subtype in the study by Tothill *et al*.[5]. An additional MRP4 siRNA duplex, MRP4.7, which has similar knockdown efficiency as MRP4.6 siRNA (Figure 3E), was included in these studies to achieve a greater reduction of MRP4 expression than that achieved with MRP4.5 siRNA. Colony forming ability of COV318 cells was significantly impaired following MRP4 depletion with either MRP4.6 and MRP4.7 siRNA, with these two siRNAs being markedly more effective than MRP4.5 (Figure 3E, 3F). In contrast to observations with HEY cells, MRP4 knockdown dramatically reduced migratory ability of the C5-like COV318 cells (Figure 3G) although the effect of MRP4 in cell invasion was unable to be examined due to the poor invasive ability of COV318 cells in Transwell chambers.

To gain insight into the growth effects apparent in the colony assays, we investigated the impact of MRP4 suppression on cell viability, cell cycle and proliferation. We observed that MRP4 suppression decreased the number of viable cells over time compared to control cells in 27/87, HEY and COV318 cell lines when measured daily by trypan blue exclusion assay (Figure 4A). However, cell cycle analysis by flow cytometry and proliferation assay by BrdU incorporation

showed that neither cell cycle progression (Figure S2A) nor proliferation rate (Figure S2B) were affected by MRP4 suppression compared with control in any of the cell lines. We thus further tested whether reduced colony formation could be explained by an increase in apoptosis in 27/87, HEY and COV318 cells by monitoring changes in two of the apoptotic markers: cleavage of PARP, a nuclear DNA-binding protein that recognizes DNA strand breaks, and γ phosphorylation on residue serine 139 of histone variant H2AX (γH2AX), a marker for double-strand breaks in response to DNA damage upon apoptosis [41]. Western blot analysis showed the induction of cleaved PARP at 48hr post-transfection in all three cell lines and induction of γH2AX in HEY by 48hr and in 27/87 and COV318 cells after 72hr transfection of MRP4 siRNA (Figure 4B). These results suggest that apoptosis accounts for decreased EOC cell growth after MRP4 suppression in all three EOC cell lines.

MRP4 suppression decreases EOC tumor growth in vivo

As MRP4 suppression was found to affect the malignant phenotypes of "high-Myc" EOC cells *in vitro*, we sought to study whether MRP4 plays a role in EOC tumor growth *in vivo* using an established HG-SOC patient-derived xenograft cell line, WEHICS27. These cells were determined to express MRP4 and N-Myc protein at similar levels to COV318 cells (Figure S1) and were previously reported to engraft successfully in mice [37]. For sustained suppression of MRP4, WEHICS27 cells were transduced with doxycycline-dependent inducible MRP4 shRNA lentiviral vectors, shMRP4.6 and shMRP4.7, encoding shRNA sequences equivalent to the MRP4.6 and MRP4.7 siRNA duplexes, or with empty vector as a control. Knockdown

of MRP4 following 72hr induction with doxycycline was confirmed by Western blot in both cell lines although MRP4 suppression was less complete with shMRP4.7 than shMRP4.6 (Figure 5A). MRP4 suppression reduced the colony forming ability of WEHICS27 cells, with the degree of growth inhibition correlating with the efficacy of knockdown (Figure 5B). When the same cells were allowed to form subcutaneous tumors in mice, and then administered either control diet or a diet supplemented with doxycycline 7 days later, silencing of MRP4 with shMRP4.6 reduced tumor growth and extended survival time from 12 to 16.5 days in the shMRP4.6 (log-rank P=0.0007; Figure 5C). MRP4 suppression was well-maintained in individual shMRP4.6 tumors harvested at 1000mg, as confirmed by western blotting (Figure 5D) and immunohistochemical staining for MRP4 (Figure 5E). MRP4 suppression with shMRP4.7 was again less complete and did not significantly affect tumor growth rate or overall survival (Figure 5F, 5G).

MRP4 regulates chemotherapy resistance to irinotecan in human endometrioid EOC cell, 27/87.

Given the observed associations above, we next assessed whether MRP4 confers resistance to chemotherapeutic drugs, including the MRP4 substrate drugs, topotecan and irinotecan that comprise some second-line regimens for platinum/paclitaxel-refractory ovarian cancer patients. The effect of MRP4 suppression on drug sensitivity of the shMRP4-transduced 27/87 cells was determined by means of colony formation assays. Sustained MRP4 suppression significantly induced sensitivity to SN38, the active metabolite of irinotecan, resulting in 2.4 and 2.7-fold

sensitization for shMRP4.6 and shMRP4.7 transduced cells, respectively, as compared to the control groups (Table 1, Figure S3A). Furthermore, we observed a trend for increased sensitivity to topotecan following MRP4 depletion whereby shMRP4.6 and shMRP4.7-expressing 27/87 cells were 1.6 and 1.4-fold more sensitive to topotecan, respectively (Table 1, Figure S3B) when compared to the cells with no doxycycline induction, although this did not reach statistical significance in either case. As expected, we found no effect of MRP4 suppression on sensitivity to cisplatin, a drug that is not subject to MRP4-mediated efflux [42, 43] (Table 1, Figure S3C). Therefore, in addition to the biological impact of MRP4 in specific EOC subtypes, these data indicate the potential for MRP4 to influence response to MRP4 substrate drugs in the disease.

Discussion

Prior observations have revealed either drug-dependent or independent roles for MRP4 in cancer cells [23-25, 27, 44] and separate studies have indicated its regulation by Myc family transcription factors [28, 44]. Despite the frequent development of drug resistance and Myc aberrations in EOC, the biological roles of MRP4 and its relationship to Myc, especially within specific subsets of this heterogeneous disease, have been poorly understood. The current study provides the first demonstration that MRP4 has significant impact on patient outcome in distinct histological and molecular EOC subtypes, in which Myc onco-proteins are highly expressed. Moreover, MRP4 not only contributes to a range of malignant characteristics in EOC, including cell/tumor growth, migration, and invasion, it is also able to confer resistance

to pharmacological substrates, irinotecan and topotecan, which highlights this transporter as a potential novel therapeutic target for EOC.

We previously demonstrated that MRP4 is a downstream target gene of the Myc transcription factors, N-Myc and c-Myc [28], and that in childhood neuroblastoma where these oncoproteins play a critical role in tumor progression [45, 46], MRP4 can significant impact the malignant phenotype and clinical outcome of neuroblastoma apart from any function in drug efflux [27, 35, 44]. A single prior study by Bagnoli *et al*. reported that in a cohort of EOC consisting of all histological subtypes MRP4 expression was associated with disease relapse after adjustment for established clinical prognostic factors such as stage, residual disease and histotype in multivariate analysis [47]. In the present study, our detailed analysis in different histological and molecular subtypes revealed that high MRP4 expression is associated with poor prognosis in particular histological (endometrioid) or molecular (C5/proliferative serous) subtypes, with each subtype exhibiting higher than average *MYCN* expression [7, 48]. These findings well reflect the complexity of EOC, and indicate that MRP4 may play an important role in progression of high-*MYCN* EOC tumors, as previously observed in neuroblastoma [44]. In addition, our findings showing that transient suppression of MRP4 led to strongly impaired cell growth of 27/87 and HEY cells, each of which exhibit overexpression of *MYC* rather than *MYCN*, suggest that MRP4 can also be functionally activated upon Myc dysregulation in EOC.

Oncogenic Myc has been shown to be responsible for deregulated cell proliferation and resistance to apoptosis in a wide range of cancer cell types [49, 50], thus facilitating tumor growth and malignant progression. The present study suggests these oncogenic effects may, in part, be mediated by MRP4. Although the precise mechanism underlying the role of MRP4 in tumor

cell biology is unknown, recent studies have proposed that MRP4 plays relevant roles in cell growth, apoptosis and/or migration through transporting signaling molecules, such as cAMP and prostaglandin PGE2, in several cancer types. In this regard, MRP4 inhibition caused increased intracellular cAMP, resulting in cell cycle arrest and apoptosis in human leukemia cells or differentiation of leukemic stem cells [24, 51]. On the other hand, MRP4 inhibition decreased the expression levels of PGE2 signaling pathway genes and reduced metastatic potential of breast cancer cells but had no effect on primary tumor growth *in vivo* [52]. However, a direct link between MRP4 and these endogenous substrates in EOC biology is still lacking, and hence elucidation of the underlying mechanism responsible for the malignant phenotypic changes by MRP4 suppression warrants further investigation.

This study has provided evidence indicating that MRP4 is functionally active as a drug transporter in EOC cells, particularly for irinotecan and topotecan. These two MRP4 substrate drugs are topoisomerase I inhibitors, which are not affected by the resistance enzyme O^6-methyl-guanine DNA methyl transferase [53] and thus have been considered to be excellent treatment alternatives for platinum and/or taxane resistant tumors. Whilst topotecan is a well-studied agent for second-line therapy in platinum-resistant EOC [42], irinotecan is yet to be widely adopted for EOC treatment. However multiple preclinical studies have demonstrated positive effects of irinotecan alone or in combination with other agents including paclitaxel and bevacizumab in recurrent EOC [43]. Nonetheless, responses to topotecan or irinotecan are often only partially effective and not durable and the mechanisms behind resistance to these inhibitors remain poorly understood. Our data thus suggest that MRP4 inhibition is a potential sensitizer to irinotecan and topotecan chemotherapies in EOC.

In conclusion, our results indicate that MRP4 contributes to multiple malignant properties of EOC cells and influences outcome in subsets of Myc-associated EOC tumors. MRP4 also regulates sensitivity to chemotherapeutics such as irinotecan and topotecan in EOC, suggesting that inhibition of MRP4 combined with either of these anti-tumor drugs may be more effective approaches for treatment of EOC with high *MYC/MYCN* and MRP4 expression. Hence, our work for the first time sheds light on a novel role of MRP4 in EOC cell biology and also suggests MRP4 to be a potential therapeutic target within certain EOC tumors.

Data Accessibility

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to ethical restrictions.

Disclose of Potential Conflicts of Interest

The authors disclose no potential conflicts of interest to this work. A deFazio has received grant funding from AstraZeneca, not related to this work. D Bowtell has received research support from Roche-Genetech, AstraZeneca and BeiGene and consulting from Exo Therapeutics, not related to this work. CL Scott has received research funding from Eisai and Sierra Oncology, not related to this work.

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Tables

Table 1. IC50 values of SN38, Topotecan and Cisplatin in MRP4-suppressed 27/87 cells

	shRNA	Vector			MRP4.6			MRP4.7		
		Mean IC50 (nM) (95% CI), Fold sensitization								
	Doxycycline	+	-	Fold	+	-	Fold	+	-	Fold
Drug	**SN38**	3.1 (1.9 – 5.1)	3.2 (2.2 – 4.7)	1.0	3.2 (1.8 – 5.8)	1.3 (0.8 – 2.3)	**2.4***	4.8 (2.7 – 8.3)	1.8 (1.2 – 2.6)	**2.7****
	Topotecan	9.0 (5.5 – 15.0)	11.4 (5.8 – 22.3)	0.8	9.1 (5.7 – 14.6)	5.8 (3.5 – 9.6)	1.6	9.3 (5.7 – 15.0)	6.6 (3.9 – 11.1)	1.4
	Cisplatin	744.7 (610.9 – 907.8)	835.2 (719.3 – 969.8)	0.9	887.4 (807.2 – 975.6)	890.3 (782.2 – 1013.0)	1.0	943.3 (822.8 – 1081.0)	895.0 (806.9 – 992.8)	1.1

*P<0.05, **P<0.005

Figures

Figure legends

Figure 1. Prognostic impact of *ABCC4* mRNA expression in histological and molecular subtypes of EOC. Kaplan-Meier analysis of *ABCC4* expression was conducted for progression-free survival (PFS; A, C and E) and overall survival (OS; B, D, and F) in 80 endometrioid EOC (A and B), 150 serous EOC (C and D), and 14 "C5-type" serous EOC (C and D) tumors. P values were obtained from the log-rank test and HR and 95% CI values from the Cox regression model in each case.

Figure 2. Comparison of *MYC*/c-Myc and *MYCN*/N-Myc expression levels between histological and molecular subtypes of EOC. (A) RT-qPCR for *MYCN* mRNA expression levels was performed in 80 endometrioid and 150 serous tumors including 14 tumors determined to belong to "C5" molecular subtype. *MYCN* mRNA expression levels in endometrioid or C5 serous tumors were compared to those in the overall serous tumors. P values were calculated using one-way ANOVA followed by post-hoc analysis. *P<0.05, **P<0.005. (B) Examples of tumors with positive staining for MRP4, c-Myc and N-Myc are shown. Tumor morphology is shown in the H&E stained tissues (insets). Photos were taken at 400x magnification.

Figure 3. MRP4 suppression reduced growth, migration and invasion of endometrioid and serous EOC cell lines. (A) siRNA-mediated MRP4 knockdown using two distinct MRP4 siRNA (MRP4.5, MRP4.6; 20nM siRNA; 48 hr after transfection) resulted in a decrease of MRP4 protein expression in 27/87 and HEY ovarian cancer cell lines, as monitored by Western blotting. Forty-eight hours post transfection, cells were re-seeded for (B) colony formation, (C) migration or (D) invasion assays. The number of cells migrated (C) or invaded (D) in five fields (stained in red) were counted for each experiment. Non-invaded cells were stained in blue/green. (E) siRNA-mediated MRP4 knockdown using any of three distinct MRP4 siRNA (MRP4.5, MRP4.6 or MRP4.7; 20nM siRNA; 48 hr after transfection) resulted in a decrease of MRP4 protein expression in COV318 ovarian cancer cells, as monitored by Western blotting. Forty-eight hours post transfection, cells were re-seeded for (F) colony formation or (G) migration. All experiments were repeated at least twice. P values were derived using one-way ANOVA with Dunnett's multiple comparison test. *P<0.05, ****P<0.0001

Figure 4. Effect of MRP4 knockdown on cell viability and apoptosis. (A) After siRNA-mediated MRP4 transfection (MRP4.6 or MRP4.7 siRNA) or non-targeting empty vector, cell viability was determined by the trypan blue exclusion method up to 72 hr (HEY) or 96 hr (27/87 and COV318). P values were derived using two-way ANOVA with Dunnett's multiple comparison test. *P<0.05, **P<0.005, ***P<0.0005 (B) siRNA mediated suppression of MRP4 and expression of apoptotic marker proteins, cleaved PARP and γH2AX, were confirmed by Western blot analysis 48 and 72hr post-transfection. Total actin was used as a loading control for each sample and experiments were performed at least twice.

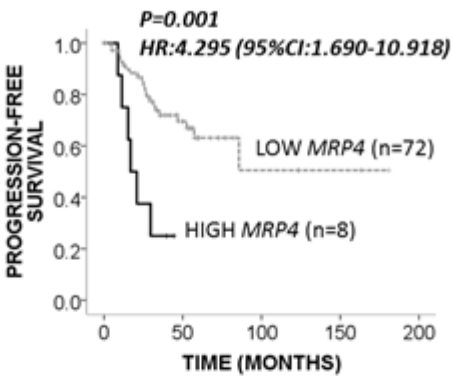
Figure 5. Effect of sustained MRP4 knockdown on colony formation of human HGSOC patient-derived cell line (WEHICS27) *in vitro* and on xenografted WEHICS27 tumor growth *in vivo*. (A) Inducible MRP4 suppression was achieved with two independent RNAs (MRP4.6 and MRP4.7) after the addition of doxycycline (DOX) (72hr time-point) in WEHICS27 cells. All experiments were repeated at least twice. (B) Sustained knockdown of MRP4 reduced colony forming ability of WEHICS27cells. P values were derived using one-way ANOVA with Dunnett's multiple comparison test. *P<0.05, ****P<0.0001 (n=4). NSG mice was subcutaneously injected with WEHICS27 cells expressing shRNA knockdown clones (MRP4.6 and MRP4.7) and doxycycline (DOX) treatment commended 7 days after engraftment (n=10 per group). (C) MRP4 suppression using MRP4.6 slowed the tumor growth in individual mice and extended survival (log-rank P=0.0007). (D) Representative western blot of MRP4 protein showed that DOX-induced MRP4 suppression by MRP4.6 (lane 6-10) was maintained *in vivo* to the experimental end-point. Total actin was used as a loading control. (E) Examples of tumors with staining for MRP4 are shown. Tumor morphology is shown in the H&E stained tissues. Photos were taken at 400x magnification. (F) MRP4 suppression using MRP4.7 did not affect tumor growth and mouse survival (log-rank P=0.9254). (G) Representative western blot of MRP4 protein showing DOX-induced MRP4 suppression *in vivo* by MRP4.7 at the experimental end-point (lane 6-10). Total actin was used as a loading control.

Novelty and Impact:

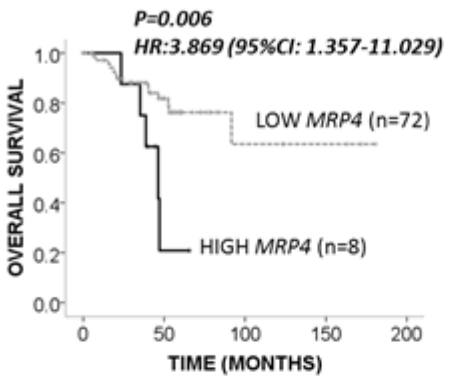
Epithelial ovarian cancer (EOC) generally has an extremely poor prognosis, and new therapies are urgently needed. In this study, the authors examined a multidrug-resistance efflux pump called MRP4, which is regulated by the Myc oncogene. They found that increased expression of MRP4 was associated with poor prognosis, and that siRNA knockdown of MRP4 led to decreased growth, migration, and invasion of EOC cells in vivo. These results suggest that MRP4 may provide a valuable therapeutic target for certain EOC tumors.

Endometrioid

A. *Overall: PFS*

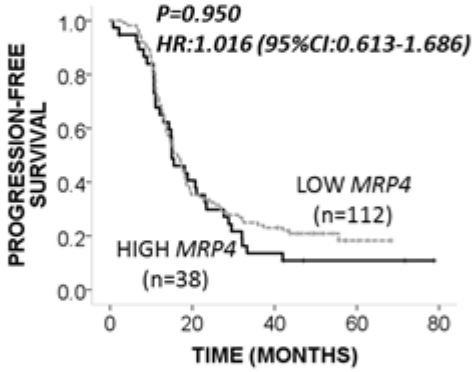


B. *Overall: OS*

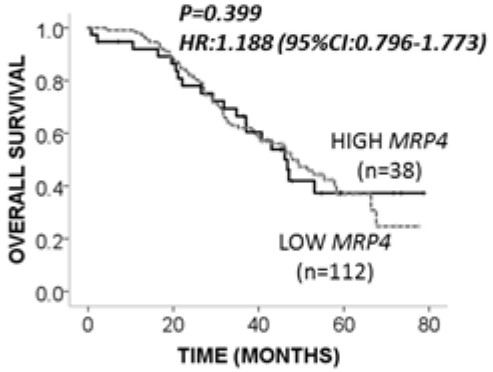


Serous

C. *Overall: PFS*

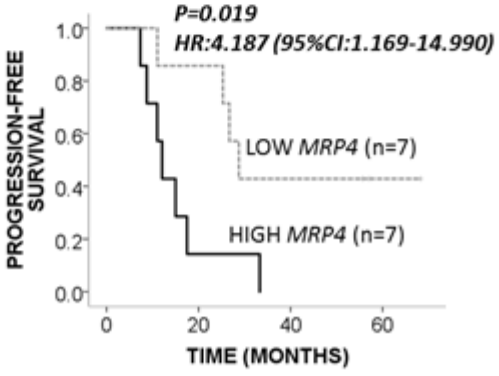


D. *Overall: OS*

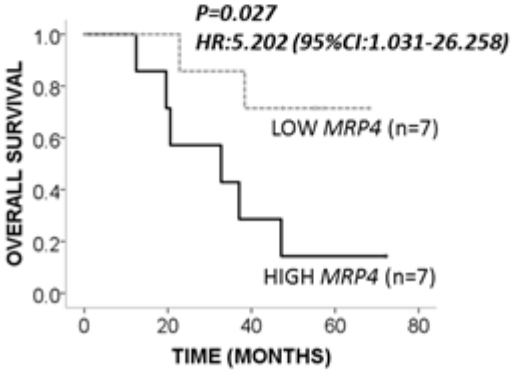


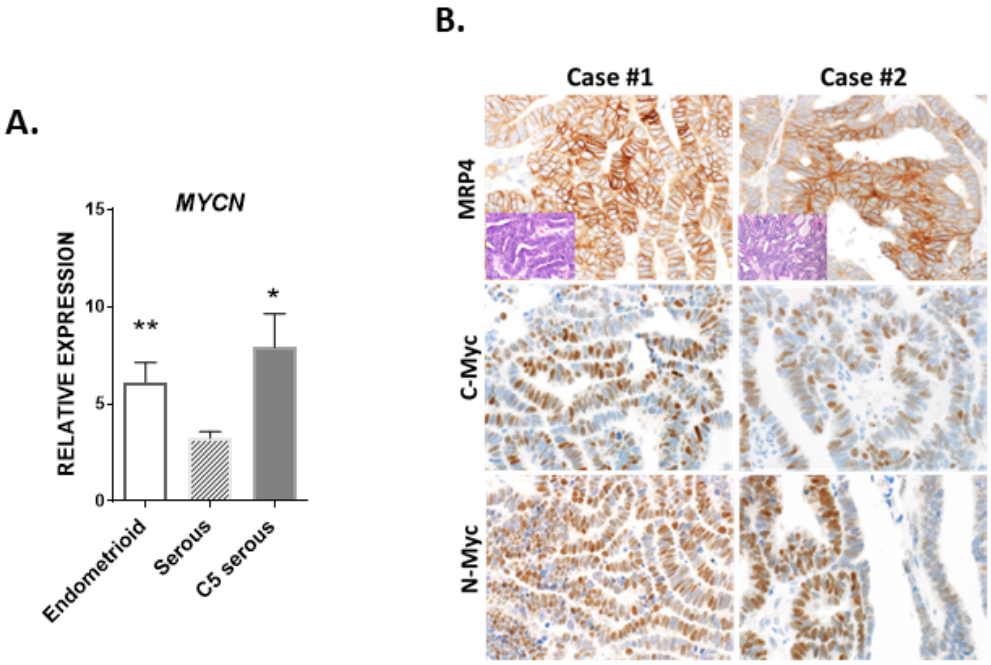
Serous C5

E. *C5: PFS*

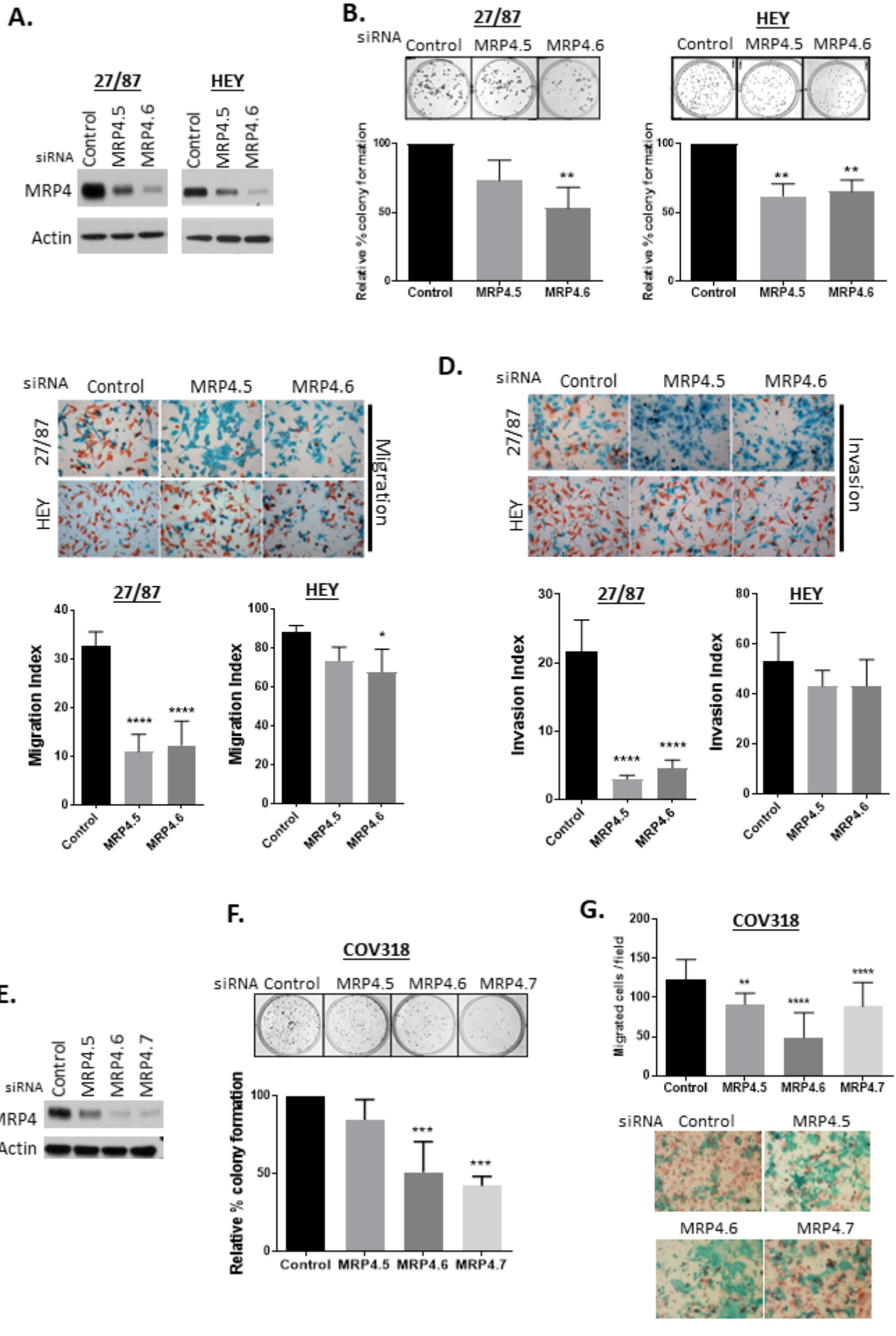


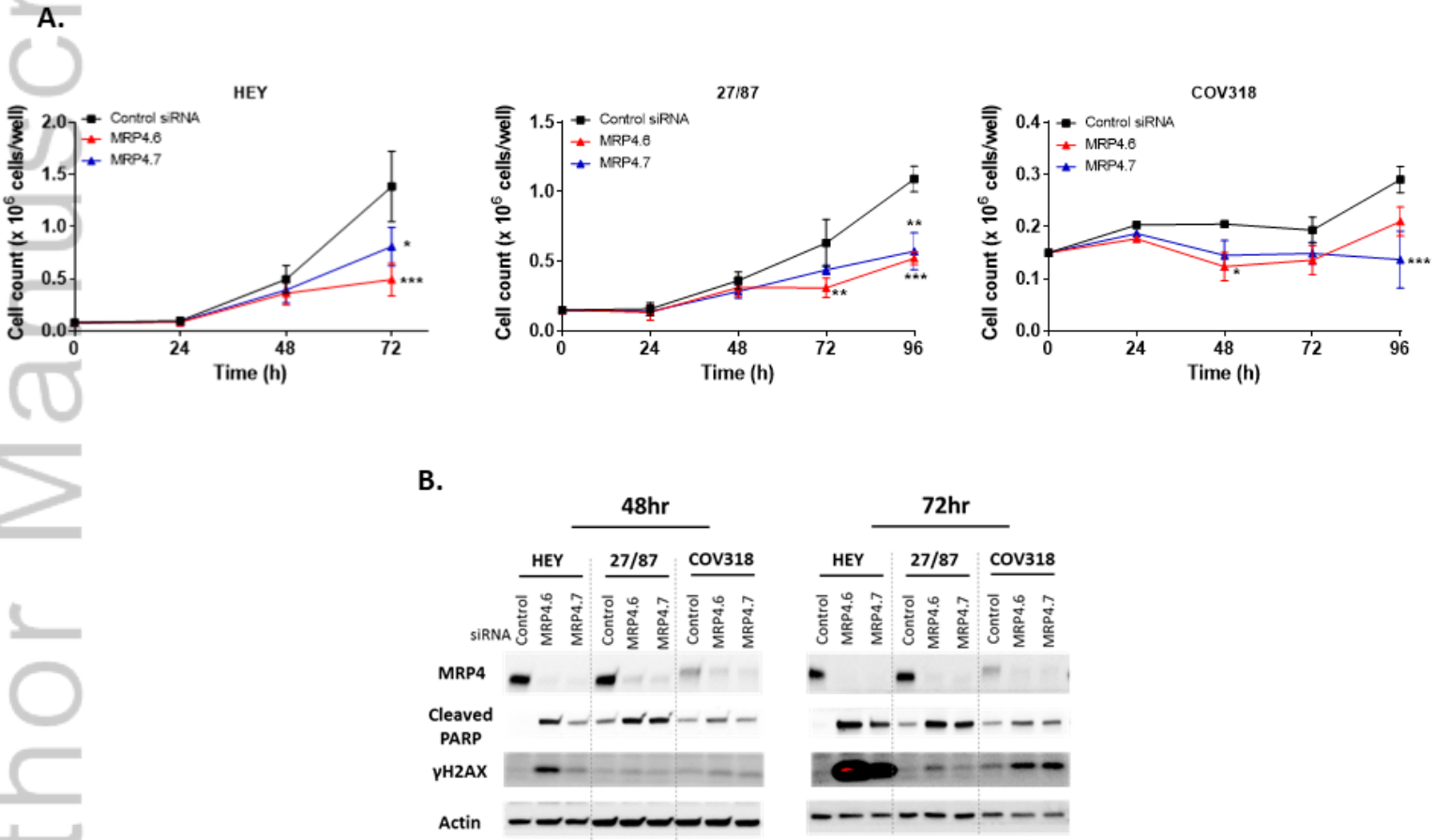
F. *C5: OS*



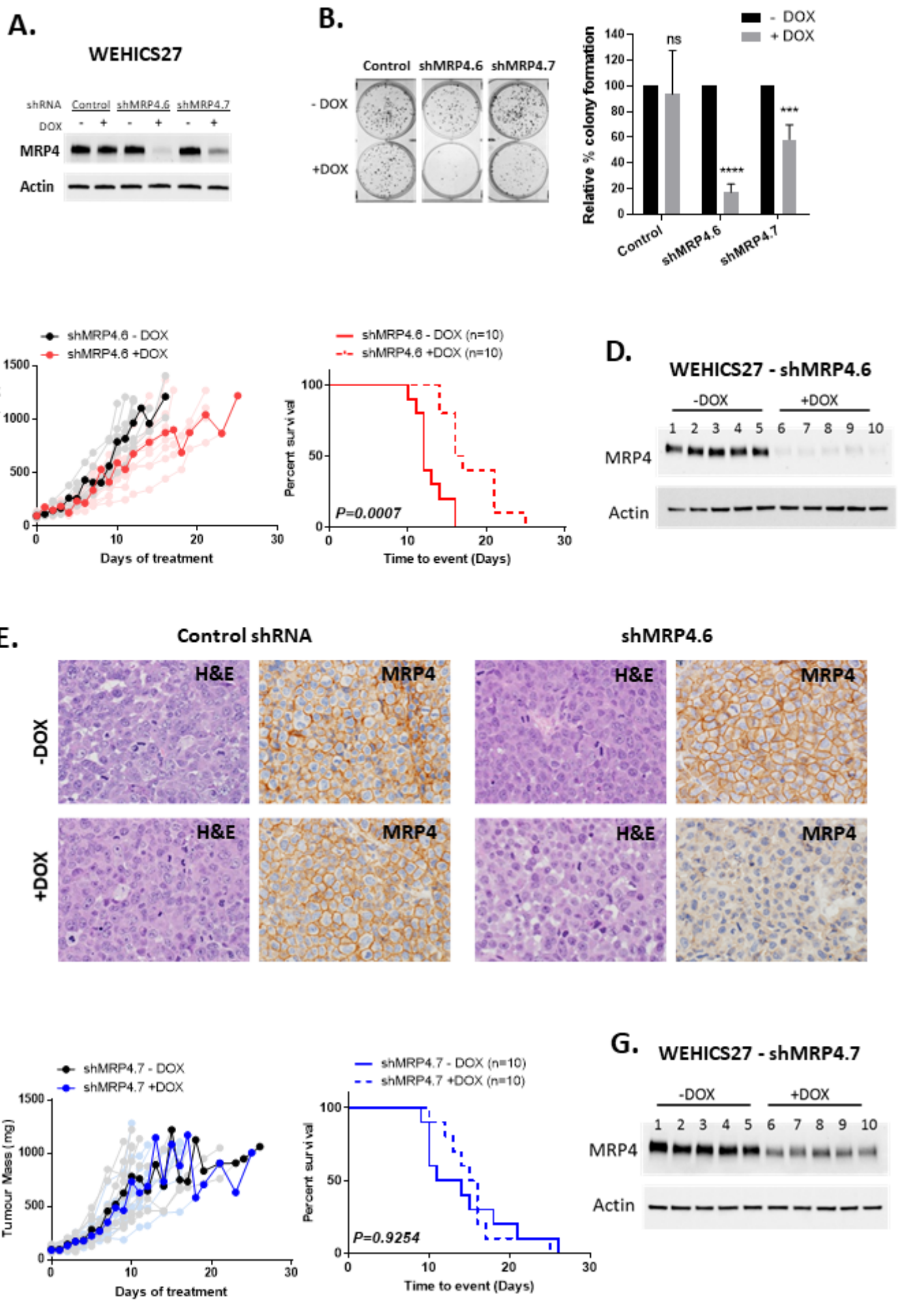


IJC_33005_Figure 2..tif





IJC_33005_Figure 4..tif



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