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Original Articles

ABCG1 and Pgp identify drug resistant, self-renewing osteosarcoma cells

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ABSTRACT

Patients with osteosarcoma (OST) frequently relapse with drug resistant disease, consistent with the hypothesis that tumours contain a cancer stem-like cell (CSCs) population that survives chemotherapy to re-populate the tumour at local or metastatic sites. We describe a dual functional approach to isolate OST-CSCs and identify the ABC transporter proteins driving this population to reveal potential targets for the development of new treatments.

OST-CSCs were isolated by selection in doxorubicin (OST-EC50 cells) and based on the ability to produce progeny from a single cell (HOS-EC50.SR cells). Pgp expression was increased in OST-EC50 cells, inducing resistance to doxorubicin, etoposide, vincristine and actinomycin D ($p < 0.05$). Increased expression of ABCG1 and Pgp protein in the HOS-EC50.SR cells induced resistance to etoposide and doxorubicin ($p < 0.01$), which was directly correlated with ABCG1 expression ($r > 0.88$, $p < 0.001$).

Pgp expression is increased in both the HOS-EC50 cells where it mediates MDR and the HOS-EC50.SR populations, whereas ABCG1 was only upregulated in the self-renewing drug resistant HOS-EC50.SR cells. Targeting ABCG1 and Pgp may eradicate the drug resistant self-renewing OST-CSCs, leading to improved outcomes for patients with OST.

1. Introduction

Osteosarcoma (OST) is the most common primary malignant tumour of the bone [1], frequently presenting in young people between the ages of 10–14 years and in adults over 65 years [2]. OST patients are treated with a combination of surgery, radiotherapy and high dose chemotherapy [3] which led to improved 5 year survival of patients with localised disease (51–68% [4–8]). However, there has been no increase in survival in the last 40 years [9] and the outcome for patients presenting with metastasis at diagnosis (14% [10]) has remained poor (< 30% survival at 5 years [9]).

Despite the intensive treatment, only 42–60% of patients achieve > 90% tumour necrosis [6,7] (a biomarker of response and clinical outcome [11]), reflecting an inherent resistance to therapy. Consistent with an incomplete response, 30–50% of patients with localised disease and 80% of those with metastasis at diagnosis will ultimately relapse [12]. Reflecting the development of acquired multidrug resistance (MDR) in response to treatment, relapsed OST are often unresponsive to chemotherapy (> 40% [13,14]), resulting in poor 5 year survival rates of < 16–20% [9,10,12]. One mechanism of acquired MDR is the overexpression of ABC transporter proteins, such as p-glycoprotein (Pgp, ABCB1), which induce MDR through the active efflux of chemotherapeutic agents from cells to reduce the efficacy of the treatment and enabling cell survival. In OST, high expression of Pgp has been linked with a worse disease progression [15–18].

Cells with an aggressive MDR metastatic phenotype have been described in a variety of cancers [19–25], including OST [26–29] and are often referred to as cancer stem-like cells (CSCs). Additional characteristics of a CSC include increased expression of stem cell markers such as sex determining region Y-box 2 (SOX2), octamer-binding transcription factor 4 (OCT4), homeobox transcription factor Nanog (NANOG) [30], increased migration often linked with metastasis [31] and decreased cell growth, which may reduce response to cytotoxic chemotherapy [32]. Therefore, we have hypothesised identification and eradication of the OST-CSC using targeted therapy in combination with standard chemotherapy, will prevent tumour recurrence and improve the overall survival of patients with OST.

Like embryonic, mesenchymal [33] and hematopoietic stem cells [34], CSCs are reported to overexpress ABC transporter proteins [35–38], consistent with their ability to survive chemotherapeutic insult. Such ABC transporter efflux activity has been employed to isolate bone CSCs from cell lines [35–38], as has the expression of cell surface

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Abbreviations

ABC transporter	ATP binding cassette transporter
ANOVA	Analysis of variance
CSC	Cancer stem-like cell
EC50	Half maximal effective concentration
ES	Ewing's sarcoma
FCS	Foetal calf serum
LIMMA	Linear Models for Microarray Data
MDR	Multi-drug resistance
NANOG	Homeobox transcription factor Nanog
OCT4	Octamer-binding transcription factor 4
OST	Osteosarcoma
Pgp	P-glycoprotein/ABCB1
PPIA	Peptidylprolyl isomerase A
SOX2	Sex determining region Y-box 2
SR	Self-renewing

proteins, such as CD133 [39]. However, several studies have described self-renewing ability in CD133 negative cells, both *in vitro* [40–42] and *in vivo* [43], suggesting some cell surface protein markers fail to robustly identify the complete CSC population [27]. OST-CSCs have also been identified in cell lines and patient derived cultures based on sphere formation [27–29,44,45], although these studies have not examined the progeny producing efficiency of a single cell.

To isolate putative OST-CSCs we have therefore selected cells capable of both surviving doxorubicin treatment and the ability to produce progeny from a single cell. We have then examined the ABC transporter profile of these cells with a view to identifying potential ABC transporter targets for the development of CSC-specific therapeutics.

2. Materials and methods

2.1. Cell lines and primary OST cell cultures

The HOS (fibroblastic and epithelial) and MG-63 (fibroblastic) cell lines were obtained from ATCC (Manassas, USA) and cultured in Eagle's Minimum Essential Medium (Sigma-Aldrich, UK) and the U-2OS (epithelial; ATCC) cells in McCoy's (Sigma-Aldrich) plus 10% foetal calf serum (FCS, Harlan Sera-Lab, UK) and 2 mM glutamine (Sigma-Aldrich). Of the patient derived OST cell cultures, 3/6 were derived from osteoblastic tumours and 1/6 was of chondroblastic origin; the subtype of 2 OSTs was unknown.

The substrate adherent Ewing's sarcoma (ES) cells TC-32, the breast adenocarcinoma MCF-7, and the neuroblastoma SK-N-SH cells were cultured as previously described [46]. The TC-32 ES cells contain an *EWSR1-Fli1* gene re-arrangement and express CD99, characteristic of ES. A431 and HEK293 (Professor Knowles, University of Leeds, UK) were cultured in DMEM (Sigma-Aldrich, UK) containing 10% FCS, 2 mM glutamine and the hepatocyte carcinoma cell line, HepG2 (Dr. Ewan Morrison, University of Leeds) in RPMI 1640 (Sigma-Aldrich) containing 10% FCS and 2 mM glutamine.

Informed consent and ethical approval were obtained for the use of patient derived OST cells (IRAS167880), which were isolated from tumour biopsies from 6 patients by macerating the tumour and culturing cells in Leeds Antibiotic Media (RPMI 1640 containing 10% FCS, 2 mM glutamine, 100 units of penicillin and 0.1 mg/ml streptomycin (Thermo Fisher Scientific, UK)). All cell lines and primary cultures are yeast, bacterial and mycoplasma-free (EZ-PCR mycoplasma test kit; Geneflow, UK).

2.2. Selection of drug resistant OST cells

To identify the half maximal effective concentration (EC50) of doxorubicin, cells were treated with doxorubicin (7–448 nM, Sigma-Aldrich) for 48 h and viable cell number counted using trypan blue exclusion (Vi-cell, Beckman Coulter, UK) [46]. To select OST cells that were resistant to doxorubicin, we maintained cells in the cell line specific EC50 of doxorubicin for up to 6 months; HOS (14 nM), MG-63 (28 nM) and U-2OS (28 nM) (Fig. 1Ai). OST-EC50 cells were re-challenged with 10-fold EC50 of doxorubicin until the OST-EC50 cell population was stable; after 33, 159 and 31 days respectively. The effect of doxorubicin (3.5–448 nM), vincristine (4 nM), etoposide (420 nM), actinomycin D (1.6 nM) (Sigma-Aldrich), fenretinide (5 μM, National Cancer Institute [47]) on viable cell number examined [46].

2.3. Characterisation of OST, OST-EC50, OST.SR and OST-EC50.SR cells

2.3.1. Migration

After 4 days, spheroids were lifted into 24-well gelatin (0.1%) coated plates (BD Biosciences, UK) containing culture media (600 μl) and visualised using light microscopy (Olympus CKX41, UK) for up to 72 h. The images were analysed using Volocity® software (Perkin Elmer, UK) and the Migration Index (MI) calculated.

2.3.2. Viable cell growth

The morphological features of cells were visualised by light microscopy (Olympus CKX41) and cell doubling times were determined by counting viable cell number with time (24 h-72 h) using the trypan blue exclusion assay and the automated Vi-cell [46].

2.3.3. Cell cycle analysis

Cells were fixed in ethanol overnight at −20 °C and then incubated with propidium iodide (40μg/ml in PBS containing sodium citrate (3.8 mM, Sigma-Aldrich) and 1.5U of RNase Cocktail Enzyme Mix (Thermo Fisher Scientific)) for 3 h at 4 °C protected from light before analysis on the FACSCalibur (BD Biosciences). The percentage of cells in G0/G1, G2/M and S phase was determined using the FlowJo Cell Cycle Platform (FlowJo, Tree Star, Inc., USA).

2.4. Profile of ABC transporter proteins in OST, OS-EC50 and OST.SR cells

RNA was extracted from cells using the RNeasy Mini Kit (Qiagen,

Table 1
The EC50 value and fold increase in resistance to doxorubicin of OST-EC50 cells.

Cell line	EC50 (nM) (± SEM)	Fold increase in resistance to doxorubicin (p value, n = 3)
HOS-EC50	61.7 ± 0.4	4 ($p < 0.0001$)
MG-63-EC50	101.8 ± 0.3	4 ($p < 0.001$)
U-2 OS-EC50	169.3 ± 0.4	6 ($p < 0.0001$)

The fold increase in resistance to doxorubicin was calculated as the doxorubicin EC50 value of the parental cells expressed as a ratio of the doxorubicin EC50 value of the OST-EC50 cells and compared using a non-parametric Mann-Whitney two-tailed *t*-test. Results are given as the mean ± SEM of three independent experiments.

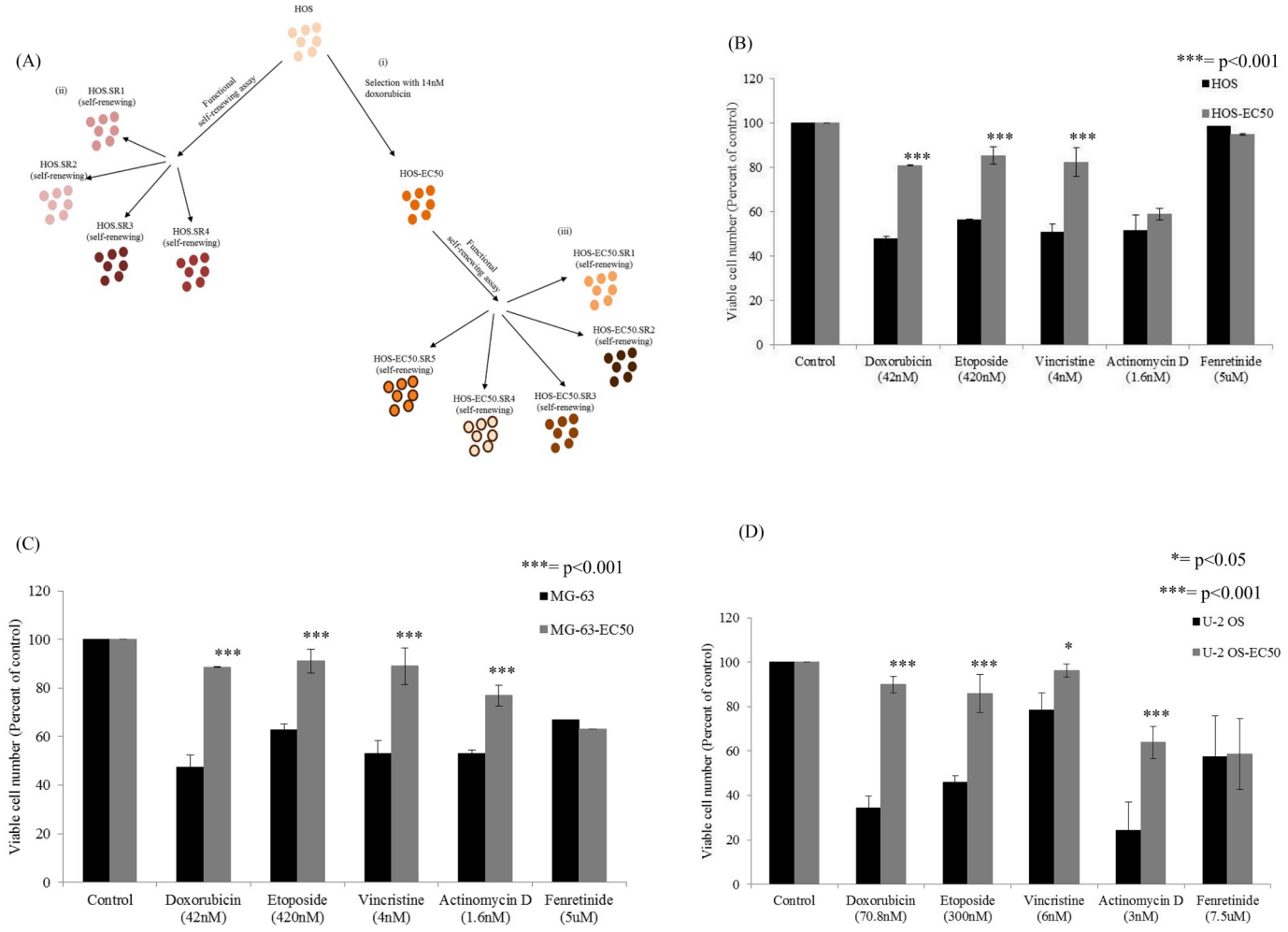


Fig. 1. Generation of multi-drug resistant OST-EC50 and self-renewing HOS.SR and HOS-EC50.SR cells. (A) Experimental strategy for (i) the selection of OST cells by continuous culture in the corresponding EC50 of doxorubicin and the generation of (ii) HOS.SR and (iii) HOS-EC50.SR cultures from the HOS and HOS-EC50 cells respectively, using a functional self-renewing assay from a single cell. The percentage of viable (B) HOS, HOS-EC50, (C) MG-63, MG-63-EC50, (D) U-2OS, U-2OS-EC50 cells following treatment with a single concentration of doxorubicin (42 nM), etoposide (420 nM), vincristine (4 nM), actinomycin D (1.6 nM), fenretinide (5 μM). * = $p < 0.05$, *** = $p < 0.001$, non-parametric Mann-Whitney two-tailed *t*-test. EC50 = effective concentration, OST = osteosarcoma, SR = self-renewing, SEM = standard error of the mean.

UK) and 1 μg converted to cDNA by reverse transcription [46]. Expression of 50 ABC transporter mRNAs was evaluated using the TaqMan® Human ABC Transporter Array (Thermo Fisher Scientific) [46]; expression reported relative to the optimal endogenous control gene, Peptidylprolyl isomerase A (PPIA [48–50]), using the comparative Ct method [51].

Differences in mRNA expression were determined using Linear Models for Microarray Data (LIMMA; Bioconductor [52]), reporting an adjusted p-value (Q-value). ABC transporter mRNAs were validated using single RTqPCR assays (10 ng RNA, Supplementary Table 1) if they ranked in the top 10 differentially expressed genes by LIMMA and there was a change in mean Ct value of > 2 between populations [53], excluding Ct values > 35 [54]. If a significant difference in mRNA expression was observed following analysis in a single RTqPCR assay, protein expression of the target was firstly validated using Western blot [46], followed by immunofluorescence and microscopy (Supplementary materials and methods).

2.5. Determining the self-renewing ability of OST, OST-EC50, OST.SR and OST-EC50.SR cells and generation of daughter progeny cell cultures

A single cell (Poisson distribution probability of $\lambda < 1 = 0.9$) was seeded onto each well of 10 Primeria™ 96 well plates and the number of wells containing ≥5 cells recorded at 21 days using light microscopy (Olympus CKX41). Where possible, self-renewing (SR) cell populations were propagated further in 25 cm^2 and then 75 cm^2 Primeria™ tissue culture flasks to establish daughter cell cultures (Fig. 1AII and Fig. 1AIII).

To determine if stable cell populations with the ability to self-renew have been established, a single cell from each daughter cell culture was serially transplanted into each well of 10 Primeria™ 96 well plates and again, the number of wells containing ≥5 cells recorded after 21 days using light microscopy (as above).

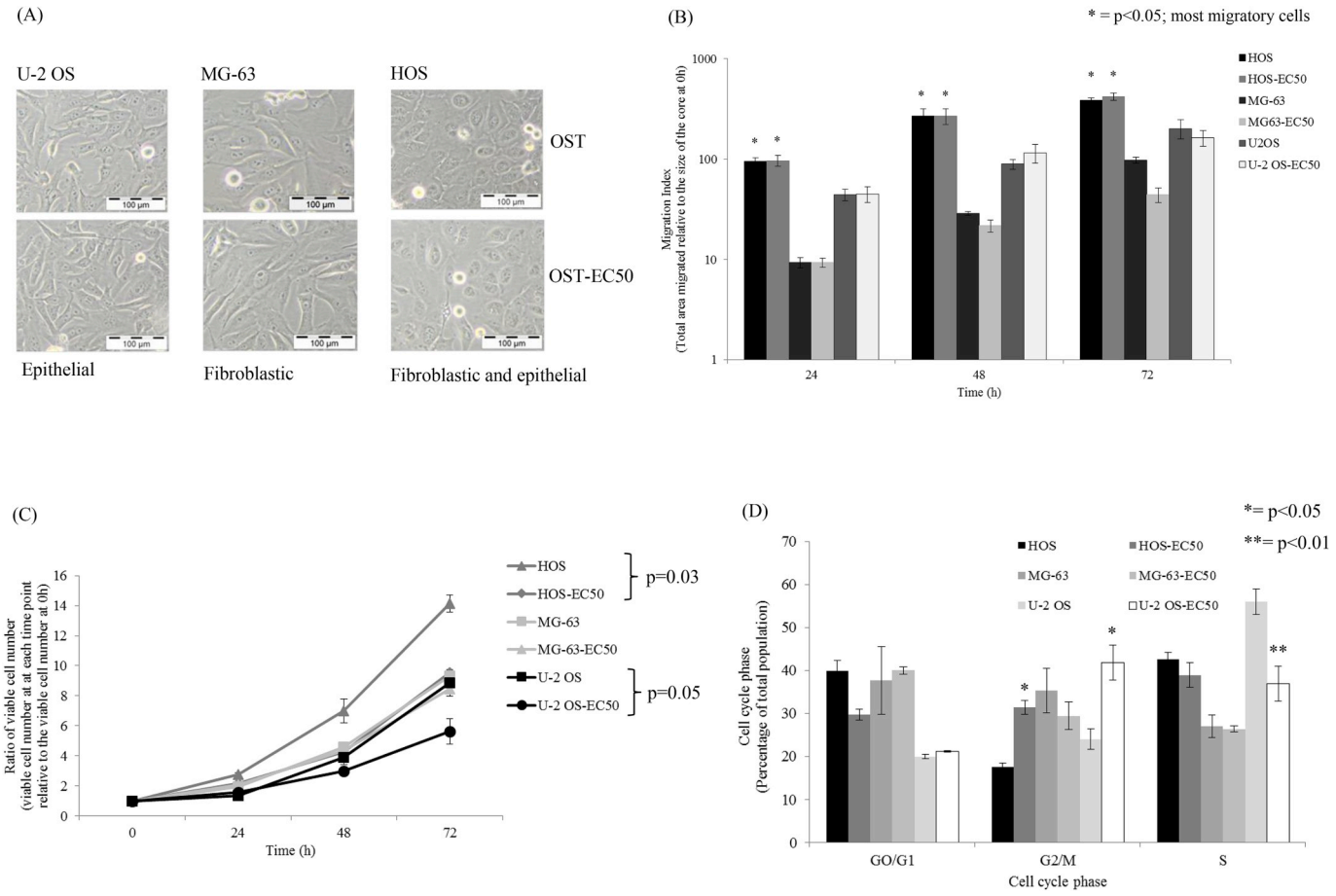


Fig. 2. The morphology, growth and migration of OST and OST-EC50 cells. (A) Cells were imaged by light microscopy. Scale bar = 100 µm. (B) The migration index (MI) of cells. The total migrated area was expressed relative to the spheroid core at each time point (mean ± SEM). The HOS and HOS-EC50 cells were the most migratory cell populations. (C) The increase in viable cell number over time (72 h) was determined by trypan blue exclusion and expressed as a ratio of the starting mean viable cell number at 0 h (mean ± SEM, non-parametric Mann-Whitney two-tailed *t*-test). (D) The percentage of cells in each cell cycle phase was determined by flow cytometry (mean ± SEM). * = $p < 0.05$, ** = $p < 0.01$, ANOVA and a Tukey's post-hoc test. EC50 = effective concentration, MI = migration index, OST = osteosarcoma, SEM = standard error of the mean.

2.6. Functional evaluation of efflux activity in OST, OST-EC50 and OST-EC50.SR4 cells

Cells were loaded with 0.05 µM of non-fluorescent calcein-AM for 30min [46]. Calcein-AM is converted to fluorescent calcein-F by intracellular esterases and removed from the cell by ABC transporter protein efflux activity [46]. To compare the efflux activity of OST, OST-EC50 and OST.EC50.SR4 cells the percentage of calcein-F effluxed from cells over 1 h was determined using the Attune NxT Flow Cytometer (Thermo Fisher Scientific), analysing 10 000 events. Unlabelled control samples were included to correct for autofluorescence.

2.7. Statistical analysis

Significant differences were determined using linear regression and compared using the extra sum of squares F test, a non-parametric Mann-Whitney two-tailed *t*-test or ANOVA with a Tukey's post-hoc test. Correlations were determined using a Pearson's correlation coefficient (r). EC50 values were calculated using regression analysis, applying a global best fit line. Statistical analyses were performed using Graphpad PRISM 7.03 (USA).

A hierarchical tree predicting cell derivation based on the expression of ABC transporter protein mRNAs, was generated using the cellTree package (Version 1.10.0 [55]) applying a Latent Dirichlet Allocation model fit using a Gibbs sampling method.

3. Results

3.1. Multidrug resistance of OST and OST-EC50 cells

Doxorubicin resistant HOS-EC50, MG63-EC50 and U-2OS-EC50 cells were established after 33, 159 and 31 days respectively ($p < 0.001$, Table 1). All OST-EC50 populations were also more resistant to etoposide and vincristine ($p < 0.05$, Fig. 1B–D) and the MG-63-EC50 (Fig. 1C) and U-2OS-EC50 (Fig. 1D) cells were also resistant to actinomycin D ($p < 0.001$). Interestingly, the effect of fenretinide on OST-EC50 viable cell number was unchanged (Fig. 1B–D).

3.2. Characterisation of OST and OST-EC50 cells

There was no change in the morphology (Fig. 2A), MI (Fig. 2B) or expression of the stem cell markers NANOG, OCT-4, SOX-2 (data not shown) in the OST-EC50 cells compared to the OST cells. However, the cell doubling time ($p < 0.05$, Fig. 2C) and the percentage of cells

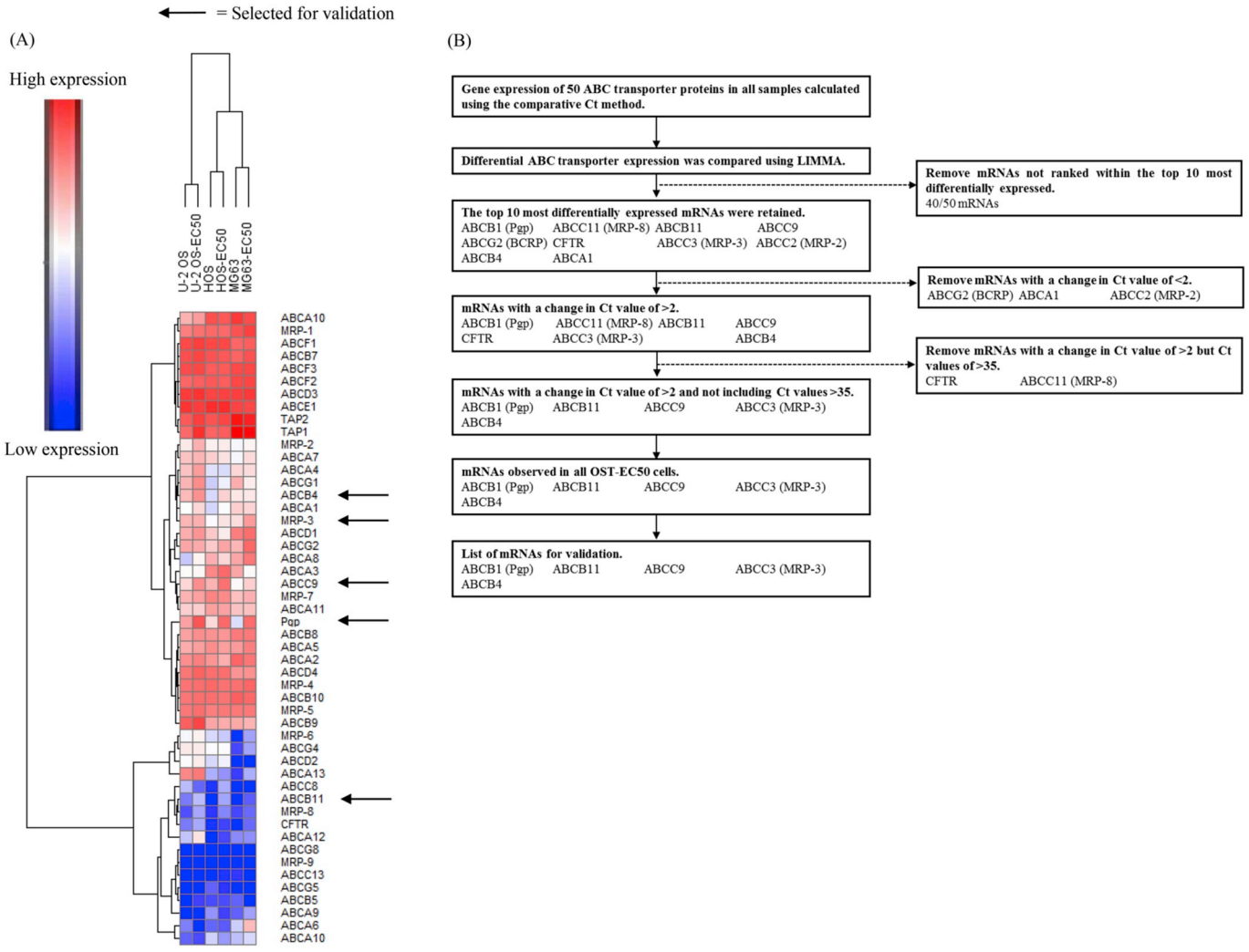


Fig. 3. Differential expression of ABC transporter proteins in OST-EC50 cells and the corresponding OST cells. (A) Heatmap visualisation of the ABC transporter mRNA expression profile. The heatmap was generated using GENE-E (Broad Institute, USA), clustering cell lines and ABC transporter genes hierarchically, using one minus Pearson correlation for the cell lines and Euclidean distance for ABC transporter genes with clustering for complete linkage. Solid black arrow = selected for validation. Red = high expression, blue = low expression. (B) Details of the strategy employed to select differentially expressed mRNAs for further validation from the TaqMan® Human ABC Transporter Array. (C) Pgp mRNA expression profile of OST and OST-EC50 cells. RNA (10 ng of OST, OST-EC50 RNA and 10 ng of SK-N-SH RNA included as a positive control) was analysed by RTqPCR and presented as $2^{-\Delta\Delta Ct}$ by normalisation of Pgp Ct values to PPIA and the positive control cell line SK-N-SH. The fold increase in Pgp mRNA expression is reported as the $2^{-\Delta\Delta Ct}$ of the OST-EC50 cells relative to the $2^{-\Delta\Delta Ct}$ of the matched parental OST cells (± SEM). (D) Protein expression of Pgp in OST and OST-EC50 cells, detected by Western blot. Equal protein loading was confirmed by expression of β-actin. Expression of Pgp is reported as a ratio of densitometry value of the Pgp protein band relative to that of the β-actin band and the fold increase in Pgp expression reported as the normalised Pgp expression in the OST-EC50 cells relative to that of the matched parental OST cells (mean ± SEM). (E) ABCC9 mRNA expression profile of OST and OST-EC50 cells. RNA (10 ng of OST, OST-EC50 RNA and 1 µg of TC-32 RNA included as a positive control) was analysed by RTqPCR and presented as $2^{-\Delta\Delta Ct}$ by normalisation of ABCC9 Ct values to PPIA and the positive control cell line TC-32. (F) Protein expression of ABCC9 in OST and OST-EC50 cells, detected by Western blot. Equal protein loading was confirmed by expression of β-actin. * = $p < 0.05$, ** = $p < 0.01$, non-parametric Mann-Whitney two-tailed *t*-test. EC50 = effective concentration, OST = osteosarcoma, PPIA = Peptidylprolyl isomerase A.

arrested in the mitotic G2/M phase ($p < 0.05$, Fig. 2D) was significantly increased in the HOS-EC50 and U-2OS-EC50 cells. In contrast, there was no significant difference in the increase in viable cell number with time (cell growth; $p > 0.05$, Fig. 2C; see Supplementary Fig. 1 for individual growth curves) or cell cycle (Fig. 2D) of MG-63 and MG-63-EC50 cells. Confirming MDR was not a direct product of deceased cell growth in the HOS-EC50 and U-2OS-EC50 cells, there was no correlation between the EC50 of doxorubicin (Fig. 1B–D) and cell cycle phase ($R^2 = 0.5$, $p = 0.10$) or doubling time ($R^2 = 0.6$, $p = 0.03$).

3.3. Differential expression of ABC transporter proteins in MDR OST-EC50 cells

Since MDR has been attributed to increased ABC transporter expression in OST, we have compared the ABC transporter profile of OST and OST-EC50 cells (details in Supplementary Table 2). Hierarchical clustering revealed parental OST and daughter OST-EC50 cells grouped together (Fig. 3A). Most likely reflecting the morphology of the cells (Fig. 2A), the fibroblastic MG-63 and HOS populations clustered independently from the epithelial U-2OS cell cultures (Fig. 3A). Pgp, ABCB11, ABCC9, MRP-3 and ABCB4 mRNAs were differentially expressed in parental and OST-EC50 cells, identifying them for further investigation (Fig. 3A and B, Table 2).

3.4. Confirmation of increased Pgp expression in OST-EC50 cells

Expression of Pgp was significantly increased in all OST-EC50 populations compared to the matched OST cells at both the mRNA

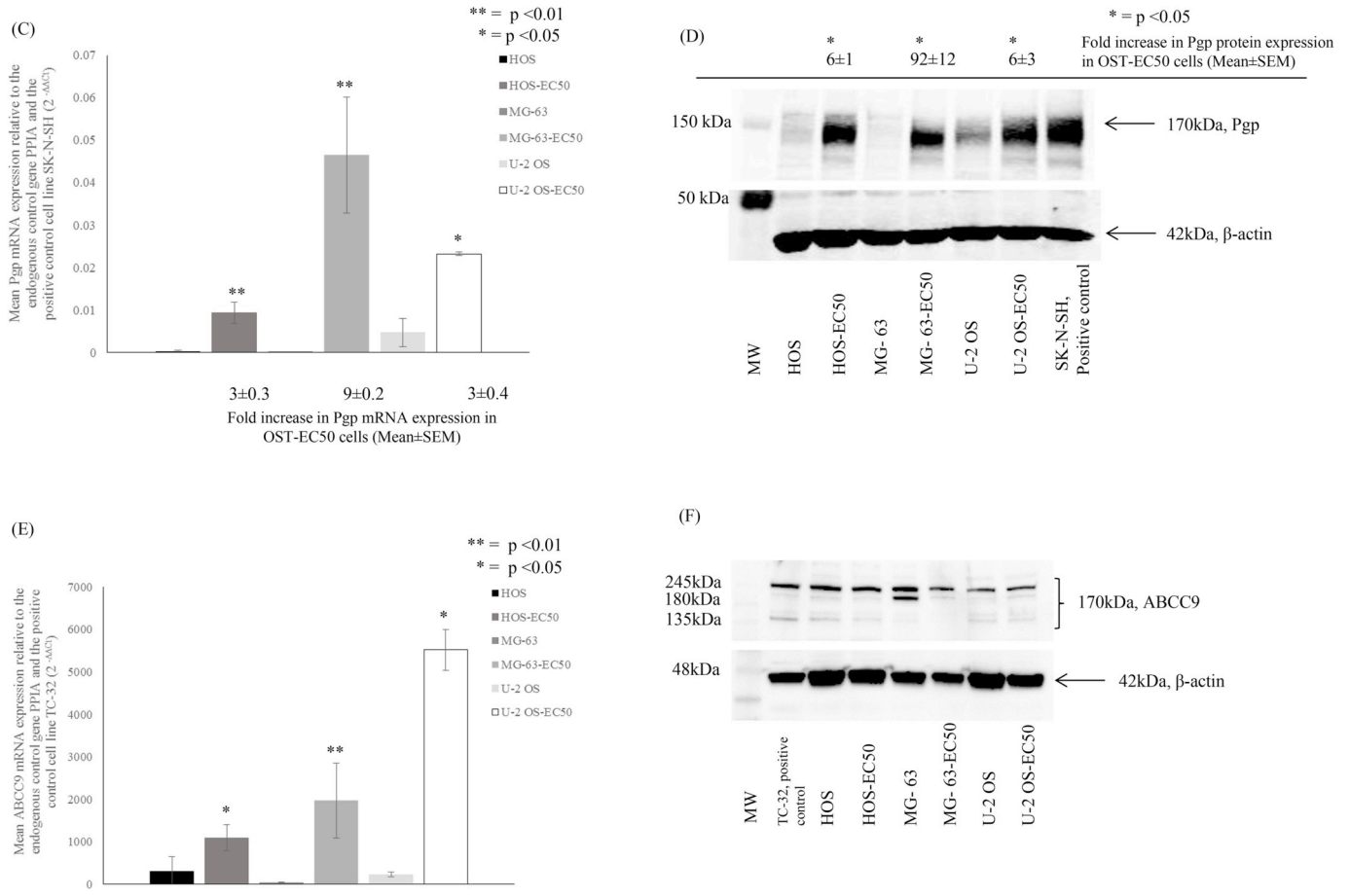


Fig. 3. (*continued*)

Table 2
The top 10 differentially expressed ABC transporter proteins ranked based on the significant differences in expression between OST and OST-EC50 cells.

ABC transporter	Mean fold difference in expression ($Log2^{-\Delta Ct}$)	Adjusted P value
ABCB1 (Pgp)	1.4	0.41
ABCC11 (MRP-8)	0.8	0.41
ABCB11	1.1	0.94
ABCC9	0.7	0.99
ABCG2 (BCRP)	0.4	0.99
CFTR	0.6	0.99
ABCC3 (MRP-3)	0.4	0.99
ABCC2 (MRP-2)	0.3	0.99
ABCB4	0.5	0.99
ABCA1	0.3	0.99

RNA (1 μg) was analysed by RT-qPCR using the TaqMan® Human ABC Transporter Array and mRNA levels ($2^{-\Delta Ct}$) in OST and OST-EC50 cells compared using LIMMA. Positive $Log2^{-\Delta Ct}$ = increased expression in OST-EC50 cells. **Bold** = ABC transporter selected for validation in a single RTqPCR assay after the removal of mRNAs with a mean change in Ct value of < 2 or Ct values > 35.

($p < 0.05$, Fig. 3C) and protein level ($p < 0.05$, Fig. 3D) and the MG-63-EC50 cells had the greatest fold increase in Pgp expression. Increased expression of Pgp in the OST-EC50 cells is consistent with resistance to the Pgp substrates doxorubicin, etoposide, vincristine and actinomycin D and sensitivity to the non-Pgp substrate, fenretinide (Fig. 1B–D) [46].

In contrast, although ABCC9 mRNA expression was increased in OST-EC50 cells ($p < 0.05$, Fig. 3E), this was not reflected in the levels of ABCC9 protein (Fig. 3F). Furthermore, there was no change in the mRNA profile of ABCB4, ABCB11 and MRP-3 in all OST-EC50 cell lines compared to the corresponding parental population ($p > 0.05$, Supplementary Fig. 2); when analysed in a single tube assay, increased ABCB4 and ABCB11 expression approached significance in the HOS-EC50 and the MG-63-EC50 cells, respectively.

3.5. Characterisation of OST.SR and OST-EC50.SR cells

In addition to MDR, CSCs must be able to re-populate the tumour and therefore be capable of self-renewal [32]. Of the OST cells examined, the HOS cells produced the most progeny from a single cell (HOS 22 ± 1% $p < 0.05$, MG-63 9 ± 4%, U-2OS 13 ± 2%). However, progeny production was decreased in the MDR HOS-EC50 cells (8 ± 0.7%, $p < 0.0001$), suggesting selection in doxorubicin is insufficient to isolate OST-CSCs. Therefore, we expanded and propagated daughter progeny from both single HOS (Fig. 1Aii) and MDR HOS-EC50 (Fig. 1Aiii) cells for further investigation.

Consistent with the isolation of OST-CSCs, HOS.SR1, HOS-EC50.SR4 and HOS-EC50.SR5 cells produced significantly more cultures from a single cell than corresponding parental cultures ($p < 0.05$, Fig. 4AB, Table 3). Two distinct groups with a self-renewing efficiency of > 30% (group I) and < 10% (group II, Fig. 4B) were identified in the HOS-EC50.SR populations, reflecting the heterogeneity of the daughter progeny (Table 3).

The HOS-EC50.SR cells were more resistant to doxorubicin ($p < 0.01$) and etoposide ($p < 0.01$, Fig. 4C) compared to the HOS.SR

Table 3
Phenotypic characteristics of the HOS populations.

	HOS	HOS-EC50	HOS-EC50.SR
HOS.SR	**M SR** p = 0.93 **M doubling time** p = 0.22 **M migration** p = 0.25 **I SR** HOS.SR1 p < 0.0001 **I doubling time** NS p = > 0.05 **I migration** HOS.SR2 p < 0.05 (decreased), 3/4 NS p > 0.05		**M SR** p = 0.55 **M doubling time** p = 0.41 **M migration** p = 0.32 **I SR** HOS.SR1 significantly different to HOS.SR2-4 p < 0.01 and all populations in HOS.EC50.SR group II p < 0.0001 All HOS-EC50 group I populations significantly different to all group II populations p < 0.0001 and also HOS.SR2-4 p < 0.05 **I doubling time** NS p = > 0.05 **I migration** HOS.SR2 p < 0.05 (decreased), 7/8 NS p > 0.05
HOS-EC50.SR		**M SR** p = 0.81 **M doubling time** p = 0.9 **M migration** p = 0.09 **I SR** HOS-EC50.SR4 p < 0.0001, HOS-EC50.SR5 p < 0.05 **I doubling time** NS p = > 0.05 **I migration** NS p = > 0.05	

Results shown as fold change and p value. M = median values compared (non-parametric Mann-Whitney two-tailed *t*-test), I = individual populations compared (analysis of variance and a Tukey's post-hoc test), SR = self-renewal, NS = no populations were significantly different, Migration = Migration at 72 h, group I = self-renewing efficiency of > 30% (Fig. 4B), group II = self-renewing efficiency of < 10% (Fig. 4B).

cells, although there was no difference in the response to actinomycin D (p = 0.90) and fenretinide (p = 0.55, Supplementary Fig. 3). Furthermore, resistance to vincristine was not significantly increased in the HOS-EC50.SR cells (p = 0.06, Supplementary Fig. 3), suggesting the MDR profile does not entirely mirror that of the HOS-EC50 cells.

There was no difference in the doubling time (Fig. 4D) or MI (Fig. 4E) when comparing between the individual SR cultures, the median or with the HOS or HOS-EC50 cells respectively (p > 0.05, Table 3, Supplementary Fig. 4, Supplementary Fig. 5). Furthermore, correlation analysis confirmed both MI and cell growth were independent of both resistance to doxorubicin or etoposide and progeny producing efficiency from a single cell (R^2 < 0.3, p > 0.32).

3.6. ABCG1 is upregulated in putative OST-CSCs (OST-EC50.SR cells) and mRNA levels correlate with the response to topoisomerase II poisons

Since OST-CSCs have been isolated based on ABC transporter protein expression and activity [35–38], we have compared the ABC transporter protein profile in 2 HOS.SR and 2 HOS-EC50.SR cell populations (details in Supplementary Table 3). Hierarchical clustering revealed all SR cultures grouped independently of the HOS and HOS-EC50 cells (Fig. 5A). Direct comparison of the HOS.SR and HOS-EC50.SR ABC transporter profiles identified ABCA1, ABCA9 and ABCG1 for further investigation (Fig. 5BC, Table 4). Increased median ABCG1 mRNA expression in the HOS-EC50.SR populations was approaching significance, compared to the HOS.SR cells (p = 0.06, Fig. 5D), and was highly significant compared to the HOS-EC50 (p < 0.0001) and HOS populations (p < 0.0001). However, there was no difference in ABCG1 mRNA expression between the HOS and either the HOS.SR cells (p = 0.96) or the MDR HOS-EC50 cells (p = 0.86), suggesting increased ABCG1 expression is a feature of the MDR SR cell.

Furthermore, mRNA expression of ABCG1 was correlated with response to doxorubicin and etoposide (r = 0.88, p < 0.001 and r = 0.94, p < 0.0001, respectively; Fig. 5E), suggesting these agents may be ABCG1 substrates. Consistent with this hypothesis, ABCG1 mRNA expression did not significantly correlate with response to vincristine (r = 0.52, p = 0.08), actinomycin D (r = 0.34, p = 0.1) or fenretinide (r = 0.03, p = 0.25, Supplementary Fig. 6). Increased mRNA expression of ABCA9 and ABCA1 in the HOS-EC50.SR cells did not validate in a single tube RTqPCR assay (p = 0.99 and p = 0.55 respectively, Fig. 5D) and in contrast to ABCG1, mRNA levels of ABCA9 and ABCA1 did not correlate with response to chemotherapy (r < 0.66, p > 0.05; data not shown).

Therefore, we have examined expression of both ABCG1 and Pgp in the HOS, HOS-EC50 and also the putative OST-CSCs (HOS-EC50.SR4 cells; highest ABCG1 mRNA expression and chemotherapy resistance (Fig. 5E)). Consistent with our previous observations (Fig. 3), plasma membrane and intracellular Pgp expression was increased in the HOS-EC50 cells (mean intensity component pixel value = 2104 ± 182) compared to the original HOS population (mean intensity component pixel value = 1444 ± 424; p = 0.00002). Although Pgp mRNA was not significantly upregulated in the HOS-EC50.SR cells (Q value > 0.21, fold change < 1), Pgp protein was increased (Fig. 5F, mean intensity component pixel value = 1726 ± 278; p = 0.005), suggesting Pgp may be regulated by protein stabilisation. Punctate ABCG1 expression co-localised with DAPI labelling of cell nuclei (Fig. 5F), consistent with expression in the nucleus, and was increased in the HOS-EC50.SR4 cells (mean intensity component pixel value = 1172 ± 175) compared to both HOS (mean intensity component pixel value = 605 ± 166, p = 0.02) and HOS-EC50 populations (Fig. 5F, mean intensity component pixel value = 902 ± 109, p = 0.04). Importantly, efflux of the ABC transporter substrate calcein-F was significantly increased in the HOS-EC50.SR4 cells (32 ± 8%), compared to the HOS-EC50 (20 ± 6%, p < 0.01) and the HOS cells (19 ± 4%, p < 0.01), consistent with the hypothesis that ABCG1 has a direct role in the efflux of substrates from the OST-CSCs.

Suggesting targeting these transporters may be an interesting therapeutic strategy in OST, high expression of both Pgp and ABCG1 was also observed in all patient derived OST cell cultures examined (6/6; Fig. 5G). Furthermore, ABCG1 expression in the patient derived OST cell cultures was increased above the levels observed in the HOS cells (mean intensity component pixel value of 6/6 patient derived OST cell cultures = 995 ± 207, range 1335-821, p = 0.007) and was primarily localised to the cytoplasm of patient derived cells, although co-localisation of ABCG1 with Pgp was also observed in the plasma membrane of these cells (Fig. 5G).

A hierarchical tree displaying the predicted order of HOS cell population derivation based on the ABC transporter mRNA profile positioned the HOS.SR cultures as the root and first node (Fig. 6A), supporting our hypothesis that these cells represent the most primitive cell population. Moreover, the HOS population was a descendant of the HOS.SR progeny and reflecting the increased ABCC9 and Pgp mRNA

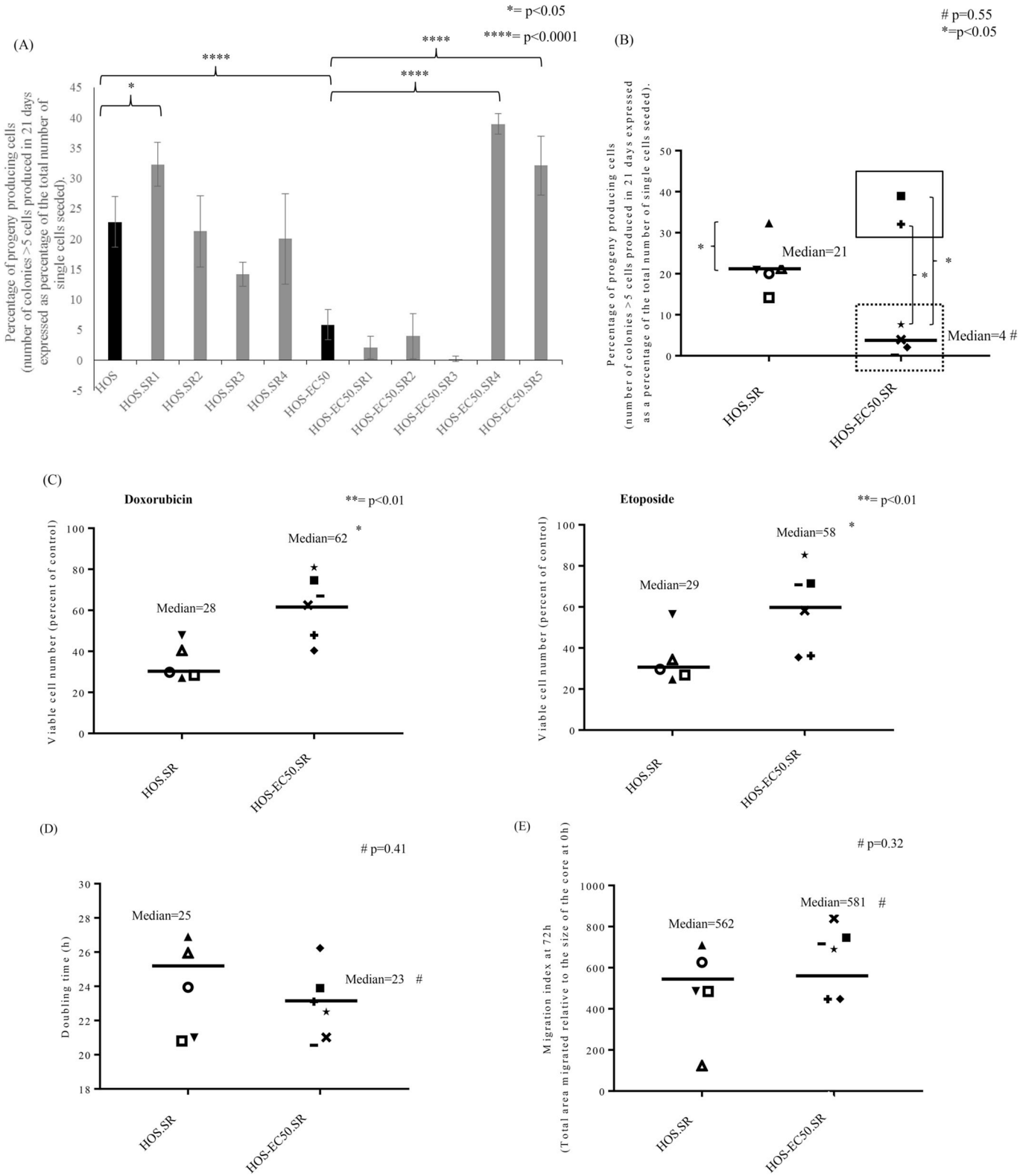


Fig. 4. The phenotype of HOS-EC50.SR and corresponding HOS.SR cells. (A, B) The percentage of progeny producing cells after 21 days. Cell colonies were calculated as the mean number of cell populations containing > 5 cells expressed as a percentage of the total number of individual cells seeded. There was no difference in the median number of progeny produced between the HOS-EC50.SR and corresponding HOS.SR cells (p = 0.55). Solid square = group I, dashed square = group II. (C) Viable cell number remaining following incubation with doxorubicin (42 nM) and etoposide (420 nM) for 48 h. (D) The mean population doubling time (hours). Viable cell number was determined by trypan blue exclusion. (E) The migration index (MI) of cells from an established spheroid at 72 h and the total migrated area was expressed relative to the spheroid core at each time point. Median values compared using a non-parametric Mann-Whitney two-tailed *t*-test and individual cultures compared using ANOVA and a Tukey's post-hoc test. - = median value, # = p > 0.05, * = p < 0.05, *** = p < 0.001, **** = p < 0.0001. EC50 = effective concentration, OST = osteosarcoma.

expression (Fig. 3), the HOS-EC50 cells were positioned directly downstream. The HOS-EC50.SR cells were reported as descendants of the HOS.SR cells, suggesting their ABC transporter profile is more consistent with a SR population than the HOS-EC50 MDR profile (Fig. 6A).

4. Discussion

For the first time, we report increased expression of both ABCG1 and Pgp ABC transporter proteins in putative OST-CSCs with both increased resistance to topoisomerase II poisons and self-renewing ability (Fig. 6B).

Validating our bifunctional approach, doxorubicin selection alone was not sufficient to identify OST-CSCs, further selection of cells using a functional assay was required to isolate cells with increased self-renewing capacity. This is in keeping with the expected increased self-renewing ability of a CSC and supports the clinical expectation that CSCs must evade chemotherapy and subsequently have the ability to repopulate the tumour [30–32]. This functional approach has the advantage that it is not dependent on protein expression or activity which have previously failed to robustly isolate the entire drug resistant, self-renewing cell population [27].

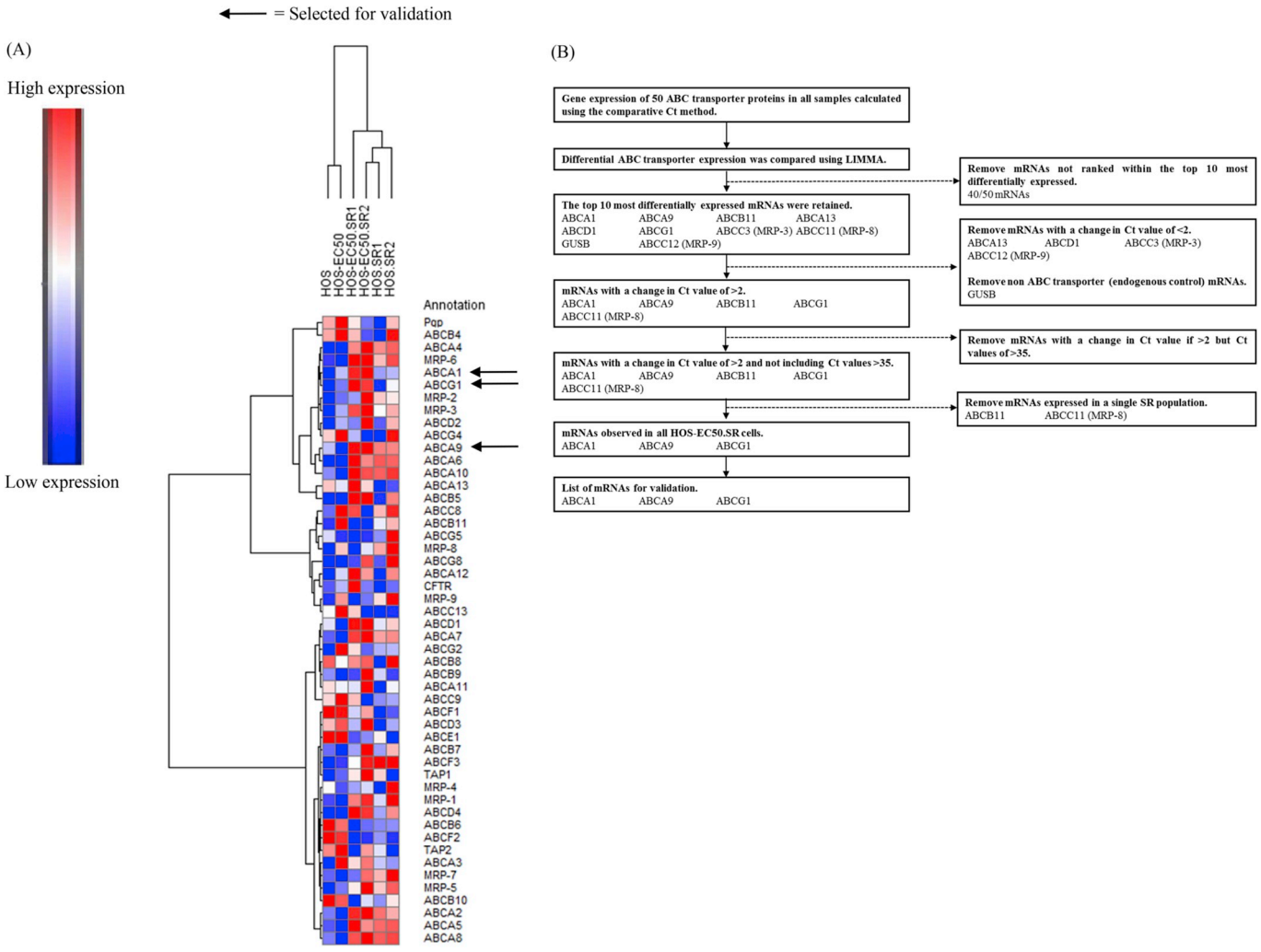
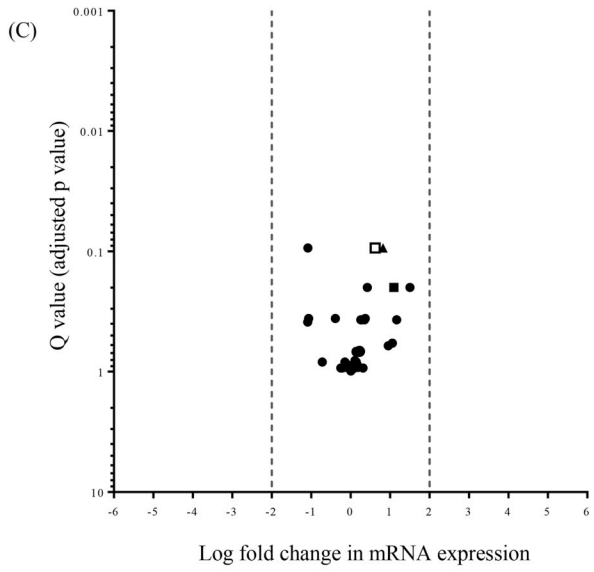
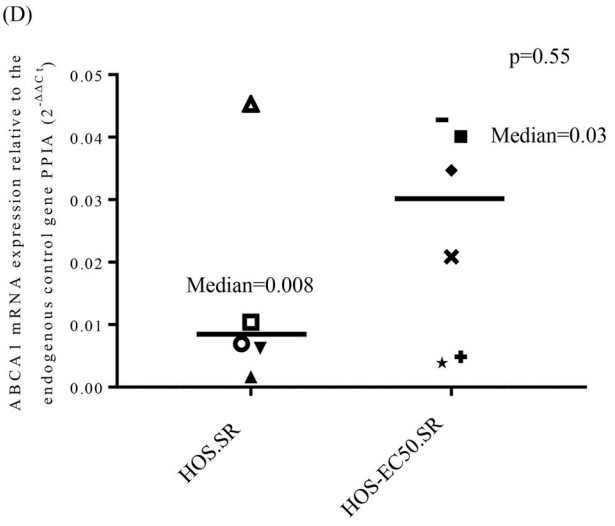
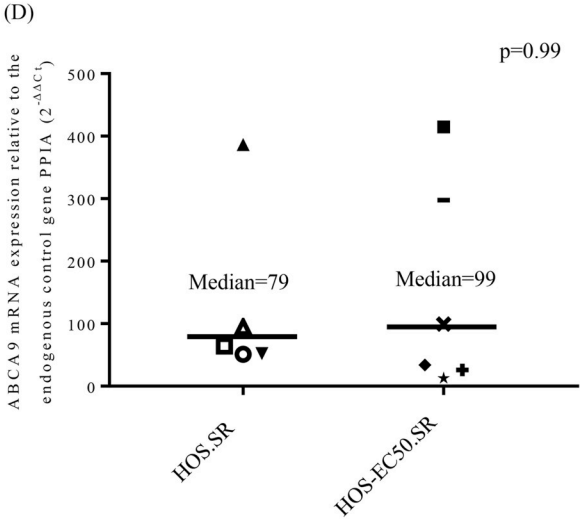
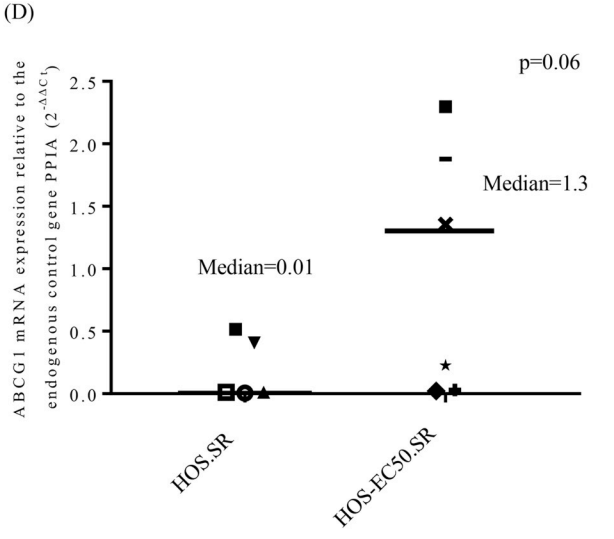


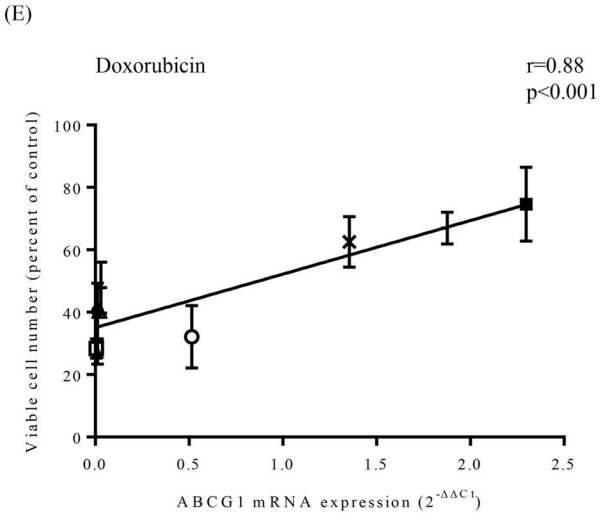
Fig. 5. Differential ABC transporter expression profiles of the HOS, HOS-EC50, HOS.SR and HOS-EC50.SR cells. (A) Heatmap visualisation of the ABC transporter mRNA expression profile. The heatmap was generated using GENE-E (Broad Institute, USA), clustering cell lines and ABC transporter genes hierarchically, using one minus Pearson correlation for the cell lines and Euclidean distance for ABC transporter genes with clustering for complete linkage. Solid black arrow = selected for validation. Red = high expression, blue = low expression. (B) Details of the strategy employed to select differentially expressed mRNAs for further validation from the TaqMan® Human ABC Transporter Array. (C) Volcano plot displaying the Q value (significance; adjusted p value) on the y axis and the mean Log fold change in mRNA expression between the HOS.SR and HOS-EC50.SR cells on the x axis. A threshold of 2 log fold change is shown as a dashed line. Filled square = ABCG1, filled triangle = ABCA1, open square = ABCA9. (D) RNA (10 ng of HOS.SR, HOS-EC50.SR RNA, 10 ng of HepG2 RNA as a positive control for ABCA1, 10 ng and 1 μg of HEK293 RNA as a positive control for ABCG1 and ABCA9 respectively) was analysed by RTqPCR and presented as $2^{-\Delta\Delta Ct}$ by normalisation of target Ct values to PPIA and the appropriate positive control cell line. - = median $2^{-\Delta\Delta Ct}$ compared using a non-parametric Mann-Whitney two-tailed *t*-test. (E) Correlation of ABCG1 mRNA expression and the viable cell number remaining after incubation with doxorubicin and etoposide (linear regression and Pearson's correlation coefficient (R)). (F) Protein expression of Pgp and ABCG1 in the HOS, HOS-EC50 and HOS-EC50.SR4 populations and (G) patient derived OST cells. Cells were fixed and stained with antibodies to visualise Pgp (red) and ABCG1 (green) expression. Nuclei were labelled with DAPI. For each cell population, the three different fluorescent stains, the merged image and a higher magnification image are shown, representative of each population analysed. White solid arrows = ABCG1 expression (green), white dashed arrows = Pgp expression (red), yellow arrow = plasma membrane co-localisation of Pgp and ABCG1, N = nuclear expression, PM = plasma membrane expression, C = cytoplasmic expression, EC50 = effective concentration, OST = osteosarcoma, PPIA = Peptidylprolyl isomerase A, SR = self-renewing, scale bar = 10um.

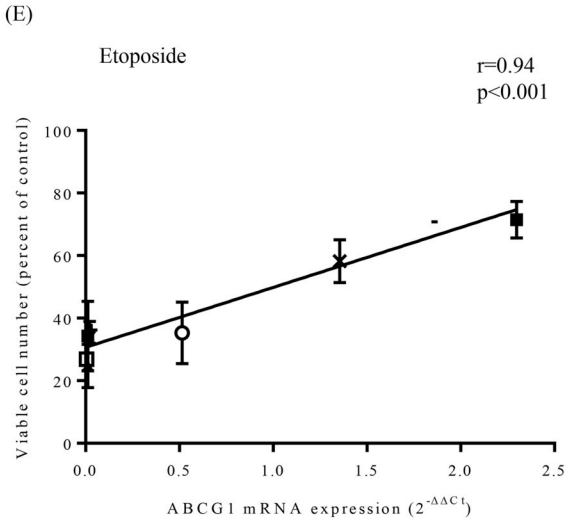












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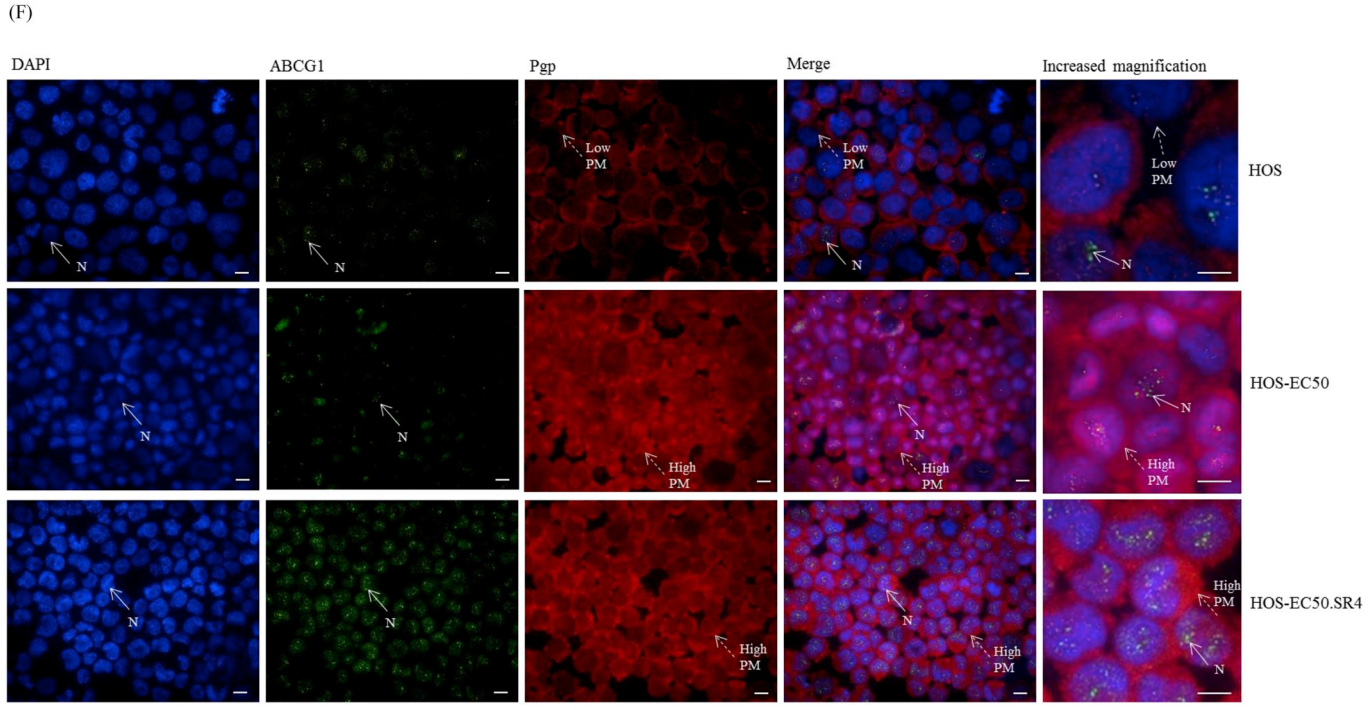

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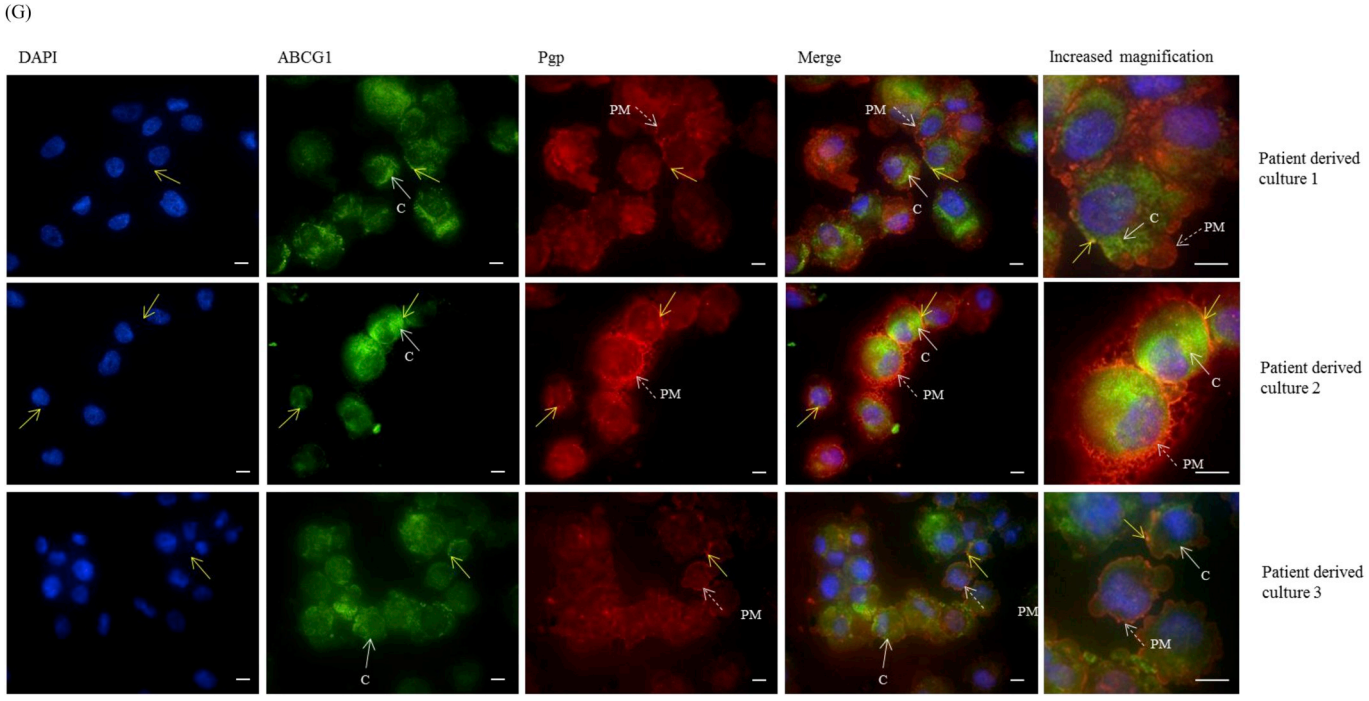

Fig. 5. (*continued*)

Suggesting ABCG1 is associated with both MDR and a self-renewing phenotype, expression was increased in the putative OST-CSCs (HOS-EC50.SR) compared to the drug sensitive HOS.SR cells. Furthermore, the response of the OST-CSCs to doxorubicin and etoposide correlated with ABCG1 mRNA expression, suggesting these chemotherapeutics are substrates for ABCG1. Supporting this observation, ABCG1 was observed in the nucleus of the HOS populations, the organelle targeted by topoisomerase II poisons and is consistent with its transport from the nucleus via the endosomal network to the plasma membrane [56]. In agreement with the traditional role of ABCG1 to regulate cellular

Table 4
The top 10 differentially expressed ABC transporter proteins ranked based on the significant differences in expression between the HOS.SR and the HOS-EC50.SR cells.

ABC transporter	Mean fold difference in expression ($Log2^{-\Delta Ct}$)	Adjusted P value
ABCA1	0.8	0.10
ABCA9	0.6	0.10
ABCB11	1.0	0.10
ABCA13	1.5	0.22
ABCD1	0.4	0.22
ABCG1	1.0	0.22
ABCC3 (MRP-3)	0.3	0.37
ABCC11 (MRP-8)	1.0	0.37
GUSB	0.3	0.37
ABCC12 (MRP-9)	0.3	0.38

RNA (1 μg) was analysed by RT-qPCR using the TaqMan® Human ABC Transporter Array and mRNA levels ($2^{-\Delta Ct}$) in HOS.SR and HOS-EC50.SR cells (LIMMA). Positive $Log2^{-\Delta Ct}$ = increased expression in HOS-EC50.SR cells. **Bold** = ABC transporter selected for validation in a single RTqPCR assay after the removal of mRNAs with a mean change in Ct value of < 2 or Ct values > 35. SR = self-renewing.

cholesterol [57,58] we have demonstrated increased efflux of the ABC transporter substrate calcein-F in OST-CSCs with increased ABCG1 expression. Since ABCG1 is expressed in the plasma membrane and nucleus of OST-CSCs we are currently investigating the impact of the subcellular localisation of ABCG1 on chemotherapeutic response [59,60] and the apoptosis [61] of cancer cells.

Consistent with nuclear ABCG1 expression, ABC transporters in the outer membranes of the cell nucleus [62] and mitochondria [46,63,64], have been reported to induce MDR by effluxing compounds from these organelles into the cytoplasm and subsequently intracellular vesicles for extracellular efflux, thereby protecting the organelle from damage, promoting cell survival. However, in the patient derived OST cells, ABCG1 expression was predominantly observed in the cytoplasm but also co-localised with Pgp in the plasma membrane of patient derived OST cells (nuclear ABCG1 was not detected), highlighting the importance of examining the expression profile of putative biomarkers or therapeutic targets in patient samples and further suggesting ABCG1 may have a role in MDR by a direct efflux mechanism.

In agreement with the nuclear localisation of ABCG1, suggesting an additional role independent of drug efflux, ABCG1 has been linked with the regulation of both endoplasmic reticulum stress proteins [61,65] and macrophage viability following oxidative stress [66]. This suggests perhaps ABCG1 may also regulate MDR by combating cellular stress and is consistent with reports suggesting a non-efflux role for other ABC transporter proteins, such as regulation of tumorigenesis (MRP1 [57,67]), migration (MRP1 [67,68], ABCG1 [69]) and tissue homeostasis (MRP8, CFTR [70]).

ABCG1 has been shown to upregulate expression of the conventional CSC markers, such as CD133 and aldehyde dehydrogenase in lung cancer CSCs [69] and high expression has been linked with tumour growth and overall survival of glioma [61], breast [71] and non-small cell lung cancer [72] patients. However, ABCG1 has yet to be linked with MDR or a CSC phenotype in OST. Previous studies investigating OST-CSCs have focussed efforts on the most commonly implicated MDR ABC transporter proteins, MRP1 [73,74], BCRP [75] and Pgp [76–78], although only the latter has been associated with OST patient outcome [15–18]. In this study, MRP1 and BCRP expression was significantly increased in the putative OST-CSCs (adjusted p = 0.035 and 0.079, respectively) but the proteins were ranked 20th and 23rd respectively based on adjusted p value and so were not investigated further following our validation strategy. Similar to the current study, OST-CSC populations generated from MG-63 sarcospheres of 1000 vincristine selected OST cells [79] upregulated BCRP and MRP1 expression. Furthermore, the sarcosphere derived OST-CSCs were also heterogeneous in phenotype and genotype [79], which is consistent both with observations from the current study describing a range of ABC transporter expression and self-renewing ability and also the heterogeneous pathology of OST [80].

Pgp was the only ABC transporter upregulated at the protein level in the OST-EC50 cells established following 31–159 days of doxorubicin treatment, which is consistent with previous studies (> 31 days, [81,82]). Moreover, increased Pgp mRNA [81,83–93], protein [94] expression and as a result, resistance to the chemotherapeutics used in the treatment of OST such as doxorubicin, etoposide, vincristine, actinomycin D [15–18,45,95–98], cisplatin [99,100] and methotrexate [92] has been widely reported. Since many OST are resistant to chemotherapy [13] and high Pgp expression is an adverse prognostic factor [15–18], it is likely this ABC transporter has a role in driving MDR in OST [13,14]. In addition to ABC transporter overexpression, a variety of additional mechanisms of MDR have been described both in bone cancer cells and putative CSCs, such as drug inactivation, enhanced DNA repair [44,45], modulation of apoptosis regulatory genes [101], autophagy, miRNA dysregulation, decreased reactive oxygen species [102,103], which must also be considered when profiling OST-CSCs for predictive targets for therapy.

In summary, our observations are in keeping with previous OST-CSC reported profiles. Since doxorubicin selection is not sufficient to identify cells with increased progeny producing ability, we advocate a dual functional approach to isolate putative OST-CSCs with self-renewing ability [32]. However, it is not possible to examine the self-renewing ability of the OST-CSCs isolated from HOS cells *in vivo* since this OST cell line is not tumorigenic [104,105]; genetic [104–106] or chemical [106] modification is required for the growth of these cells in a xenograft model.

In addition to increased Pgp expression, ABCG1 was the only ABC transporter overexpressed uniquely in the OST-CSC population and was associated with resistance to topoisomerase II poisons. The expression of ABC transporter proteins was consistent with previous OST-CSC reported profiles, although this is the first study to employ a screening RTqPCR approach examining expression of 50 ABC transporter proteins in OST-CSCs. Since the expression level was enhanced in patient derived OST cells, we suggest targeting ABCG1 in OST represents a viable strategy to eradicate the drug resistant self-renewing cells which when employed in combination with standard chemotherapy targeting the bulk OST cells may improve survival for OST patients. Compounds capable of inhibiting the ATPase activity of both Pgp (verapamil, [107]) and ABCG1 (Cyclosporine A, L-thyroxine and benzamil; [108]) are commercially available, although these inhibitors target a number of additional ABC transporter proteins such as ABCG4 [109,110], ABCC1 and ABCG2 [111,112]. These inhibitors are therefore of limited value to investigate the impact of targeting Pgp and ABCG1 in combination with chemotherapy. We are currently evaluating the functional relevance of these ABC transporters using classical knock-in/knock-out studies. The prognostic significance of ABCG1 in patient samples will also be determined as part of a multi-variate analysis to evaluate the independent prognostic value and clinical relevance of ABCG1 in OST.

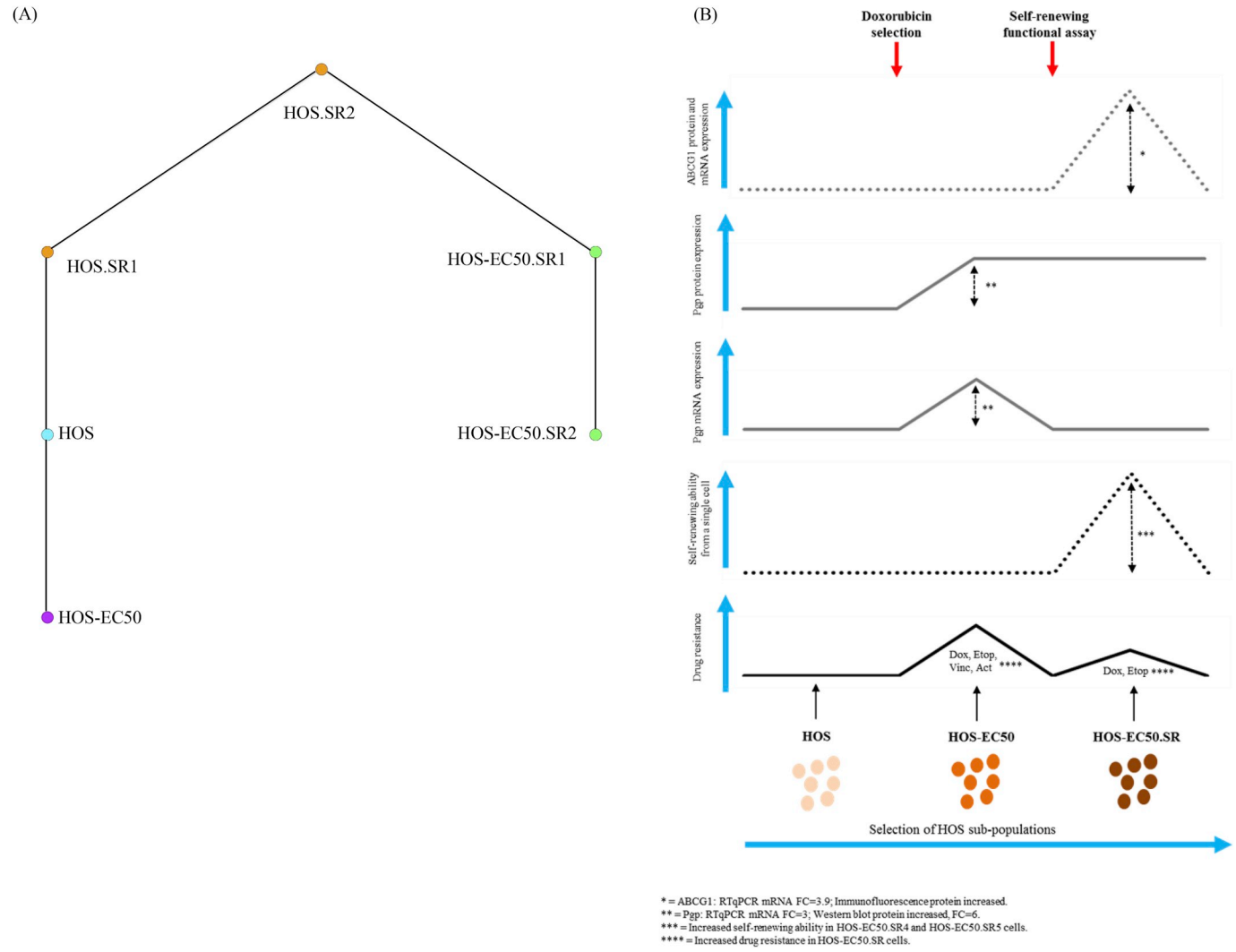

Fig. 6. The relationship between ABC transporter protein profile and the phenotypes of OST cells. (A) Hierarchical tree predicting the order of development of the HOS, HOS-EC50, HOS.SR1, HOS.SR2, HOS-EC50.SR1 and HOS.EC50.SR2 based on ABC transporter protein mRNA expression. The tree was visualised using the cellTree package applying a Latent Dirichlet Allocation model fit using a Gibbs sampling method. Orange circle = HOS.SR cells, green circle = HOS-EC50.SR, blue circle = HOS cells, purple circle = HOS-EC50. (B) Summary of the changes in the phenotype and ABC transporter protein expression of the HOS-EC50 and HOS-EC50.SR cells, compared to the original HOS population from which they were originally derived. Horizontal blue arrow = selection of HOS cells using a dual functional approach (selection in doxorubicin and self-renewal from a single cell), vertical blue arrow = increasing ABC transporter mRNA, protein expression, self-renewing ability and drug resistance. FC = fold change, Dox = doxorubicin, Etop = etoposide, Vinc = vincristine, Act = actinomycin D, SR = self-renewing, black line = drug resistance, dashed black line = self-renewing ability, grey line = Pgp mRNA and protein expression, dashed grey line = ABCG1 mRNA expression. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Author declarations of interest

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.011.

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