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This is a pre print version of the following article:

Original Citation:

Availability:

This version is available http://hdl.handle.net/2318/1711962 since 2019-12-30T22:01:52Z

Published version:

DOI:10.1002/ijc.32419

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14 July 2024

Wnt/IL-1β/IL-8 autocrine circuitries determine chemoresistance in mesothelioma stem cells by inducing ABCB5

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Keywords: malignant pleural mesothelioma; cancer stem cells; chemoresistance; ABCB5

Abbreviations: ABC: ATP binding cassette; AC: adherent cells; ALDH: aldehyde dehydrogenase; ANOVA: analysis of variance; ChIP: chromatin immunoprecipitation; CSF1R: colony-stimulating-factor-1-receptor; DAPI: 4',6-diamidino-2-phenylindole dihydrochloride; FBS: fetal bovine serum; FFPE: formalin-fixed paraffin-embedded; FITC: fluorescein isothiocyanate; GFP: green fluorescence protein; i.p.: intraperitoneally; KO: knocked-out; GSK3β: glycogen synthase kinase 3β; LDH: lactate dehydrogenase; LRP6: Low-density lipoprotein receptor-Related Protein 6; MPM: malignant pleural mesothelioma; myc-i: 5-[(4-Ethylphenyl)methylene]-2-thioxo-4-thiazolidinone; Nanog: octamer-binding transcription factor 4; OS: overall survival; PFA: paraformaldehyde; PS: penicillin-streptomycin; RLU: relative luminescence units; SASP: senescence-associated secretory phenotype; SC: stem cell; s.c.: subcutaneously; SHH: Sonic Hedgehog; SOX: Sex determining region Y-box 2; TBP: TATA-Box Binding Protein; TBS: Tris-buffered saline; TRITC: tetramethylrhodamine isothiocyanate; TTP: time to progression; UPN: Unknown Patient Number

Novelty and impact

The molecular bases of chemoresistance in malignant pleural mesothelioma are poorly known. We identified ABCB5 as a key determinant of chemoresistance in the stem cells of primary mesotheliomas and as a negative prognostic factor in patients. ABCB5 expression is controlled by interconnected Wnt/IL-1β/IL-8/β-catenin/c-myc-driven autocrine loops. Disrupting these loops improves the

sensitivity towards first-line chemotherapy in stem cells. Overall, ABCB5 may have a translational relevance as a new therapeutic target and predictive biomarker in mesothelioma.

Abstract

Malignant pleural mesothelioma (MPM) is a tumor with high chemo-resistance and poor prognosis. MPM stem cell (SC) are known to be chemo-resistant, but it is unknown if and how stemness-related pathway determine chemoresistance. Moreover, there no predictive markers of SC-associated chemoresistance.

We generated MPM SC cultures from primary samples and compared the gene expression and chemo-sensitivity profile of SC and differentiated/adherent cells (AC) of the same patient.

SC were more resistant to cisplatin and pemetrexed than AC and have the drug efflux transporter ABCB5 up-regulated. ABCB5-knocked-out (KO) SC clones were re-sensitized to the drugs in vitro and in patient-derived xenografts. ABCB5 was transcriptionally activated by the Wnt/GSK3β/β-catenin/c-myc axis, that also increased IL-8 and IL-1β production. IL-8 and IL-1β-KO SC clones reduced the c-myc-driven transcription of ABCB5 and re-acquired chemosensitivity. ABCB5-KO clones had lower IL-8 and IL-1β secretion and c-myc transcriptional activity, suggesting that either Wnt/GSK3β/β-catenin and IL-8/IL-1β signaling drive c-myc-mediated transcription of ABCB5. ABCB5 correlated with lower time-to-progression and overall survival in MPM patients treated with cisplatin and pemetrexed.

Our work identified multiple autocrine loops linking stemness and chemoresistance in MPM. ABCB5 may represent a new target to chemo-sensitize MPM SC and a potential biomarker to predict the response to the first-line chemotherapy in MPM patients.

Introduction

Malignant pleural mesothelioma (MPM) is an asbestos related tumor characterized by three histotypes, i.e. epithelioid, biphasic and sarcomatous, mostly diagnosed in advanced stage and with an overall dismal prognosis. Platinum-based (cisplatin/carboplatin) chemotherapy in combination with anti-folate agents (pemetrexed, raltitrexed), is a standard of care for advanced stage disease.[1] Immunotherapy, targeted therapies-based approaches [2] and tumor microenvironment-targeting approaches [3] are still under development. All treatments produce only partial responses, because of the strong chemoresistance of MPM.[1]

Tumor initiating cells or stem cells (SC) represent a small sub-population of tumor bulk, but they are the main responsible for tumor mass renewal, recurrence and chemoresistance.[4]

MPM SC were first identified from commercial cell lines as a side population, ranging from 0.05 to 1.32%, positive for CD133, CD9, CD24, CD26, CD44, octamer-binding transcription factor 4^+ (Oct4), Nanog, Sex determining region Y-box 2^+ (SOX2), ATP Binding Cassette Transporter G2 (ABCG2), aldehyde dehydrogenase (ALDH).[5-11] A shared feature of SC is their resistance to cisplatin and pemetrexed.[6-9,11,12] The CD24- and CD26-downstream signaling [13], the senescence-associated secretory phenotype (SASP) kinase/STAT3 axis [7] and the colony-stimulating-factor-1-receptor (CSF1R)/Akt/β-catenin axis [8] contribute to the resistance to pemetrexed.

Until now, there are no report linking classical stemness pathways, such as Wnt-, Notch-, Sonic Hedgehog (SHH)-dependent pathways, and chemoresistance, nor investigating the clinical implications of these linkages.

In different tumors the chemoresistance of SC have been related to the over-expression of multiple ABC transporters that efflux a broad spectrum of chemotherapeutic and targeted-therapy agents.[4] ABCG2 only has been reported in MPM SC, but it does not efflux cisplatin and pemetrexed.[14]

The aim of the present work is to investigate whether classical stemness pathways and ABC transporters are implicated in the chemoresistance of MPM SC derived from patients, in order to identify new biomarkers predictive of poor response to the first-line chemotherapy, and possible druggable targets.

Material and methods

Chemicals

Fetal bovine serum (FBS), penicillin-streptomycin (PS) were supplied by Sigma Chemical Co. (St. Louis, MO), HAM F12 and DMEM medium were from Life Technologies (Milano, Italy), plastic ware for cell culture was from Falcon (BD Biosciences, Bedford, MA). Electrophoresis reagents were from Bio-Rad Laboratories (Hercules, CA). The protein content of cell monolayers and cell lysates was assessed with the bicinchoninic acid kit (Sigma Chemical Co.). Cisplatin and pemetrexed as the other reagents, if not otherwise specified, were purchased from Sigma Chemical Co.

Cells. Primary human MPM samples (3 epithelioid MPM, 1 sarcomatous MPM, 2 biphasic MPM) were obtained from diagnostic thoracoscopies, from the Biological Bank of Mesothelioma, S. Antonio e Biagio Hospital, Alessandria, Italy. Tissue was digested in medium containing 1 mg/ml collagenase and 0.2 mg/ml hyaluronidase for 1 h at 37°C. From each sample differentiated/adherent cells (AC) and SC were obtained. AC were cultured in HAM F12 medium supplemented with 10% FBS, 1% PS. SC were generated from AC by maintaining them in HAM F12/DMEM medium supplemented 1% PS, 20 ng/ml of EGF, 20 ng/ml of β-FGF, 4 μg/ml of IGF, 0.2% B27 (Invitrogen). After two weeks, SC were isolated by cell sorting of ALDH^{bright} (ALDEFLUORTM kit, StemCell Technologies, Vancouver Canada), Oct4/POUF5F1^{+} (rabbit #2750; Cell Signaling Technologies, Danvers, MA), Nanog^{+} (rabbit mAb #4903; Cell Signaling Technologies), SOX2^{+} (rabbit, #poly6308;BioLegend, San Diego, CA), ABCG2^{+} (mouse clone 5D3; Santa Cruz Biotechnology Inc., Santa Cruz, CA) cells, using a Cell Sorter BD FACSAria III (Becton Dickinson, Bedford, MA). All patients were identified with Unknown

Patient Numbers (UPN). The Ethical Committee of Biological Bank of Mesothelioma approved the study (#9/11/2011). The mesothelial origin of the isolated cells was confirmed by positive immune-staining, as detailed previously [15], and authenticated by the STR analysis method. Cells were used until passage 6. *Mycoplasma spp*. contamination was checked by RT-PCR weekly; contaminated cells were discharged.

Stemness functional assays. Self-renewal and clonogenicity assay were performed as reported.[16] For *in vivo* tumorigenicity assay, 1×10^8 AC or SC (3 mice/each UPN AC or SC), mixed with 100 µl Matrigel, were injected subcutaneously (s.c.) in 6-week-old female NOD-SCID-γ Balb/C mice (Charles River Laboratories Italia, Calco), housed (5 per cage) under 12 h light/dark cycle, with food and drinking provided *ad libitum*. Tumor growth was measured daily by caliper, according to the equation $(LxW^2)/2$, where L=tumor length and W=tumor width, up to 30 weeks. The number of tumors developed and tumor volume are reported in **Supplementary Table 3**.

qRT-PCR and high-throughput PCR arrays. Total RNA was extracted and reverse-transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories). qRT-PCR was performed using IQ™ SYBR Green Supermix (Bio-Rad Laboratories). The primer sequences, designed with qPrimerDepot software (http://primerdepot.nci.nih.gov/), were: *ABCB5*: 5'-ATTGGAGTGGTTAGTCAAGAGCC-3', 5'-AGTCACATCATCTCGTCCATACT-3'; *ABCB1*: 5'-TGCTGGAGCGGTTCTACG-3', 5'-ATAGGCAATGTTCTCAGCAATG-3'; *FGF4*: 5'-TACCTCACAAGCCCTGAAAC-3', 5'-GGAGACCTACAAACACCATCTT-3'; *VEGFA*:5'-ATCTTCAAGCCATCCTGTGTGC-3', 5'-GCTCACCGCCTCGGCTTGT-3'; *c-myc*: 5'-CTGAGGAGGAACAAGAAGATGAG-3', 5'-CTTGGACGGACAGGATGTATG-3'; *IL-1β: 5'*-ATGATGGCTTATTACAGTGGCAA-3', 5'-GTCGGAGATTCGTAGCTGGA-3'; *IL-8*: 5'-ACTGAGAGTGATTGAGAGTGGAC-3', 5'-AACCCTCTGCACCCAGTTTTC-3'; *actin*: 5'GCTATCCAGGCTGTGCTATC-3'; 5'-TGTCACGCACGATTTCC-3'. PCR arrays were carried out on 1 µg cDNA, using Human Cancer

Stem Cells RT^2 Profiler PCR Array, WNT Signaling Pathway RT^2 Profiler PCR Array, WNT Signaling Targets RT^2 Profiler PCR Array (Bio-Rad Laboratories) as per manufacturer's instructions. Data analysis was performed using the PrimePCR™ Analysis Software (Bio-Rad Laboratories).

Cytotoxicity and viability assays. The release of lactate dehydrogenase (LDH) in the extracellular medium, used as a sensitive index of drug cytotoxicity [16], was measured by spectrophotometry, using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments, Winooski, VT). The results were expressed as percentage of extracellular LDH versus total (intracellular plus extracellular) LDH. Cell viability was measured by the ATPlite Luminescence Assay System (PerkinElmer, Waltham, MA), as per manufacturer's instructions, using a Synergy HT Multi-Detection Microplate Reader. The relative luminescence units (RLU) of untreated cells were considered as 100% viability; results were expressed as a percentage of viable cells versus untreated cells.

Flow cytometry analysis. 1×10^6 cells were rinsed and fixed with 2% w/v paraformaldehyde (PFA) for 2 min, permeabilized using 0.1% v/v Triton-X100 for 2 min on ice, washed three times with PBS and stained with the following antibodies: anti-Oct4, anti-Nanog; anti-SOX2; anti-ABCG2; ABCB5 (rabbit SAB1300315; Sigma Chemical Co.); anti-Frizzled 1 (mouse clone E-7; Santa Cruz Biotechnology Inc.); anti-Low-density lipoprotein receptor-Related Protein 6 (LRP6, rabbit EPR2423(2); Abcam, Cambridge, UK) for 1 h on ice, followed by an AlexaFluor 488-conjugated secondary antibody (Millipore, Billerica, MA) for 30 min. 1×10^5 cells were analyzed with EasyCyte Guava™ flow cytometer (Millipore), equipped with the InCyte software (Millipore). Control experiments included incubation with non-immune isotype antibody.

Immunofluorescence analysis. 5×10^5 AC were seeded onto glass coverslips in 6 well plates overnight. SC were collect by cyto-spinning. All cells were fixed using 4% PFA for 15 minutes, washed with PBS, permeabilized with 1% Triton X-100 for 5 minutes, washed with PBS and incubated for 24 h with anti-ABCB5 antibody (Sigma Chemicals Co.) or anti c-myc antibody (clone 9E10.3; Millipore), diluted

1:100 in 1% FBS/PBS at 4 °C. Samples were washed five times with PBS and incubated for 1 hour with fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibodies (Sigma Chemicals Co.), diluted 1:50. After this step, cells were incubated with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), diluted 1:1000 in PBS for 5 min, washed four times with PBS and once with deionized water. The cover slips were mounted with Gel Mount Aqueous Mounting and examined with a Leica DC100 fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany). For each experimental point, a minimum of five microscopic fields were examined.

Generation of knocked-out clones. SC were knocked-out (KO) for *ABCB5, IL-1β* or *IL-8* using respective CRISPR/Cas9-green fluorescence protein (GFP)-plasmids (KN415604, KN402079, KN202075; Origene, Rockville, MD). Non-targeting (scrambled) CRISPR/Cas9 plasmid was used as control of specificity. Cells were seeded at 1×10^5 cells/ml in PS-free medium. 1 µg of CRISPR/Cas9 KO plasmid was used as per manufacturer's instructions. Transfected cells were sorted by isolating GFP-positive cells. Knocking-out efficacy was verified by qRT-PCR or immunoblotting. Stable KO-clones were generated by culturing cells for 6 weeks in medium containing 1 µg/ml puromycin.

***In vivo* chemo-sensitivity assay**. 1×10^7 SC, stably transfected with a non-targeting (scrambled) CRISPR/Cas9 vector or with *ABCB5*-targeting CRISPR/Cas9 vector, were inoculated s.c. in 9-weeks old NOD-SCID-γ Balb/C female mice. Tumor volume was monitored by caliper and calculated according to the equation: equation $(LxW^2)/2$, where L=tumor length and W=tumor width. When tumor reached the volume of 50 mm^3, animals were randomized in the following groups (n= 6/for each group) and treated as it follows at day 1, 7, 14, 21, 28, 35 after randomization: 1) scrambled vehicle, i.e. animals bearing a scrambled-SC tumor receiving 200 µl solution saline i.p.; 2) scrambled cisplatin plus pemetrexed, i.e. animals bearing a scrambled-SC tumor, receiving 5 mg/kg cisplatin

intraperitoneally (i.p.) and 10 mg/kg pemetrexed i.p; 3) KO vehicle, i.e. animals bearing a *ABCB5* KO-SC tumor receiving 200 µl solution saline i.p.; 2) KO cisplatin plus pemetrexed, i.e. animals bearing a *ABCB5* KO-SC tumor, receiving 5 mg/kg cisplatin i.p. and 10 mg/kg pemetrexed i.p. Tumor volumes were monitored daily by caliper and animals were euthanized at day 48 after randomization with zolazepam (0.2 ml/kg) and xylazine (16 mg/kg). Animal care and experimental procedures were approved by the Bio-Ethical Committee of the Italian Ministry of Health (#122/2015-PR).

Immunoblotting. Cells were rinsed with ice-cold lysis buffer (50 mM Tris, 10 mM EDTA, 1% v/v Triton-X100; pH 7.5), supplemented with the protease inhibitor cocktail set III (Calbiochem. La Jolla, CA), 2 mM phenylmethylsulfonyl fluoride, 1 mM Na_3VO_4. Cells were then sonicated (10 bursts of 10 sec, 4°C, 100 W; Labsonic sonicator, Hielscher, Teltow, Germany) and centrifuged at 13000×g for 10 min at 4°C. 20 µg protein extracts were subjected to 4-20% gradient SDS-PAGE and probed with the following antibodies, all diluted 1:1000 in Tris-buffered saline (TBS)-Tween non-fat dry milk 5%: anti-glycogen synthase kinase 3β (GSK3β, rabbit mAb#9315; Cell Signaling Technologies), anti-phospho(Tyr279/Tyr216)GSK3β (mouse, clone 5G-2F; Millipore), anti-β-catenin (rabbit mAb#8480, Cell Signaling Technologies), anti-phospho(Ser33/37/Thr41)-β-catenin (rabbit #9561; Cell Signaling Technologies), anti-β-tubulin antibody (mouse clone D-10; Santa Cruz Biotechnology). Blotting was followed by the peroxidase-conjugated secondary antibody (Bio-Rad). The membranes were washed with TBS/Tween 0.01% v/v and proteins were detected by enhanced chemiluminescence (Bio-Rad Laboratories). To detect ubiquitinated β-catenin, 100 µg protein extracts were immuno-precipitated overnight with the anti-β-catenin antibody, using 25 µl of PureProteome Magnetic Beads (Millipore). Immunoprecipitated samples were then probed with an anti-mono/polyubiquitin antibody (mouse clone FK-2; Axxora, Lasuanne, Switzerland). Blot images were acquired with a ChemiDoc™ Touch Imaging System device (Bio-Rad Laboratories). Nuclear extraction was performed using the Nuclear Extraction Kit (Active Motif, Rixensart, Belgium). 10 µg of nuclear proteins were subjected immunoblotting and

analyzed for β-catenin or TATA Box Binding Protein (TBP, mouse clone 1TB18; Santa Cruz Biotechnology Inc.) expression.

Chromatin Immunoprecipitation (ChIP). ChIP samples were prepared as previously reported [17] using a ChIP-tested anti-c-myc antibody (mouse clone 9E11; Abcam). The putative c-Myc binding site on *ABCB5* promoter was validated with the Matinspector software (https://www.genomatix.de/matinspector.html). Primer sequences were: 5'-CACAACTTCAAGTGGTAGCATG-3'; 5'-CCATTCTACCCAGTGAAATG-3'. Primers used as negative internal controls for a non-specific 10000 bp upstream sequence were: 5'-GTGGTGCCTGAGGAAGAGAG-3'; 5'-GCAACAAGTAGGCACAAGCA-3′. The immunoprecipitated products were amplified by qRT-PCR.

GSK3β, RhoA and RhoA kinase activity. The kinase activity of GSK3β was measured on the protein immunopurified from cell extracts by a radiometric assay, using the GSK-3β Activity Assay Kit (Sigma Chemicals. Co), as per manufacturer's instructions. Results were expressed as count per minute (cpm)/mg cellular proteins. Rho-GTP, considered an index of active RhoA, and RhoA kinase activity, were measured by spectrophotometric methods, using the G-LISA RhoA Activation Assay Biochem Kit (Cytoskeleton, Denver, CO) and the CycLex Rho Kinase Assay Kit (CycLex, Nagano, Japan) respectively. Results were expressed U absorbance/mg cell proteins, according to titration curves prepared with serial dilutions of Rho-GTP positive control (Cytoskeleton) and recombinant RhoAK (MBL, Woburn, MA, USA).

Cytokine production. IL-1β and IL-8 levels were measured in the culture supernatants using the human IL-8(CXCL8) TMB ELISA Development Kit (PeproTech, London, UK) and the human IL-1β/IL-1F2 ELISA kit (DuoSet ELISA, R&D Systems, Minneapolis, MN), as per manufacturer's instructions.

Immunohistochemistry. The formalin-fixed paraffin-embedded (FFPE) samples of patients with confirmed histological diagnosis of MPM, treated with cisplatin/carboplatin plus pemetrexed as first line therapy, were analyzed for the expression of ABCB5 (rabbit ab203120; Abcam). ABCB5 was considered positive when a weak-to strong membrane or cytosolic positivity was shown. The tumor proportion positivity was recorded. Patients were divided into $ABCB5^{low}$ and $ABCB5^{high}$, if the tumor proportion of ABCB5 IHC staining was respectively below or equal/above the median value.

Statistical analysis. All data in the text and figures are provided as means±SD. The results were analyzed by a one-way analysis of variance (ANOVA), using Statistical Package for Social Science (SPSS) software (IBM SPSS Statistics v.19). $p<0.05$ was considered significant. The Kaplan-Meier method was used to calculate the time to progression (TTP: time from the start of treatment to the first sign of disease's progression) and overall survival (OS: survival from the beginning of chemotherapy until patients' death). Log rank test was used to compare the outcome of $ABCB5^{low}$ and $ABCB5^{high}$ groups. The sample size was calculated with the G*Power software (www.gpower.hhu.de), setting $\alpha \leq 0.05$ and $1-\beta=0.80$. Researchers analyzing the results were unaware of the treatments received.

Results

ABCB5 determines chemo-resistance in malignant pleural mesothelioma-derived stem cells

Six MPM samples, representative of the 3 main MPM histotypes (i.e. epithelioid, sarcomatous and biphasic), were obtained from patients with annotated clinical data (**Supplementary Table 1**) and histopathological characterization (**Supplementary Table 2**). From each patient, AC and SC were obtained (**Figure 1a**). Sorted SC had strong positivity for the general stemness markers ALDH, Oct4, Nanog, SOX2 and ABCG2 (**Figure 1b-c**). For all the histotypes, SC showed significantly higher self-renewal (**Figure 1d**), *in vitro* clonogenicity (**Figure 1e**) and *in vivo* tumorigenicity (**Supplementary Table S3**) compared to AC, displaying the key phenotypic and functional properties of SC.

Targeted-gene expression analysis confirmed that general stemness markers were up-regulated, developmental and differentiation markers were either up- or down-regulated. Notch-related genes were mostly down-regulated, while genes associated to Wnt and SHH pathways were mostly up-regulated. No clear signatures of increased proliferation, survival, epithelial-mesenchimal transition, adhesion and migration differentiated SC from AC (**Figure 1e**; **Supplementary Table S4**).

SC were significantly more resistant to cisplatin and pemetrexed than AC, as suggested by the lack of any increase of extracellular LDH (**Figure 2a**), and by the absence of any change in cell viability (**Figure 2b**). Since ABCB5, a stemness gene that induces resistance to 5-fluouracile in colon cancer side-population cells [18], to taxanes, Vinca alkaloids, doxorubicin, etoposide/teniposide and dacarbazine in melanoma initiating cells [19], to carboplatin in Merkel cell carcinoma [20], was significantly up-regulated in 9 out of 9 SC compared to AC (**Figure 1e**), we investigated its role in MPM SC chemoresistance. ABCB5 was significantly up-regulated in all the histotypes of MPM SC as mRNA (**Figure 2c**) and protein, either in cytosol (**Figure 2d)**, i.e. the newly synthesized protein moving from endoplasmic reticulum to plasma-membrane, or on cell surface (**Figure 2e),** i.e. the active protein form. *ABCB5*-KO SC (**Figure 2f**) dramatically rescued the sensitivity to the cisplatin and pemetrexed, in terms of increased cell damage (**Figure 2g**) and reduced viability (**Figure 2h**). SC-patient derived xenografts of epithelioid and sarcomatous MPM were resistant to the combination of cisplatin and pemetrexed. By contrast, chemotherapy significantly reduced the tumor growth of the same SC tumors KO for *ABCB5* (**Figure 2i-j**). Overall, these data indicate that ABCB5 contributes to the chemo-resistance in MPM SC.

The canonical Wnt/GSK3β/β-catenin pathway is up-regulated in malignant pleural mesothelioma-derived stem cells

Previous findings demonstrate that Wnt pathway induces chemoresistance by upregulating ABC transporters [16,21-23], but the up-regulation of ABCB5 has not been yet investigated. Since some Wnt-related genes were up-regulated in MPM SC (**Figure 1e**), we focused on their possible involvement in the chemoresistance mediated by ABCB5. Notable, most Wnt ligands, Wnt-receptors belonging to Frizzled family, activating Wnt-transducers of Wnt-canonical pathway were significantly up-regulated in SC, whereas most soluble Wnt inhibitors and negative transducers were down-regulated (**Figure 3a**; **Supplementary Table S5**). In keeping with this signature, Frizzled 1 receptor, but not the co-receptor LRP6, was higher in SC (**Figure 3b**). Phospho(Tyr279/Tyr216)GSK3β, i.e. the active GSK3β, and phospho(Ser33/Ser37/Thr41)-β-catenin, i.e. the protein primed for ubiquitination and proteasomal degradation, and ubiquitinated β-catenin were all lower in SC (**Figure 3c-d**), indicating an increased activity of the Wnt/GSK3β/β-catenin axis. SC had higher β-catenin translocated in the nuclease (**Figure 3e**), corresponding to the transcriptionally active form. Consistently, several target genes of Wnt canonical pathway resulted up-regulated in SC, as demonstrated by global gene expression profile (**Figure 3a**; **Supplementary Table S5**) and qRT-PCR validation of selected β-catenin-target genes, unrelated for biological functions, such as the drug efflux transporter *ABCB1*, the growth factor *FGF4*, the pro-angiogenetic factor *VEFGA*, the oncogenic factor *c-myc* (**Figure 3f**).

The GSK3β/β-catenin/c-myc axis up-regulates ABCB5 in malignant pleural mesothelioma-derived stem cells

c-myc is a target gene of Wnt/GSK3β/β-catenin axis [24] and a transcriptional factor for *ABCB5*.[25] To test if it may represent the possible link between Wnt canonical pathway and ABCB5 in MPM, we treated and SC with the c-myc inhibitor 5-[(4-Ethylphenyl)methylene]-2-thioxo-4-thiazolidinone (myc-i), and AC with the GSK3β inhibitor LiCl, that inhibited the phosphorylation activity of GSK3β (**Supplementary Figure S1**) and activates Wnt canonical pathway (16). In LiCl-treated AC, c-myc was more translocated into the nucleus (**Figure 4a**) and more bound to *ABCB5* promoter (**Figure 4b**).

Consistently, the transcription of *ABCB5* was increased (**Figure 4c**) and the cytotoxicity exerted by cisplatin and pemetrexed was reduced (**Figure 4d**). Myc-i prevented the nuclear translocation of c-myc in SC (**Figure 4a**), reduced c-myc binding on *ABCB5* promoter (**Figure 4b**) and *ABCB5* mRNA levels (**Figure 4c**), re-sensitized SC to the cytotoxic effects of cisplatin and pemetrexed (**Figure 4d**). These data provide the proof of concept that ABCB5 is under the control of GSK3β/β-catenin/c-myc axis and that the inhibition of this pathway chemo-sensitizes MPM SC.

Although the gene of *RhoA*, a non-canonical Wnt-transducer, was up-regulated in MPM SC (**Figure 3a**) and RhoA was more active in SC than in AC (**Supplementary Figure S2a**), the RhoA/RhoA kinase axis was not involved in the upregulation of ABCB5: indeed, when RhoA kinase was inhibited by Y27632 (**Supplementary Figure S2b**), neither c-myc binding to *ABCB5* promoter (**Supplementary Figure S2c**) nor ABCB5 mRNA (**Supplementary Figure S2d**) were modified compare to untreated MPM SC.

Wnt-driven autocrine production of IL-8 and IL-1β contributes to up-regulate ABCB5 in malignant pleural mesothelioma-derived stem cells

In melanoma initiating cells, ABCB5 secretes IL-1β that stimulates ABCB5-negative cells to increase the production of IL-8: IL-8 in turn up-regulates ABCB5 in initiating cells.[19] To explore whether an IL-1β/IL-8 loop is active also in MPM, we first screened the expression of cytokine genes in MPM cultures. Among the 84 cytokines mRNAs detectable in MPM cells, IL-8 and IL-1β were the highest expressed in SC compared to AC (**Figure 5a**). The higher mRNA levels (**Figure 5b-c**) were paralleled by the higher amount of both cytokines in the culture medium (**Figure 5d-e**) of SC (termed SC scr in Figure 5). The production of IL-8 and IL-1β was controlled by Wnt/GSK3β/β-catenin/c-myc axis: indeed, AC treated with LiCl increased the production of IL-8 and IL-1β, while SC treated with myc-i reduced the amount of both cytokines (**Figure 5f-g**). Our results are consistent with previous findings

reporting that IL-8 and IL-1β are targets of β-catenin [26,27] and c-myc. [28,29] Interestingly, *IL-8*-KO and *IL-1β*-KO SC clones, characterized by nearly undetectable levels of cytokines mRNA (**Figure 5b-c**) and protein (**Figure 5d-e**), had lower binding of c-myc to *ABCB5* promoter (**Figure 5h**) and lower ABCB5 mRNA (**Figure 5i**). Accordingly, *IL-8* and *IL-1β*-KO SC clones were significantly more sensitive to cisplatin and pemetrexed cytotoxicity (**Figure 5j**).

Also *ABCB5*-KO clones (**Supplementary Figure S3a**) had a lower secretion of IL-8 and IL-1β (**Supplementary Figure S3b-c**). Of note, *ABCB5*-KO SC had a lower binding of c-myc to the promoter of *ABCB5* compared to parental (scr) SC: the binding was increased by exogenous IL-8 and IL-1β (**Supplementary Figure S3d**), added at a concentration that restored IL-8 and IL-1β to levels comparable to parental SC (**Supplementary Figure S3e-f**).

These results suggest that multiple autocrine loops promote chemoresistance mediated by ABCB5. Firstly, Wnt/GSK3β/β-catenin/c-myc induced *ABCB5* transcription. Secondly, β-catenin- and c-myc-induced up-regulation of IL-8 and IL-1β increased c-myc transcriptional activation of *ABCB5*. Thirdly, ABCB5 promoted the secretion of IL-8 and IL-1β that in turn up-regulated c-myc binding to *ABCB5* promoter (**Figure 5k**).

ABCB5 is predictive of poor response to chemotherapy in patients with malignant pleural mesothelioma

In the FFPE MPM samples of 37 patients (32 epithelioid, 3 sarcomatous, 2 biphasic MPM), treated with cisplatin plus pemetrexed (**Supplementary Table S6**), ABCB5 was detected in isolated cells or clusters (**Figure 6a**), in particular in plasma-membrane (**Figure 6b**). The median staining intensity of ABCB5 in MPM cells (**Supplementary Table S6**) was used to dichotomize patients in ABCB5^{low} and ABCB5^{high}. As shown in **Figure 6c** and **6d**, ABCB5^{high} group had significantly lower TTP and OS,

suggesting that ABCB5 expression is predictive of poorer response to the first-line chemotherapy and poorer outcome in MPM patients.

Discussion

Few strategies – e.g. targeting the CD26-dependent activation of EGFR/ERK axis [13], using natural killer lymphocytes against tumor cells with active Wnt and Akt pathway [32] – have been evaluated pre-clinically as potential treatment options against MPM SC. However, the molecular mechanisms linking stemness and chemoresistance are not clarified in MPM, limiting the possibilities of a targeted chemosensitizing treatment.

We identified ABCB5 as a crucial efflux transporter mediating resistance to cisplatin and pemetrexed in MPM SC, under the control of the highly conserved, stemness-related Wnt pathway.

Previous findings reported a constitutive activation of the Dishevelled/GSK3β/β-catenin pathway in MPM [31-33] in commercial cell lines or tumor bulk. We demonstrated that the canonical Wnt/GSK3β/β-catenin pathway was specifically activated in MPM SC, leading to the up-regulation of several β-catenin-target genes involved in proliferation, invasion, angiogenesis and chemoresistance.

Wnt pathway inhibitors emerged as anti-proliferative strategies and chemosensitozers in MPM. [34-38] However, no studies investigated if the chemosensitization was due to the specific targeting of SC.

Our work indicated that ABCB5 induces constitutive resistance cisplatin and pemetrexed in SC derived from MPM patients. We cannot exclude a priori that other ABC transporters expressed in MPM SC, such as ABCG2 and ABCB1, another target of Wnt pathway [16], mediate chemoresistance. However, cisplatin and pemetrexed are not substrates of these transporters.[12] By contrast, ABCB5 is known to induce resistance to carboplatin [20] and to recognize a broad spectrum of chemotherapeutic drugs.[19] Hence, it may determine a multidrug resistant phenotype in MPM SC.

We propose that inter-connected mechanisms up-regulate ABCB5 in MPM SC. Firstly, Wnt/GSK3β/β-catenin/c-myc axis induced ABCB5 transcription, as demonstrated by the pharmacological inhibition of Wnt/GSK3β/β-catenin/c-myc axis. Secondly, IL-8/c-myc and IL-1β/c-myc axis up-regulated ABCB5, as demonstrated by IL-8 and IL-1β-KO clones. Partially in disagreement with our data, Wilson and colleagues proposed that ABCB5 is upstream to Wnt pathway in melanoma initiating cells, where ABCB5 and Wnt pathway are upregulated by IL-8 and IL-1β.[19] In MPM SC the pharmacological inhbition of Wnt/c-myc axis demonstrated that this axis controls the production of both IL-8 and IL-1β, suggesting that IL-8 and IL-1β are targets and a controllers of β-catenin/c-myc transcriptional program in MPM SC. Differently from melanoma, that requires a paracrine cooperation between ABCB5-positive and ABCB5-negative cells to maintain high levels of IL-1β and IL-8, and chemoresistance [19], MPM SC adopted a completely autocrine system. Indeed, ABCB5-KO clones had lower secretion of IL-8 and IL-1β cells, suggesting that ABCB5 controls the secretion of both cytokines, and lower c-myc binding to ABCB5 promoter, restored by exogenous IL-8 and IL-1β. These results support the hypothesis that Wnt/GSK3β/β-catenin/c-myc/ABCB5 axis, IL-8/c-myc/ABCB5 axis and IL-1β/c-myc/ABCB5 axis are part of feed-forward circuitries that maintain chemoresistance in MPM SC.
The clinical meaning of ABCB5 was validated in a retrospective series of MPM patients treated with platinum-derivatives and pemetrexed, since patients highly expressing ABCB5 had significantly lower TTP and OS. ABCB5 was positively associated with tumor progression and recurrence in oral squamous cell carcinomas [39], but – to the best of our knowledge – this is the first time that ABCB5 emerged as a potential marker of chemoresistance. The patients series analyzed was limited. Since ABCB5 can be easily detected by immunohistochemistry analysis, its expression is being evaluated in a larger cohort of MPM patients, to strengthen its predictive value.
Our study provides the first evidence of a molecular link between the classical stemness-related Wnt pathway and the chemoresistance related to ABC transporters, namely ABCB5, in MPM. Targeting

Wnt-pathway or IL-8/IL-1β signaling may be chemosensitizing strategies against MPM SC. Moreover, we suggest to include the analysis of ABCB5 levels in the diagnostic assessment of MPM patients, as a potential stratification marker identifying patients more resistant to the first-line chemotherapy.

Competing interests

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by Italian Association for Cancer Research (IG15232 to CR); Italian Ministry of University and Research (Future in Research program RBFR12SOQ1 to CR; Basic research Funding program, FABR2017 to LR and CR); Italian Ministry of Health (GR-2011-02348356 to LR; Italian Mesothelioma Network-CCM2012 to GVS) University of Torino (Intramural Grant 2015 to SN and CR; Intramural Grant 2016 to LR; Intramural Grant 2017 to JK); Fondazione Cassa di Risparmio di Torino (2016-2443 to CR). VM is a PhD fellow of Erasmus Mundus-ERAWEB Action 2 program. ICS is a post-doctoral research fellow supported by the Fondazione Franco e Marilisa Caligara for Multidisciplinary Sciences, Torino, Italy. TA-S is a fellow of Trialect Inc., Global Medical Education Program. The funding institutions had no role in the study design, data collection and analysis, or in writing the manuscript.

We are grateful to Mr. Costanzo Costamagna, Dept of Oncology, University of Torino, and to Dr. Erika Ortolan, Department of Medical Sciences, University of Torino, for the technical assistance.

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Figures legends

Figure 1. Isolation and characterization of malignant pleural mesothelioma stem cells

a. Morphological analysis of adherent cells (AC) and stem-enriched cultures (SC) derived from one epithelioid (Epi), one sarcomatous (Sar) and one biphasic (Bip) patient-derived malignant pleural mesothelioma (MPM), by contrast-phase microscope. Magnification: 20× objective lens (0.52 numerical aperture); 10× ocular lens. Bars: 100 μm. **b.** Percentage of aldehyde dehydrogenase $(ALDH)^{bright}$-positive cells, measured by flow cytometry. Dot plots show one epithelioid MPM sample. Similar results were obtained on all the other MPM analyzed. The analysis was performed on viable cells according to cell morphology (R1 gate, left plots). R2 gate (right plots) was designed to identify $ALDH^{bright}$-positive cells, as per manufacturer's instruction. **c.** Flow cytometry analysis of stemness markers Oct4, Nanog, SOX2 and ABCG2 in AC and SC epithelioid, sarcomatous and biphasic MPM. The histograms are representative of one patient per each histotype. **d.** Self-renewal assay. AC and SC were diluted and seeded at a density of 1 cell/well; cells were counted weekly until day 48. Data are presented as means±SD of all MPM samples (n=3 experiments, 12 wells/sample). *$p<0.001$:SC vs AC (days: 35-48). **e**. Clonogenic assay. AC and SC were seeded at a density of 100 cells/well; the spheres or adherent colonies were counted weekly, until day 48. Data are presented as means±SD of all MPM samples (n=3 experiments, 6 wells/sample). *$p<0.001$:SC vs AC (days: 35-48). **f.** Hitmap of stemness-related genes in SC. The expression of each gene in the corresponding AC was considered 1 (not shown in the figure). The whole list of up- or down-regulated genes is reported in the Supplementary Table S4.

Figure 2. ABCB5 determines resistance to cisplatin and pemetrexed in malignant pleural mesothelioma stem cells

AC and SC MPM were grown in fresh medium (ctrl), incubated with cisplatin (Pt, 25 μM) or pemetrexed (PMX, 5 μM). **a-b.** Release of LDH, measured spectrophotometrically after 24 h in

duplicates, and cell viability, measured using a chemiluminescence-based method after 72 h in quadruplicates. Data are presented as means±SD of the pool of UPN 1-6. *p<0.01:Pt/PMX-treated cells vs ctrl cells; °p<0.005:SC vs AC. **c.** ABCB5 mRNA as determined by qRT-PCR in triplicates. Data are presented as means±SD of UPN 1-6 pool. *p<0.001:SC vs AC. **d.** Representative immunofluorescence analysis of ABCB5. Green signal: ABCB5; blue signal: nuclear counterstaining with DAPI. Magnification: 63× objective lens (1.42 numerical aperture); 10× ocular lens. Bar: 20 μM. The micrographs are representative of one patient per each histotype. **e.** Flow cytometry analysis of surface ABCB5 in AC and SC. The histograms are representative of one patient per each histotype. **f.** SC from UPN1 (epithelioid MPM, epi) and UNP4 (sarcomatous MPM, sar) were transduced with a non-targeting scrambled vector (scr) or with a CRISPR/Cas9 ABCB5-targeting vector (KO), lysed and immunoblotted with the indicated antibodies. The AC of the corresponding patients were used as internal control of cells lowly expressing ABCB5. The figure is representative of 1 out of 3 independent experiments. **g-h**. The release of LDH was measured spectrophotometrically in duplicates, cell viability was measured using a chemiluminescence-based method in quadruplicates in UPN1 and UPN4 SC. Data are presented as means±SD (n =3). * p<0.001:ABCB5-KO cells vs scr-cells. **i.** SC from UPN1 (epitelioid MPM) or UPN4 (sarcomatous MPM) were inoculated s.c. in 9-weeks old NOD-SCID-γ Balb/C female mice, and treated as reported in Methods section. Data are means±SD (n=6/group). *p<0.005:KO Pt+PMX vs. all the other groups (day 48). **j.** Representative photos of tumors (day 48).

Figure 3. Malignant pleural mesothelioma stem cells have Wnt canonical pathway constitutively up-regulated

a. Hitmap of Wnt pathway-related genes in epithelioid (Epi), sarcomatous (Sar) and biphasic (Bip) SC. The expression of the each gene in the corresponding AC was considered 1 (not shown in the figure). The whole list of up- or down-regulated genes is reported in Supplementary Table S5. **b.** Flow

cytometry analysis of Wnt receptor and co-receptor Frizzled-1 and LRP6 in MPM AC and SC. The histograms are representative of one epithelioid (Epi, UPN1) and sarcomatous (Sar, UPN4) patient. Similar results were obtained in all the other MPM analyzed. c**.** Immunoblot analysis of phospho(Tyr279/Tyr216)GSK3 (pGSK3β), GSK3β, phospho(Ser33/Ser37/Thr41)-β-catenin (pβ-cat), β-catenin (β-cat) in whole-cell lysates of epithelioid (UPN1) and sarcomatous (UPN4) AC and SC. The β-tubulin expression was used as a control of equal protein loading. Similar results were obtained in all the other MPM analyzed. **d.** Whole-cell lysates of epithelioid (UPN1) and sarcomatous (UPN4) AC and SC were immunoprecipitated (IP) with an anti-β-catenin (β-cat) antibody, then immunoblotted (IB) with an anti-mono/polyubiquitin (UB) antibody. The β-tubulin expression was used as a control of equal protein loading. Similar results were obtained in all the other MPM analyzed. no Ab: Epi AC sample immunoprecipitated without anti-β-catenin antibody. **e.** The cytosolic and nuclear extracts from epithelioid (UPN1) and sarcomatous (UPN4) AC and SC were analyzed for the amount of β-catenin (β-cat). The expression of β-tubulin and TBP were used as a controls of equal protein loading in cytosolic and nuclear fractions. The figure is representative of 1 out of 3 experiment with similar results. **f**. RT-PCR expression of β-catenin-target genes ABCB1, FGF4, VEGFA and c-myc, measured in triplicates in AC and SC. Data are presented as means$\pm$SD of UPN 1-6 pool. *$p<0.02$ (ABCB1), $p<0.002$ (FGF4), $p<0.001$ (VEGFA), $p<0.002$ (c-myc):SC vs AC.

Figure 4. ABCB5 is controlled by Wnt/GSK3β/β-catenin/c-myc pathway

AC and SC were grown in fresh medium (ctrl). When indicated, AC were treated for 6 h (panel **a-b**) or 24 h (panel **c-d**) with the Wnt pathway activator (i.e. GSK3β inhibitor) LiCl (10 mM), SC were treated with the c-myc inhibitor 5-[(4-Ethylphenyl)methylene]-2-thioxo-4-thiazolidinone (myc-i, 250 µM). **a.** Representative immunofluorescence analysis of c-myc in AC and SC MPM cells from UPN1, grown in fresh medium (ctrl). Red signal: c-myc; blue signal: nuclear counterstaining with DAPI: Magnification: 63× objective (1.42 numerical aperture); 10× ocular lens. Bar: 20 µM. Similar results were obtained in

all the other MPM analyzed. **b**. Binding of c-myc to the ABCB5 promoter, measured by ChIP, in triplicates. Data are presented as means±SD of UPN 1-6 pool. *p<0.02:LiCl-treated/myc-i-treated cells vs ctrl cells; °p<0.02:SC vs AC. **C.** Levels of ABCB5 mRNA as determined by qRT-PCR, in triplicates. Data are presented as means±SD of UPN 1-6 pool. *p<0.005:LiCl/myc-i-treated cells vs ctrl cells; °p<0.001:SC vs AC. **d.** Cells treated as indicated above were grown in the absence (ctrl) or in the presence of cisplatin (Pt, 25 μM) or pemetrexed (PMX, 5 μM). The release of LDH was measured spectrophotometrically, in duplicates. Data are presented as means±SD of UPN 1-6 pool. *p<0.001:Pt/PMX-treated AC vs ctrl cells; °p<0.001:Pt/PMX-treated SC vs Pt/PMX-treated AC; #p<0.005:LiCl-treated or myc-i-treated, Pt/PMX-treated AC vs Pt/PMX-treated AC or SC, respectively.

Figure 5. IL-8 and IL-1β contribute to ABCB5-mediated resistance in malignant pleural mesothelioma stem cells

a. Relative expression of cytokine mRNAs in SC vs AC MPM, measured by qRT-PCR array. Data are presented as means±SD of the pool of UPN 1-6 SC. **b-c.** mRNA levels of IL-8 or IL-1β assessed by qRT-PCR, in triplicates, in SC transfected with a non-targeting scrambled vector (scr) or with a CRISPR/Cas9 IL-8- or IL-1β-targeting vectors (KO). AC (-) were included as reference. Data are presented as means±SD of UPN 1-6 pool. *p<0.002:scr-SC vs AC; °p<0.001:KO-SC vs scr-SC. **d-e.** IL-8 or IL-1β production, measured by ELISA, in duplicates. Data are presented as means±SD of UPN 1-9 pool. *p<0.05:scr-SC vs AC; °p<0.001:KO-SC vs scr-SC. **f-g.** IL-8 or IL-1β production, measured by ELISA, in duplicates, in AC and SC MPM cells were grown for 24 h in fresh medium (ctrl), treated with the GSK3β inhibitor (i.e. Wnt pathway activator) LiCl (10 mM) or the c-myc inhibitor 5-[(4-Ethylphenyl)methylene]-2-thioxo-4-thiazolidinone (myc-i, 250 μM). Data are presented as means±SD of UPN 1-6 pool. *p<0.02:LiCl-treated AC/ctrl SC vs ctrl-AC; °p<0.001:myc-i-treated SC vs ctrl-SC. **h.** Binding of c-myc to the ABCB5 promoter, measured by ChIP, in triplicates. Data are presented as means±SD of UPN 1-6 pool. *p<0.02:scr-SC vs AC; °p<0.05:KO-SC vs scr-SC. **i.** Levels of ABCB5

mRNA as determined by qRT-PCR, in triplicates. Data are presented as means±SD of UPN 1-6 pool. *$p<0.001$:scr-SC vs AC; °$p<0.01$:KO-SC vs scr-SC. **j.** SC were grown in fresh medium (24 h) or in medium containing cisplatin (Pt, 25 µM) or pemetrexed (PMX, 5 µM). The release of LDH was measured spectrophotometrically, in duplicates. Data are presented as means±SD of UPN 1-6 pool. *$p<0.005$:Pt/PMX-treated IL-8 KO/IL-1β KO-cells vs untreated (ctrl) KO-cells; °$p<0.002$:Pt/PMX-treated IL-8 KO/IL-1β KO-cells vs Pt/PMX-treated scr-cells. **k.** Proposed mechanisms of the multiple autocrine loops up-regulating ABCB5 and determining resistance to cisplatin and pemetrexed in MPM SC.

Figure 6. ABCB5 is a negative prognostic factor in malignant pleural mesothelioma patients

a-b. Representative immunohistochemistry images of ABCB5-positive cells within MPM (panel **a**: 60×, bar: 100 µm; panel **b**: 100×, bar: 100 µm). Arrow: ABCB5-strongly positive cells. **c-d.** ABCB5 staining was ranked according to staining extent of each patient (Supplementary Table 6), and median value was calculated. Patients were classified as $ABCB5^{low}$ and $ABCB5^{high}$ if the staining was low or equal/higher than the median value. Time to progression (panel **c**), for the patients with available data, and overall survival (panel **d**) probability was calculated using the Kaplan-Meier method. *$p<0.005$ (panel c); *$p<0.05$ (panel d): $ABCB5^{high}$ vs $ABCB5^{low}$ group.