International Journal of Biochemistry and Cell Biology 107 (2019) 116–127

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Long term treatment of metformin impedes development of chemoresistance by regulating cancer stem cell differentiation through taurine generation in ovarian cancer cells

Aniketh Bishnu[a,c,1], Asmita Sakpal[a,c,1], Nilanjana Ghosh[b], Priyanka Choudhury[b], Koel Chaudhury[b], Pritha Ray[a,c,*]

[a] Imaging Cell Signaling and Therapeutics Laboratory, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Navi Mumbai, 410210, India
[b] Clinical Biomarker Research Laboratory, School of Medical Science and Technology, Indian Institute of Technology Kharagpur, West Bengal, 721302, India
[c] Homi Bhabha National Institute, Mumbai, Anushakti Nagar, India

ARTICLE INFO

Keywords:
Ovarian cancer
Metformin
Chemoresistance
Cancer stem cells
Taurine

ABSTRACT

Development of resistance poses a significant challenge to effective first-line platinum based therapy for epithelial ovarian cancer patients. Cancer Stem Cells are envisaged as a critical underlying factor for therapy resistance. Thus, there is a critical need for developing approaches to diminish the enrichment of cancer stem cells and acquirement of resistance. Administration of metformin, a commonly prescribed drug against Type II diabetes exhibited promising effect in the management of ovarian cancer. However, the effect of long term administration of low dose of metformin as an adjuvant to cisplatin and paclitaxel during acquirement of chemoresistant phenotype has not been investigated so far. Using two isogenic cellular chemoresistant models (A2780 and OAW42) developed in the presence or absence of metformin, we demonstrated the ability of metformin to impede the development of resistance through increased drug sensitivity, increased proliferation, and reduced migratory abilities of the resistant cells. Metformin introduction also decreased the cancer stem cell population, expression of specific biomarkers and pluripotent genes. Further metabolic profiling of these cells using ^{1}H-Nuclear Magnetic Resonance spectroscopy revealed significant modulation in taurine and histidine levels in resistant cells developed in the presence of metformin. Intriguingly, taurine treatment considerably reduced the cancer stem cell population and chemoresistance in resistant cells, indicating a novel role of taurine in differentiation of ovarian cancer stem cells. Altogether this is the first report on the potential role of metformin for targeting the cancer stem cell population via up regulation of taurine, leading to impediment in the acquirement of chemoresistance.

1. Introduction

Metabolic reprogramming, a cumulative endpoint modification of signalling and biochemical pathways, is associated with many cancer related phenotypes. Bioenergetics, redox balance pathways, lipid, and amino acid metabolism show modulation in cancers (Hirschey et al., 2015; DeBerardinis and Chandel, 2016). Various studies demonstrated the intricate association of chronic metabolic syndromes (obesity or diabetes) with increased cancer risk (Renehan et al., 2008; Steenland et al., 1995). The cancer prevention study II reported 40–80% increased risk of cancer in obese adults (Calle et al., 2003). Further study on this cohort suggested an increased risk of site specific cancer in diabetic men and women (Campbell et al., 2012). An altered metabolic profile was also observed during cancer progression in the mouse ovarian surface epithelial cancer model (Anderson et al., 2013). Inhibition of glucose uptake or glycolysis was also reported to reduce survival and metastasis of ovarian cancer cells (Xintaropoulou et al., 2015; Zhang et al., 2013).

The major obstruction in management of epithelial ovarian cancer (EOC), the most lethal gynaecological malignancy, is acquirement of resistance to the standard regimen of combinatorial chemotherapy of cisplatin and paclitaxel (Gubbels et al., 2010). Amongst all the

* Corresponding author at: Imaging Cell Signaling and Therapeutics Laboratory, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Navi Mumbai, 410210, India.
E-mail address: pray@actrec.gov.in (P. Ray).
[1] Equal contribution.

https://doi.org/10.1016/j.biocel.2018.12.016
Received 29 October 2018; Received in revised form 20 December 2018; Accepted 25 December 2018
Available online 26 December 2018
1357-2725/

molecular mechanisms that impart relapse and resistance to standard chemotherapy, the presence of a small population of pluripotent cells termed as Cancer Stem Cells (CSC) significantly aids the process (Gasch et al., 2017; Shibue and Weinberg, 2017). These cells are highly plastic, slow proliferating, and remain irresponsive to standard therapy (Tang, 2012; Kwon and Shin, 2013). Metabolic reprogramming plays a crucial role in the maintenance and differentiation of normal and cancer stem cells. Similar to cancer cells, pluripotent stem cells and CSCs utilize aerobic glycolysis as the major source of energy generation (Liao et al., 2014; Varum et al., 2011). Various metabolites are reported to regulate differentiation of pluripotent stem cells. Carnitine and acetylcarnitine can regulate bone-marrow or adipose-derived mesenchymal stem cell differentiation by regulating mitochondrial metabolism (Lu et al., 2015). Amino acid metabolism, especially catabolism of methionine, plays a major role in maintenance of stemness by regulating histone methylation (Shiraki et al., 2014). Taurine, a derivative of cysteine and methionine metabolism, is known to regulate differentiation of mesenchymal and neuronal stem cells (Li et al., 2017; Zhou et al., 2014). However, effects of these metabolites on CSC maintenance and differentiation are yet to be explored.

Metformin, a biguanide class of drug primarily prescribed for type II diabetes, has been reported to impart anti-tumour and anti-CSC effects in various cancers (Hirsch et al., 2009; Shafiee et al., 2014; Shackelford et al., 2013). Metformin gained prominence in cancer biology since Evans et al (2005) reported that diabetic patients receiving metformin had a reduced incidence of tumour formation (Evans et al., 2005). Metformin administration was associated with better survival in 73 EOC patients (Kumar et al., 2013). Pre-treatment of SKOV3ip1 xenograft mice with metformin reduced tumour formation, and combinatorial treatment of metformin and paclitaxel reduced tumour volume by 60% in LSL-$Kras^{G12D/+}$$Pten^{loxP/loxP}$ mice (Lengyel et al., 2015). In addition to glucose metabolism, metformin treatment results in altered TCA cycle, lipid and fatty acid metabolism (Birsoy et al., 2014; Fendt et al., 2013). High dosage of metformin imparts an anti-proliferative effect on TYKNU, OV90, and OVCAR 433 cell lines by altering mitochondrial respiration and the level of mitochondrial shuttle metabolites (Hodeib et al., 2018). Studies also showed that metformin treatment decreased cellular proliferation of platinum or taxol resistant A2780 cells and reduced mRNA of inflammatory cytokines and NF-kb signalling pathway (Dos Santos Guimaraes et al., 2018). Although, these studies described various effects of metformin on cellular proliferation, drug sensitization, and tumour growth, the effect of metformin as an adjuvant to cisplatin-paclitaxel treatment during the acquirement of chemoresistance has never been explored.

In this study, we evaluated the effect of continuous treatment of low dose metformin along with cisplatin and paclitaxel during acquirement of chemoresistance in EOC cells. Along with the changes in functional properties and reduction in CSC pool, the metabolic profiling of chemoresistant cells developed in the presence and absence of metformin demonstrated alteration in specific amino acids level. Addition of taurine, one such amino acid, in the culture media significantly induced chemosensitization, and CSC to non-CSC differentiation. To the best of our knowledge, this is the first report on the role of metformin in differentiation of CSC population by regulating specific amino acids, and thereby lowering the resistance level in EOC cells.

2. Materials and methods

2.1. Reagents and antibodies

Cisplatin, Paclitaxel, Metformin, Thiazolyl **blue** tetrazolium bromide, Mitomycin, Verapamil, Taurine, anti-tubulin antibodies, and HRP conjugated secondary antibodies (anti-mouse and anti-rabbit) were purchased from Sigma Aldrich (USA). Dye Cycle **Violet** (DCV) dye, Dylight 488/633 conjugated anti-rabbit antibodies was purchased from Invitrogen (USA). Phospho and total AKT, ERK antibodies, CD 133 antibodies were purchased from Cell Signalling Technology (USA).

2.2. Cell culture and treatment

A2780, an epithelial ovarian cancer (EOC) cell line was cultured in Dulbecco's Modified Eagle's Medium, OAW42 (EOC cell line) and SKOV3 (EOC cell line) cells were cultured in Minimum Essential Medium and in Roswell Park Memorial Institute Medium respectively. All the media were supplemented with 10% FBS (Himedia, Maharashtra, India) and 1% penicillin–streptomycin (GIBCO, Carlsbad, CA).

2.3. MTT assay

To assay cell viability, the standard thiazolyl **blue** tetrazolium bromide (MTT) method was used and percent viability was counted using the formula: Absorbance (Test)/Absorbance (Control)*100 (Gaikwad et al., 2015). The percent viability was converted to percent cell death by using the formula: (Cell death % = 100%-Viable cell %)

2.4. Trypan blue exclusion assay for cell proliferation

For the Trypan **blue** assay to assess proliferation rate, cells were seeded (~20,000 cells/well) in a 6 well plate, trypsinized after specific time points, and re-suspended in PBS containing 0.4% trypan **blue**. The number of viable cells were counted using a haemocytometer (Bright line 0.1 mm deep, Sigma Aldrich, USA) and expressed as cells/ml. Each experiment was repeated thrice and the mean value was used to compare the proliferation rate among sub-sets. Doubling time (Td) was calculated using an online calculator (Roth, 2006).

2.5. Wound healing assay

Cells were cultured as confluent monolayer in 6 well plates and pretreated with mitomycin (1 μg/ml) for 2 h. A wound was created across the well with a 200 μl pipette tip. Cells were washed twice to remove non-adherent cells. Live cell images were obtained using an inverted microscope (Ziess Axiovert 200 M) for 24 h. Wound closure was quantified using Image *J* software.

2.6. Clonogenic assay

The Clonogenic assay was performed as described earlier (Gaikwad et al., 2015). Plating efficiencies (PE) and survival fractions (SF) were calculated using the formula: PE = Number of colonies/Number of cells seeded; SF = Number of colonies after treatment/ (Number of cells seeded × PE) and plotted graphically to obtain survival curves.

2.7. Western blotting

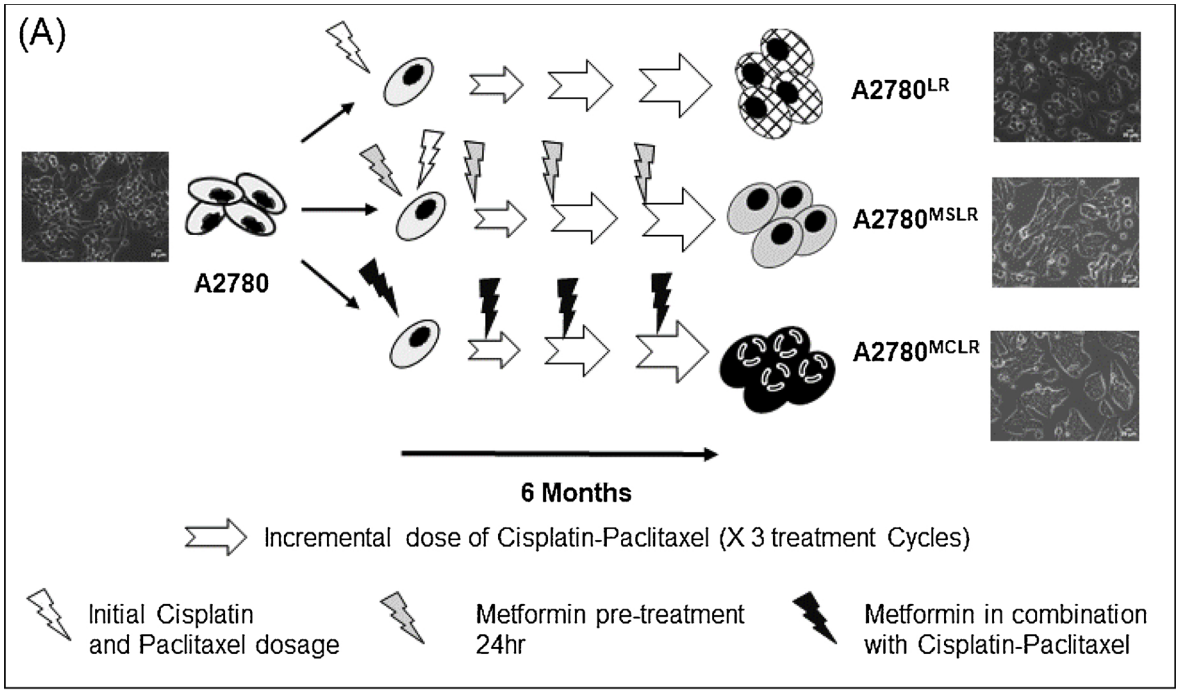
Western blotting was performed as described earlier (Singh et al., 2014).

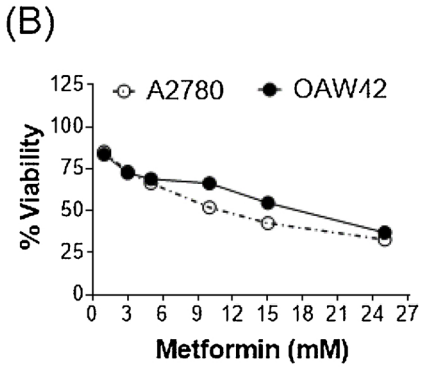
2.8. Quantitative real-time PCR

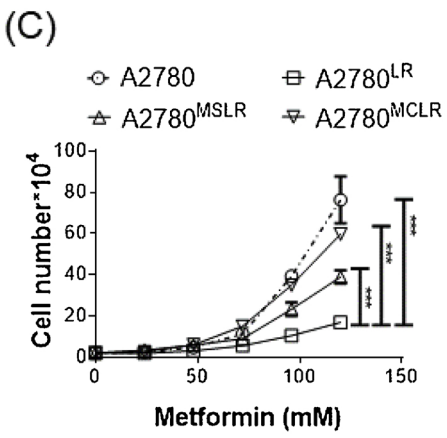
RT-PCR for pluripotent genes like Oct-4, Sox 2 and Nanog was performed with GAPDH used as an internal control following protocols described earlier (Gaikwad et al., 2013). Primer sequences of these genes are provided in Supplementary Table 2.

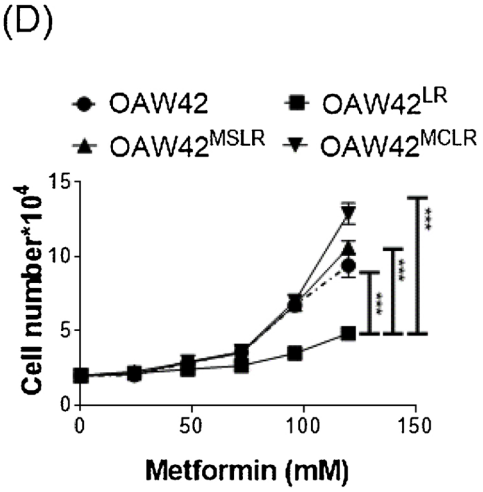
2.9. Flow cytometry

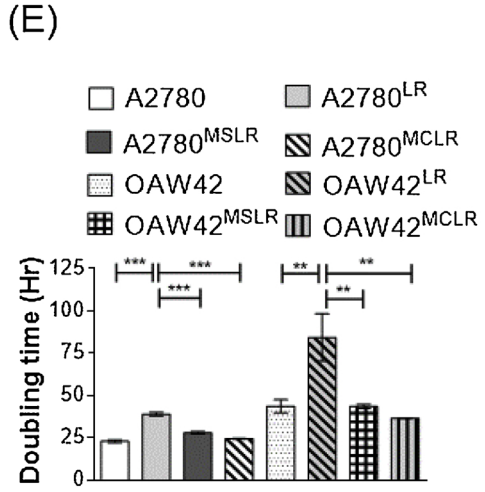
Sorting of Side and non-side population cells was performed as described earlier (Singh et al., 2016). Verapamil (50 μM), a drug transporter inhibitor, was used as a negative control for gating. Data analysis was performed using FlowJo software. Cancer stem cell











(caption on next page)

Fig. 1. Characterization of cisplatin paclitaxel resistant cells developed in the presence and absence of metformin: A) Schematic representation of the strategies for developing the cellular models. A2780^{LR} was developed by challenging the cells with incrementing doses of cisplatin and paclitaxel (each dose for 2 h). A2780^{MSLR} was developed by pre-treating the cells with metformin (2 mM) for 24 h, followed by cisplatin and paclitaxel treatment for 2 h. A2780^{MCLR} model was developed by combinatorial treatment with 2 mM metformin and cisplatin-paclitaxel for 2 h. Phase contrast microscopy represents morphological heterogeneity amongst the cell types, on acquirement of resistance in the presence and absence of metformin. B) Dose dependant decrease in cellular viability on metformin treatment in the A2780 and OAW42 cell lines. C–D) Differential proliferation rate was observed among the A2780 and OAW42 resistant cells over a span of 120 h. MSLR and MCLR cells had a higher proliferation rate than LR Cells in both the A2780 and OAW42 resistant model. E) Graphical representation of the doubling time of the cell lines, where A2780^{MCLR} and A2780^{MSLR} showed a significantly reduced doubling time while A2780^{LR} had longest doubling time. Similar reduction in doubling time was observed for OAW42^{MSLR} and OAW42^{MCLR} cells in comparison to OAW42^{LR} cells. (Data represents mean ± SD of three independent experiments, *** indicates $p < 0.0005$ as calculated by unpaired t-test) F–G) Wound healing assay indicates a significantly lower migration potential of A2780^{MCLR}, A2780^{MSLR} cells compared to A2780^{LR} cells (Data represents mean ± SD of three independent experiments, ns indicates non-significant, ** $p < 0.005$ as calculated by unpaired t-test). H–I) Western blot depicting enhanced ERK phosphorylation in MSLR and MCLR cells, and enhanced AKT phosphorylation in LR cells in both the A2780 and OAW42 resistant models. Tubulin was used as loading control.

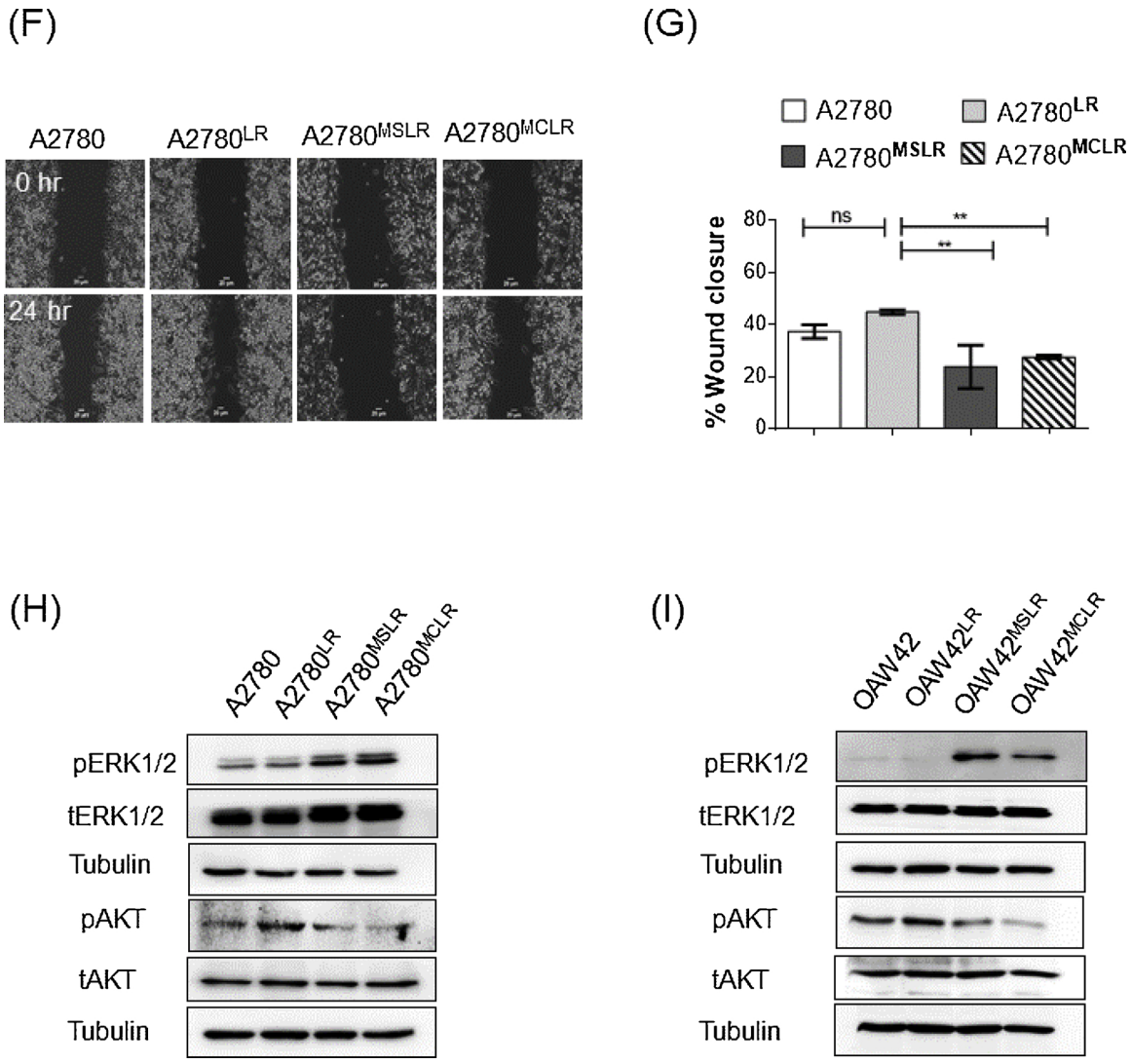


Fig. 1. (*continued*)

biomarker, CD133 staining was performed as described earlier and analysed using FlowJo software (Singh et al., 2016).

2.10. Cell metabolome quenching and metabolites extraction for NMR analysis

Metabolites were quenched and isolated as described earlier with slight modification in the number of sonication cycles (Lauri et al., 2016).

2.11. NMR experimentation

Proton Nuclear magnetic Resonance (^{1}H-NMR) spectra of all the extracted metabolites were acquired at 298 K on a 800 MHz Bruker Avance AV III spectrometer using ZGPR 1D pulse sequence for efficient water signal suppression (García-Álvarez et al., 2015). The metabolite extracts were resuspended in 600 µl of D2O with 3-(trimethylsilyl) propionic-2, 2, 3, 3, d4 acid (TSP) and loaded into 5 mm NMR tubes. The spectra were recorded with 256 transients, spectral width of

14,367.8 Hz, 32 K data points, 1.5 s relaxation delay, and an acquisition time of 1.14 s. 2D NMR spectra were obtained using TOCSY and COSY software for metabolite identification purposes. Following manual phase and baseline correction, the 1D spectra were referenced to TSP ($\delta = 0.0$ ppm) in MestReNova version 7.1.0 (Mestrelab Research, Santiago de Compostela, Spain) (Qin et al., 2013).

For minimizing the chemical shift variations, NMR spectra(s) were bucketed with a binning width of 0.02 ppm. After removal of the water signal regions (δ 4.5–5.10 ppm), the spectral regions of δ 0.5–4.5 and δ 5.10–9.0 were subjected to further multivariate and univariate analysis. Spectra were normalized using constant sum normalization using MetaboAnalyst 4.0 (Xia et al., 2012) and scaled with unit variance scaling (SIMCA 13.0.2, Umetrics, Sweden) (Vorkas et al., 2015).

2.12. Data analysis

Multivariate analysis was performed to the datasets as described elsewhere (Subramani et al., 2017; Banerjee et al., 2014; Dutta et al., 2012). Partial Least squares-discriminant analysis (PLS-DA) and Orthogonal Partial Least squares-discriminant analysis (OPLS-DA) were generated using SIMCA 13.0.2 (Umetrics, Sweden). Robustness and validation of the OPLS-DA model was confirmed by R2 and Q2 score. S-plot and variable important for projection (VIP) plot with threshold of > 0.60 and ≥ 1 respectively were used to identify significant regions discriminating the groups. The identified significant variables underwent spectral integration (MestReNova version 7.1.0, Mestrelab Research, Santiago de Compostela, Spain). These metabolite integral values were constant sum normalized and subjected to univariate analysis.

2.13. Statistical analysis

Statistical significance of data between the groups was attained using student T-test, Mann–Whitney U test, and one-way analysis of variance (Kruskal Wallis test), as applicable (GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego, California, USA). Statistical significance was set to $p < 0.05$.

3. Results

3.1. Development and characterization of cisplatin-paclitaxel resistant cellular models with metformin intervention

Dual chemo-resistant A2780 and OAW42 cellular models were developed using a pulse method with incremental dosage of cisplatin and paclitaxel treatment for 6 months (Singh et al., 2014). Cells showing approximately 10 fold higher resistance towards platinum-taxol are referred as $A2780^{LR}$ and $OAW42^{LR}$. To understand the effect of metformin on acquirement of chemoresistance, two different metformin treatment strategies were adopted: i) Sequential treatment of cells with metformin for 24 h followed by cisplatin-paclitaxel for 2 h [referred as MSLR ($A2780^{MSLR}$ or $OAW42^{MSLR}$)], and ii) Combinatorial metformin treatment along with cisplatin-paclitaxel for 2 h [referred as MCLR ($A2780^{MCLR}$ or $OAW42^{MCLR}$)]. For both the treatment strategies, the dosage of cisplatin and paclitaxel was incremented as discussed above (Fig. 1A). To avoid cytotoxicity, a lower dose of metformin (IC_{20} i.e., 2 mM) was used (Fig. 1B). The proliferation rate for both A2780 and OAW42 cellular models were measured for 120 h. Interestingly, from 96 h onwards, $A2780^{MCLR}$ and $A2780^{MSLR}$ cells showed a comparatively higher proliferation rate than $A2780^{LR}$, while A2780 had the fastest proliferative capacity (Fig. 1C). Similarly, a significant increase in the proliferation rate was observed for $OAW42^{MSLR}$ and $OAW42^{MCLR}$ cells post 72 h in comparison to $OAW42^{LR}$ cells (Fig. 1D). The Doubling time of A2780, $A2780^{MSLR}$ and $A2780^{MCLR}$ cells was measured as 23.02 ± 0.74 h, 28.05 ± 0.60 h and 24.18 ± 0.14 h respectively, while $A2780^{LR}$ exhibited longest doubling time of 39 ± 0.93 h (Fig. 1E). $OAW42^{MSLR}$ and $OAW42^{MCLR}$ cells exhibited a doubling time of 43.59 ± 1.02 h and 36.66 ± 0.11 h respectively in comparison to $OAW42^{LR}$ cells, which underwent duplication in 84.25 ± 11.42 h. The doubling time for OAW42 cells was found to be of 43.73 ± 3.11 h (Fig. 1E). We further characterized the migratory property of these resistant cells by wound healing assay. Wound repair rate was found to be similar for $A2780^{LR}$ and A2780 cells (44.80 ± 0.47% vs. 37.34 ± 1.51% wound closure), while $A2780^{MSLR}$ (23.75 ± 4.80%) and $A2780^{MCLR}$ (27.51 ± 0.40%) cells displayed significantly lower migratory potential (Fig. 1F, G). A similar trend was observed for OAW42 cellular model (data not shown). Previously, our lab has reported differential activation of PI3K-AKT and MAPK/ERK signalling at different stages of chemoresistance in EOC cells (Singh et al., 2014). In the present study, MSLR and MCLR cells of both A2780 and OAW42 cell lines, showed higher ERK1/2 phosphorylation compared to $A2780^{LR}$ and $OAW42^{LR}$ cells, while AKT phosphorylation was highest in $A2780^{LR}$ and $OAW42^{LR}$ cells in comparison to their respective metformin treated counterparts. ERK and AKT phosphorylation remained significantly less for sensitive cells of both cell lines (Fig. 1H, I).

3.2. Combined or simultaneous treatment of metformin attenuates development of chemoresistance and stemness of ovarian cancer cells

To determine whether the presence of metformin during acquirement of resistance affects the tolerance of these resistant cells towards cisplatin and paclitaxel, cell viability was measured after 72 h of chemotherapy. A combined dosage of 50 ng/ml cisplatin and 8.5 ng/ml paclitaxel was able to induce only 4.5 ± 2% cell death in $A2780^{LR}$ cells while the same dose led to 33 ± 2.73% and 39 + 0.46% cell death in $A2780^{MSLR}$ and $A2780^{MCLR}$ cells respectively (Fig. 2A). A similar chemotherapeutic insult induced 18.51 ± 2.4% and 32.26 ± 2.9% cell death in $OAW42^{MSLR}$ and $OAW42^{MCLR}$ cells respectively while inducing 6.2 ± 4.2% cell death in $OAW42^{LR}$ and 51.72 ± 3.67% cell death in sensitive OAW42 cells (Fig. 2A). Next, clonogenic potential of A2780 and its resistant counterparts $A2780^{LR}$, $A2780^{MSLR}$, and $A2780^{MCLR}$ was assessed with increasing concentrations of cisplatin and paclitaxel for 48 h. Significantly higher fraction of colonies were obtained for $A2780^{LR}$ cells (~90% and 65% surviving fraction) compared to $A2780^{MSLR}$ (~66% and 18%), and $A2780^{MCLR}$ (~19% and 17%) in both the drug concentrations tested (100 ng cisplatin + 13 ng paclitaxel/ml and 100 ng cisplatin + 42.5 ng paclitaxel/ml) (Fig. 2B, C). A similar observation was made for the OAW42 cellular model. Approximately 50% surviving fraction was observed for $OAW42^{LR}$ cells compared to 21% and 16% survival fraction for $OAW42^{MSLR}$ and $OAW42^{MCLR}$ cells respectively when treated with 250 ng Cisplatin + 20 ng Paclitaxel/ml (Fig. 2D, E). To assess the effect of metformin on the CSC fraction, we used Side Population assay to identify the CSC population based on their innate drug efflux property. $A2780^{LR}$ cells possessed highest CSC (28.6%) population while $A2780^{MSLR}$ and $A2780^{MCLR}$ had 15.1% and 9.70% of CSC. Sensitive A2780 cells had only 2.16% of CSC (Fig. 3A). $OAW42^{LR}$ cells also displayed the highest percentage of CSC population (22.1%) while $OAW42^{MSLR}$ and $OAW42^{MCLR}$ contained 4.13% and 6.79% CSC fraction respectively (Fig. 3B). The expression level of CD133, a known ovarian CSC biomarker was evaluated in both A2780 and OAW42 resistant models (Curley et al., 2009). $A2780^{LR}$ (74.4%) cells had the highest percentage of CD133 positive cells in comparison to A2780 (35%), $A2780^{MSLR}$ (28.5%), and $A2780^{MCLR}$ (35.5%) cells (Fig. 3C). A similar increase in CD133 positive cell population was observed in $OAW42^{LR}$ (9.63%) cells with respect to the OAW42 (0.51%), $OAW42^{MSLR}$ (4.29%), and $OAW42^{MCLR}$ (0.64%) cells (Fig. 3D). Expression level of the pluripotent genes, Oct 4, Sox 2 and Nanaog was also evaluated in both chemoresistant models (Klemba et al., 2018). A significantly higher relative expression of pluripotent genes like Sox2, Oct 4, and Nanog was observed in $A2780^{LR}$ cells in comparison to $A2780^{MSLR}$ and $A2780^{MCLR}$ cells (Fig. 3E). Expression of these pluripotent genes was also found to be upregulated in $OAW42^{LR}$

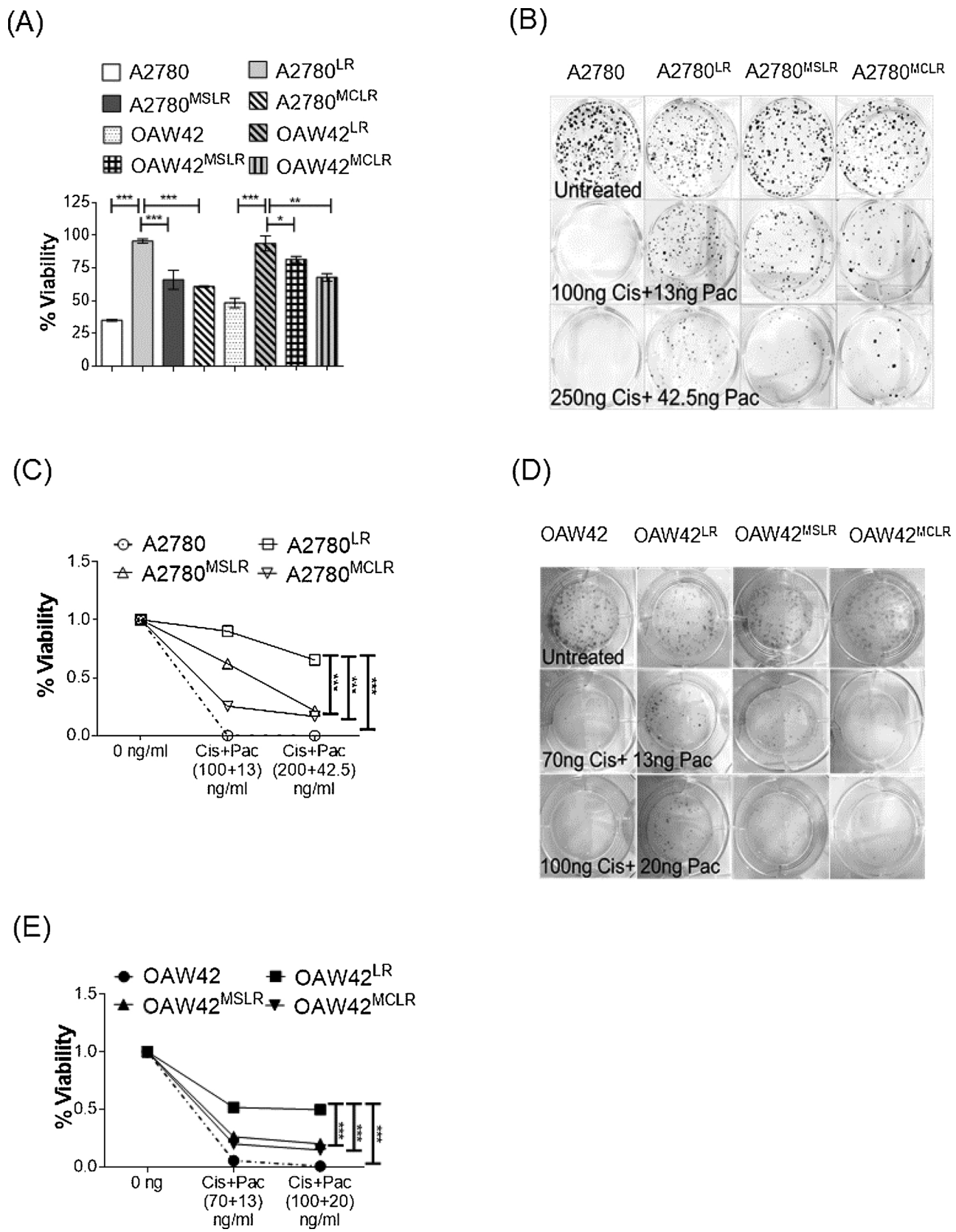


Fig. 2. Effect of metformin on chemoresistance: A) MTT assay showing significantly reduced viability of MCLR, MSLR as compared to LR cells, in response to 50 ng cisplatin + 8.5 ng paclitaxel/ml in both A2780 and OAW42 models. (Data represents mean ± SD of three independent experiments, ns indicates non-significant,* indicates $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ as calculated by unpaired t-test) B–E) Long term survival assay of A2780 and OAW42 cell models in response to increasing concentration of cisplatin-paclitaxel treatment displayed significantly higher colony forming ability in LR cells compared to MCLR and MSLR cells in both the cellular models. (Data represents mean ± SD of three independent experiments, ns indicates non-significant,* indicates $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ as calculated by unpaired t-test).

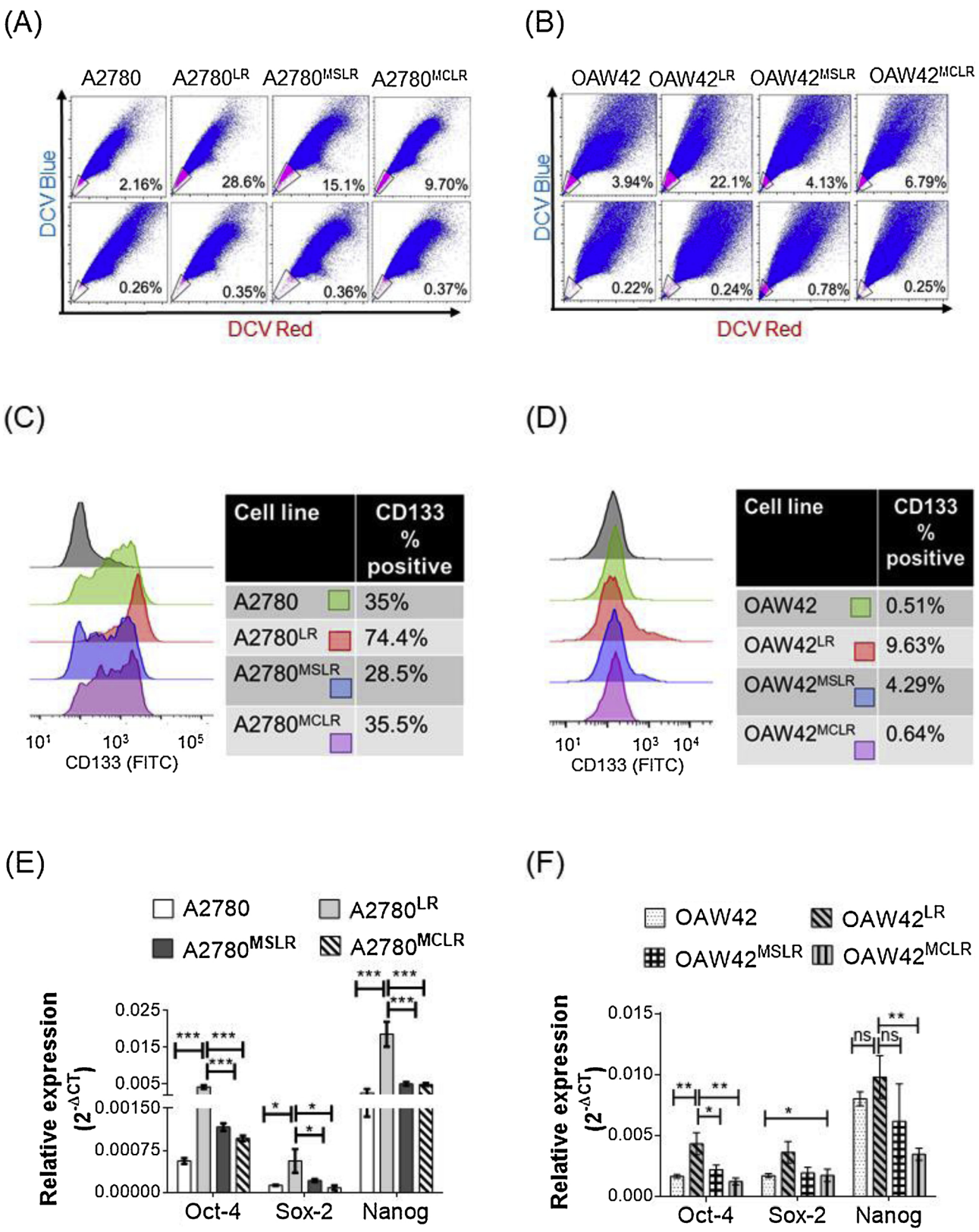
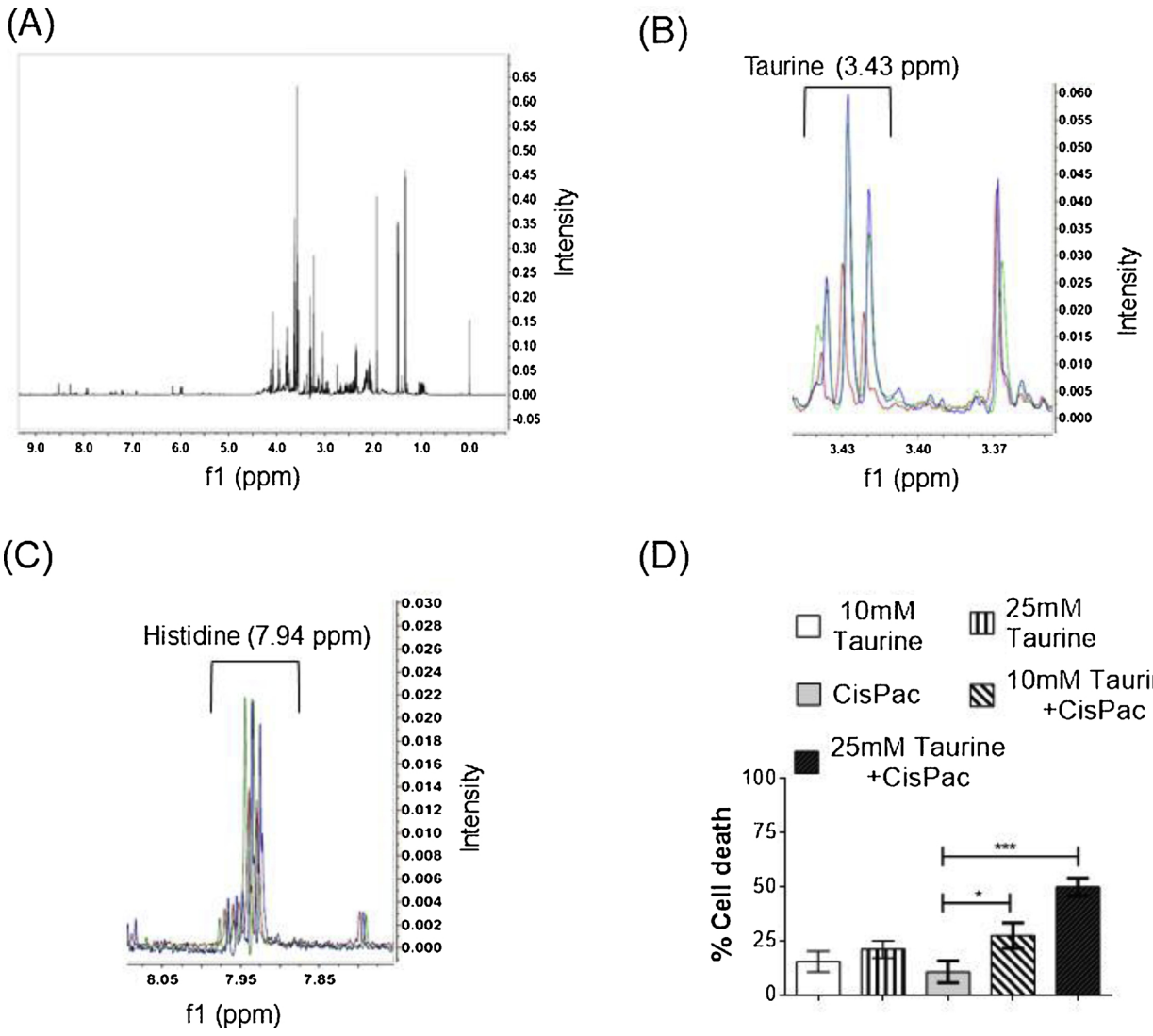


Fig. 3. Effect of metformin on stemness properties. A–B) FACS dot plot showing side population distribution (tail end of scatter) with and without verapamil in A2780 and OAW42 cellular resistant models. Both the MSLR and MCLR cells of A2780 and OAW42 cellular model had a reduced SP population compared to LR cells. C–D) Histogram showing an increased expression of CSC biomarker CD133 in LR cells of both the A2780 and OAW42 resistant model in comparison to MSLR and MCLR cells. E–F) Quantitative PCR showing the relative expression of pluripotent genes (oct-4, sox-2, nanog), in A2780 and OAW42 cellular models. GAPDH was used as an internal control. MSLR and MCLR cells show significantly reduced expression of pluripotent genes compared to LR cells. (Data represents mean ± SD of three independent experiments, ns indicates non-significant,* indicates $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ as calculated by unpaired t-test).



(caption on next page)

Fig. 4. A key metabolite, taurine affects stemness and chemoresistance of ovarian cancer cells. A) Representative 800 MHz NMR spectra (δ 0.0 − 9.5) showing metabolite profile identified from the cell lysate of A2780^{LR} cells B–C) Inset representing regions from the superimposition of the mean NMR spectra showing differential expression of taurine and histidine from cell lysate collected of A2780^{LR} (Red), A2780^{MSLR} (Blue), A2780^{MCLR} (Green) D) MTT assay showing percentage of cell death in response to taurine and cisplatin-paclitaxel. Combination of taurine + cisplatin-paclitaxel significantly sensitized A2780^{LR} cells towards platinum-taxol (Data represents mean ± SD of three independent experiments, ns indicates non-significant,* indicates $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ as calculated by unpaired t-test) E–F) FACS dot plot depicting the side population distribution in A2780^{LR} and SKOV3 cells treated with taurine. Treatment of taurine for 24 h reduced stem cell like population as compared to untreated in both the cell lines (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

cells with respect to OAW42^{MSLR} and OAW42^{MCLR} cells (Fig. 3F).

3.3. Metformin treatment brings changes in specific amino acid levels regulating stemness

Since metformin is known to affect cellular metabolism, we performed 1D ^{1}H-NMR spectroscopy from cell lysates of sensitive and resistant A2780 cells developed in the presence and absence of metformin. In order to classify the various groups, chemometric analysis was used based on differences in metabolites expression. Improved separation was achieved by utilising a higher supervised clustering method (orthogonal partial least squares discriminant analysis OPLS-DA) for one to one analysis. This optimized the classification between the groups by removing unrelated variables of the class (Figure S1-2). We also used this model to extract/identify the significant metabolites discriminating the groups using the S-plot and VIP plot. One dimensional (1D) ^{1}H-NMR peaks of the treated cells corresponding to specific metabolites were identified using two dimensional (2D) NMR experiments [Total Correlation Spectroscopy (TOCSY) and Correlation Spectroscopy (COSY)] and references of relevant literature (Subramani et al., 2017; Lauri et al., 2016). Forty one metabolites were identified in 1D ZGPR NMR spectra. The representative NMR spectra of cells with metabolites assignment are shown (Fig. 4A) along with the table containing all the identified metabolites (Table S1). Based on the significant variables identified in the S-plot and VIP scores, univariate analysis was applied to the integral values of these metabolites. Taurine and histidine were found to be the most important metabolites from both the multi and uni-variate analysis which were significantly different between the groups. Representative mean spectral integrals of these regions have been presented (Fig. 4B, C). Taurine level was found to be upregulated in A2780^{MSLR} (˜2 fold) and A2780^{MCLR} (˜1.7 fold) as compared to A2780 cells. In contrast, the level of taurine in A2780^{LR} (˜1.2 fold) cells remained almost similar to sensitive cells (Fig S3). The histidine level was upregulated by ˜3 fold and ˜1.93 fold in A2780^{MCLR} and A2780^{MSLR} respectively in comparison to A2780 cells. A2780^{LR} cells had a ˜1.5 fold increase in histidine level compared to sensitive cells (Fig S4). Since all the resistant models were developed in a synchronous fashion, changes in many metabolites were not expected. Indeed, identification of only two metabolites that were significantly elevated in resistant models (particularly in metformin treated ones) increased our confidence. Since taurine is reported to promote stem cell differentiation (Li et al., 2017, Zhou et al., 2014) and we had also observed a decrease in the percent SP population in A2780^{MCLR} and A2780^{MSLR} cells, we aimed to understand the direct effect of taurine on SP population. To prevent any cytotoxicity, a low dose of taurine was used to determine its influence on the CSC population (Fig. 4D). Taurine treatment for twenty four hours reduced the CSC population in A2780^{LR} cells. 10 mM (IC_{20}) and 25 mM (IC_{30}) of taurine treatment reduced the CSC population by 5.7% and 10.6% respectively in comparison to untreated cells (30.5% SP population) (Fig. 4E). We also tested the effect of taurine on the CSC population in SKOV3, an intrinsically resistant cisplatin cell line. Treatment of 10 mM and 25 mM of taurine reduced the percentage of CSCs to 3.31 and 1.79% respectively compared to 7.12% of untreated cells (Fig. 4F). Further combinatorial treatment of 50 ng Cisplatin + 8.5 ng paclitaxel/ml along with 10 mM and 25 mM of taurine was found to significantly increase the cell death (27.22 ± 2.62% and 49 ± 2.35% respectively) compared to only cisplatin + paclitaxel (7.61 + 2.28%) in A2780^{LR} cells (Fig. 4D).

4. Discussion

Metabolic profile of a tumour can influence therapy outcome in various ways. In EOC, acquirement of chemoresistance is a major obstacle towards effective therapy, and enrichment of CSCs may influence the process (Latifi et al., 2012). Thus, investigating and discovering novel therapeutic approaches to targeting CSCs are critical. Utilizing indigenously developed cisplatin and paclitaxel dual resistant models, either in the presence or absence of metformin, we showed that the introduction of metformin during the development of chemoresistance led to significant alteration in proliferation rate, migration, ERK and AKT kinase activation level and increased chemosensitivity possibly through reduced CSC population in both A2780 and OAW42 cells. The expression of CD133, an ovarian cancer CSC biomarker and pluripotent genes like Oct 4, Sox 2 and Nanog was also found to be significantly downregulated in these cells (Curley et al., 2009; Klemba et al., 2018). Further metabolic profiling using ^{1}H-NMR of these cells identified two amino acids (taurine and histidine) that were significantly and differentially produced among the sensitive cells, platinum-taxol resistant cells, and metformin treated platinum-taxol resistant cells (MSLR and MCLR). Intriguingly, taurine at low concentration was found to decrease the side population fraction (CSC-like population) in A2780^{LR} cells and SKOV3, an inherently cisplatin resistant cell line. Increased cell death (chemosensitization) was observed when A2780^{LR} cells were treated with the combination of platinum-taxol and taurine. Collectively, our data for the first time illustrates that metformin impedes acquirement of chemoresistance by inducing CSC differentiation through taurine generation, and maintains a more proliferative cellular state which is susceptible to chemotherapeutic intervention. We also demonstrate the role of taurine, a common health supplement in differentiation of ovarian CSCs.

Repurposing of approved drug for one pathological condition to treat another disease is a standard strategy that reduces the time and cost for developing a promising molecule to approved drug. Since detailed information on the pharmacokinetics, initial toxicity and other characteristics of this molecule is already known, a quick entry into clinical trial and speedy review by Food and Drug Administration hastens the process of integration of the molecule in health care. Vast clinical and research data support the idea of repurposing of metformin against EOC. A case control study by Kumar et al 2013 reported that the use of metformin was associated with an increased survival in EOC patients (Kumar et al., 2013). A recent report suggests that routine administration of metformin along with platinum-taxol reduces NFkβ mediated Interleukin-6 production by the stromal component of EOC tumours in patients, and thereby slows tumour growth (Xu et al., 2018). High dose of metformin (200 mg/kg in mouse or 8 mM/48 h for cells) resulted in decreased proliferation in EOC tumour xenografts or in a panel of EOC cells (Rattan et al., 2011; Chan and Miskimins, 2012). Using our cellular models, developed mimicking the real life situation of a diabetic patient suffering from EOC, we investigated the altered molecular and cellular functions that arose by continuous metformin treatment on acquirement of resistance. Surprisingly, resistant cells (MSLR and MCLR) developed in the presence of metformin showed

approx. 2.5 fold and 3.5 fold higher proliferation rate than platinum-taxol resistant (LR) cells after 72–96 hours. This discordance with earlier studies might have arisen due to the difference in doses (8 mM vs. 2 mM (for our study)), as well as the nature of treatment (one time vs. continuous) of metformin. Our data, thus indicate that a sub-lethal (IC_{20}) concentration of metformin increases the rate of cellular proliferation when applied in combination with anti-cancer drugs in a synchronous manner. A dose dependent decrease in migration potential of SKOV3 and HO-8910PM cells was reported upon metformin treatment (Wu et al., 2012). Interestingly, we also observed a slower migration rate in MSLR and MCLR cells as compared to LR cells, which might reduce the metastatic ability of these cells. Chemosensitization effect of metformin (alone or in combination with chemotherapeutic drugs) had been reported in breast, lung and prostate cancer cells (Zordoky et al., 2014; Iliopoulos et al., 2011). Treatment of metformin (1 mM or higher) for 24/48 h sensitized cisplatin resistant and paclitaxel resistant A2780 cells (Dos Santos Guimaraes et al., 2018). In accordance with these studies that used single or short duration metformin treatment, we also found a similar sensitization effect in the cells, which were made resistant towards platinum-taxol in the presence of metformin than the cells only resistant to platinum-taxol. Since cytotoxic drugs mainly target proliferative cells (Valeriote and van Putten, 1975), the reduced survival of MSLR and MCLR cells against cisplatin-paclitaxel may be attributed to their superior proliferation ability compared to LR cells, which are more quiescent in nature (Thakur and Ray, 2017). Activation of MAPK/ERK signalling fosters cell proliferation by upregulating cell cycle entry genes and negatively regulating genes that prevent the process, while upregulation of AKT signalling prevents cell cycle entry and promotes a quiescent phenotype (Yamamoto et al., 2006; Touil et al., 2013; Thakur and Ray, 2017). Contradictory reports suggest that metformin can induce ERK1/2 activation in acute myeloid and promyelocytic leukemia cells and inhibits ERK activation in pancreatic cancer cells (Huai et al., 2012; Chai et al., 2015). In this study, increased ERK1/2 activation was observed in MCLR and MSLR cells for both the A2780 and OAW42 models. In contrast, AKT activation was reduced in metformin treated cells indicating lowering of quiescence. Such a phenomenon was reported earlier (Fu et al., 2017). Thus increased activation of mitogenic ERK1/2 signalling may contribute to the proliferative nature of MSLR and MCLR cells, while in LR cells, upregulated AKT activation may promote a quiescence state and CSC enrichment.

Among several processes, presence of CSCs can also influence therapy resistance (Shibue and Weinberg, 2017). Our group has reported enrichment of CSCs with acquirement of resistance (Singh et al., 2016). A low dose of metformin had been shown to reduce ALDH positive cells and spheroid formation in A2780 and SKOV3 cells (Shank et al., 2012), and mamosphere formation in breast cancer cells (Hirsch et al., 2009). Interestingly, in our study, resistant cells that were developed in the presence of metformin had lower percentage of CSCs (higher percentage of differentiated cells) compared to resistant cells developed in metformin's absence for both A2780 and OAW42 resistant models. The level of CD133 was also decreased in MSLR and MCLR cells of both A2780 and OAW42 models. Down regulation of Nanog, Oct 4, and Sox2 expression in MSLR and MCLR cells compared to LR cells indicated differentiation of the CSC-like population to non-CSC population in the A2780 and OAW42 models. These results strongly suggest for the role of metformin in differentiation of cancer stem cell like population in EOC cells, which may ultimately account for higher drug sensitization.

Cellular metabolism can regulate CSC properties since these cells have an altered bioenergetics profile that is different from the non-CSC population (Agathocleous and Harris, 2013). Metformin treatment was found to deplete TCA cycle intermediates in breast and non-small cell lung cancer cell lines (Janzer et al., 2014; Griss et al., 2015), and target mitochondrial metabolism in EOC patients (treated with metformin prior to upfront surgery), leading to alteration in nucleotide metabolism, redox pathways and TCA cycle (Liu et al., 2016). Interestingly, we observed that prolonged exposure of metformin during development of chemoresistant model in normal culture condition led to perturbation in both purine metabolism (Histidine level) and semi-essential amino acid metabolism (Methionine/Cysteine-Taurine level) in $A2780^{MSLR}$ and $A2780^{MCLR}$ cells. Perturbation of only a few metabolites was expected since the models were developed in a synchronous fashion and the basal metabolite profile was acquired without any treatment. We also did not see any significant difference in cellular functions between the MSLR (sequential treatment) and MCLR (continuous treatment) (see Fig.1) cells, indicating a comparable final outcome for both the treatment strategies. In humans, taurine can be produced from methionine or cysteine in the presence of vitamin B_6 (Bouckenooghe et al., 2006) but does not get incorporated in proteins. Taurine plays an important role in retinal function and exhibits neuroprotective effect through calcium homeostasis (Lombardini, 1991; Menzie et al., 2014). High dosage of taurine has been reported to exert anti-cancer effect by inducing apoptosis in lung and colon cancer cells (Zhang et al., 2014; Tu et al., 2018). Taurine is also reported to enhance mesenchymal stem cell differentiation through upregulation of the MAPK/ERK pathway (Zhou et al., 2014). However, influence of taurine on CSC homeostasis has not been explored till now. Addition of taurine (10 mM and 25 mM for 24 h) to culture media reduced SP population in $A2780^{LR}$ cells from 30.5% (untreated) to 24.6% and 19.9% respectively, indicating its ability to promote differentiation of CSC like population into non-CSC like population. Similar treatment in an inherently platinum resistant SKOV3 cells with taurine significantly reduced SP population from 7.12% (untreated) to 3.31% and 1.79% respectively. Also, the MSLR and MCLR cells having a higher level of taurine displayed a significantly lower SP fraction. It is still unclear how continuous metformin treatment increases cellular levels of taurine which promotes CSC differentiation. Further studies to elucidate the mechanisms are on-going in our lab. We also observed that combination treatment of taurine (10 mM and 25 mM) with cisplatin-paclitaxel sensitized LR cells by inducing 2.65 and 4.68 fold higher cell death in comparison to cisplatin-paclitaxel alone, which may have resulted from the reduction in chemoresistant CSC population. This novel role of taurine, a common nutrient supplements, on CSCs and altogether on chemoresistance may be developed as an approach to combat chemoresistance. Thorough experimentation is needed to validate these findings in future.

5. Conclusion

Targeting CSC population is envisaged as a potential approach to overcome the shortcomings attributing to therapy failure. Compelling evidences suggest that CSCs are responsible for tumour initiation, relapse, and chemoresistance. Data presented here signifies the importance of metformin and taurine on EOC chemoresistance. Continuous presence of metformin during acquirement of resistance leads to an increased taurine level, which in turn induces CSC differentiation and alleviates resistant properties of EOC cells. The idea of metformin application along with cisplatin-paclitaxel to prevent/lessen development of resistance may open a new dimension in designing a therapeutic regimen. Similarly, application of taurine to prevent enrichment of CSCs in resistant cells may open new opportunities to tackle or subdue the relapse or recurrence of cancer.

Declaration of interest

None.

Acknowledgment

AB acknowledges ACTREC for fellowship. PR acknowledges ACTREC for funding. We also acknowledge NMR facility at Tata

Institute of Fundamental Research for all the NMR spectra collection for metabolomics profiling. We specially thank Mrs. Mamta Joshi, from Department of Chemical Sciences, Tata Institute of Fundamental Research, for her valuable suggestions in the NMR experimentations.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biocel.2018.12.016.

References

Agathocleous, M., Harris, W.A., 2013. Metabolism in physiological cell proliferation and differentiation. Trends Cell Biol. 23 (10), 484–492. https://doi.org/10.1016/j.tcb.2013.05.004.

Anderson, A.S., Roberts, P.C., Frisard, M.I., McMillan, R.P., Brown, T.J., Lawless, M.H., Hulver, M.W., Schmelz, E.M., 2013. Metabolic changes during ovarian cancer progression as targets for sphingosine treatment. Exp. Cell Res. 319 (10), 1431–1442. https://doi.org/10.1016/j.yexcr.2013.02.017.

Banerjee, P., Dutta, M., Srivastava, S., Joshi, M., Chakravarty, B., Chaudhury, K., 2014. (1)H NMR serum metabonomics for understanding metabolic dysregulation in women with idiopathic recurrent spontaneous miscarriage during implantation window. J. Proteome Res. 13 (6), 3100–3106. https://doi.org/10.1021/pr500379n.

Birsoy, K., Possemato, R., Lorbeer, F.K., Bayraktar, E.C., Thiru, P., Yucel, B., Wang, T., Chen, W.W., Clish, C.B., Sabatini, D.M., 2014. Metabolic determinants of cancer cell sensitivity to glucose limitation and biguanides. Nature 508 (7494), 108–112. https://doi.org/10.1038/nature13110.

Bouckenooghe, T., Remacle, C., Reusens, B., 2006. Is taurine a functional nutrient? Curr. Opin. Clin. Nutr. Metab. Care 9 (6), 728–733. https://doi.org/10.1097/01.mco.0000247469.26414.55.

Calle, E.E., Rodriguez, C., Walker-Thurmond, K., Thun, M.J., 2003. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. Adults. N. Engl. J. Med. 348 (17), 1625–1638. https://doi.org/10.1056/NEJMoa021423.

Campbell, P.T., Newton, C.C., Patel, A.V., Jacobs, E.J., Gapstur, S.M., 2012. Diabetes and cause-specific mortality in a prospective cohort of one million U.S. Adults. Diabetes Care 35 (9), 1835–1844. https://doi.org/10.2337/dc12-0002.

Chai, X., Chu, H., Yang, X., Meng, Y., Shi, P., Gou, S., 2015. Metformin increases sensitivity of pancreatic Cancer cells to gemcitabine by reducing CD133+ cell populations and suppressing ERK/P70S6K signaling. Sci. Rep. 5, 14404. https://doi.org/10.1038/srep14404.

Chan, D.K., Miskimins, W.K., 2012. Metformin and phenethyl isothiocyanate combined treatment in vitro is cytotoxic to ovarian cancer cultures. J. Ovarian Res. 5 (1), 19. https://doi.org/10.1186/1757-2215-5-19.

Curley, M.D., Therrien, V.A., Cummings, C.L., Sergent, P.A., Koulouris, C.R., Friel, A.M., Roberts, D.J., Seiden, M.V., Scadden, D.T., Rueda, B.R., Foster, R., 2009. CD133 expression defines a tumor initiating cell population in primary human ovarian cancer. Stem Cells 27 (12), 2875–2883. https://doi.org/10.1002/stem.236.

DeBerardinis, R.J., Chandel, N.S., 2016. Fundamentals of cancer metabolism. Sci. Adv. 2 (5). https://doi.org/10.1126/sciadv.1600200. e1600200.

Dos Santos Guimaraes, I., Ladislau-Magescky, T., Tessarollo, N.G., Dos Santos, D.Z., Gimba, E.R.P., Sternberg, C., Silva, I.V., Rangel, L.B.A., 2018. Chemosensitizing effects of metformin on cisplatin- and paclitaxel-resistant ovarian cancer cell lines. Pharmacol. Rep. 70 (3), 409–417. https://doi.org/10.1016/j.pharep.2017.11.007.

Dutta, M., Joshi, M., Srivastava, S., Lodh, I., Chakravarty, B., Chaudhury, K., 2012. A metabonomics approach as a means for identification of potential biomarkers for early diagnosis of endometriosis. Mol. Biosyst. 8 (12), 3281–3287. https://doi.org/10.1039/c2mb25353d.

Evans, J.M., Donnelly, L.A., Emslie-Smith, A.M., Alessi, D.R., Morris, A.D., 2005. Metformin and reduced risk of cancer in diabetic patients. BMJ 330 (7503), 1304–1305. https://doi.org/10.1136/bmj.38415.708634.F7.

Fendt, S.M., Bell, E.L., Keibler, M.A., Olenchock, B.A., Mayers, J.R., Wasylenko, T.M., Vokes, N.I., Guarente, L., Vander Heiden, M.G., Stephanopoulos, G., 2013. Reductive glutamine metabolism is a function of the alpha-ketoglutarate to citrate ratio in cells. Nat. Commun. 4, 2236. https://doi.org/10.1038/ncomms3236.

Fu, Y.L., Zhang, Q.H., Wang, X.W., He, H., 2017. Antidiabetic drug metformin mitigates ovarian cancer SKOV3 cell growth by triggering G2/M cell cycle arrest and inhibition of m-TOR/PI3K/Akt signaling pathway. Eur. Rev. Med. Pharmacol. Sci. 21 (5), 1169–1175.

Gaikwad, S.M., Gunjal, L., Junutula, A.R., Astanehe, A., Gambhir, S.S., Ray, P., 2013. Non-invasive imaging of phosphoinositide-3-kinase-catalytic-subunit-alpha (PIK3CA) promoter modulation in small animal models. PLoS One 8 (2). https://doi.org/10.1371/journal.pone.0055971. e55971.

Gaikwad, S.M., Thakur, B., Sakpal, A., Singh, R.K., Ray, P., 2015. Differential activation of NF-kappaB signaling is associated with platinum and taxane resistance in MyD88 deficient epithelial ovarian cancer cells. Int. J. Biochem. Cell Biol. 61, 90–102. https://doi.org/10.1016/j.biocel.2015.02.001.

García-Álvarez, Lara, Busto, Jesús H., Avenoza, Alberto, Sáenz, Yolanda, Peregrina, Jesús Manuel, Oteo, JoséA., 2015. Proton nuclear magnetic resonance spectroscopy as a technique for gentamicin drug susceptibility studies with "genus-species" Escherichia coli ATCC 25922. J. Clin. Microbiol. 53 (8), 2433–2438. https://doi.org/10.1128/jcm.00604-15.

Gasch, C., Ffrench, B., O'Leary, J.J., Gallagher, M.F., 2017. Catching moving targets: cancer stem cell hierarchies, therapy-resistance & considerations for clinical intervention. Mol. Cancer 16 (1), 43. https://doi.org/10.1186/s12943-017-0601-3.

Griss, T., Vincent, E.E., Egnatchik, R., Chen, J., Ma, E.H., Faubert, B., Viollet, B., DeBerardinis, R.J., Jones, R.G., 2015. Metformin antagonizes cancer cell proliferation by suppressing mitochondrial-dependent biosynthesis. PLoS Biol. 13 (12). https://doi.org/10.1371/journal.pbio.1002309. e1002309.

Gubbels, J.A., Claussen, N., Kapur, A.K., Connor, J.P., Patankar, M.S., 2010. The detection, treatment, and biology of epithelial ovarian cancer. J. Ovarian Res. 3, 8. https://doi.org/10.1186/1757-2215-3-8.

Hirsch, H.A., Iliopoulos, D., Tsichlis, P.N., Struhl, K., 2009. Metformin selectively targets cancer stem cells, and acts together with chemotherapy to block tumor growth and prolong remission. Cancer Res. 69 (19), 7507–7511. https://doi.org/10.1158/0008-5472.CAN-09-2994.

Hirschey, M.D., DeBerardinis, R.J., Diehl, A.M.E., Drew, J.E., Frezza, C., Green, M.F., Jones, L.W., Ko, Y.H., Le, A., Lea, M.A., Locasale, J.W., Longo, V.D., Lyssiotis, C.A., McDonnell, E., Mehrmohamadi, M., Michelotti, G., Muralidhar, V., Murphy, M.P., Pedersen, P.L., Poore, B., Raffaghello, L., Rathmell, J.C., Sivanand, S., Vander Heiden, M.G., Wellen, K.E., Team Target Validation, 2015. Dysregulated metabolism contributes to oncogenesis. Semin. Cancer Biol. 35 (Suppl), S129–S150. https://doi.org/10.1016/j.semcancer.2015.10.002.

Hodeib, M., Ogrodzinski, M.P., Vergnes, L., Reue, K., Karlan, B.Y., Lunt, S.Y., Aspuria, P.P., 2018. Metformin induces distinct bioenergetic and metabolic profiles in sensitive versus resistant high grade serous ovarian cancer and normal fallopian tube secretory epithelial cells. Oncotarget 9 (3), 4044–4060. https://doi.org/10.18632/oncotarget.23661.

Huai, L., Wang, C., Zhang, C., Li, Q., Chen, Y., Jia, Y., Li, Y., Xing, H., Tian, Z., Rao, Q., Wang, M., Wang, J., 2012. Metformin induces differentiation in acute promyelocytic leukemia by activating the MEK/ERK signaling pathway. Biochem. Biophys. Res. Commun. 422 (3), 398–404. https://doi.org/10.1016/j.bbrc.2012.05.001.

Iliopoulos, Dimitrios, Hirsch, Heather A., Struhl, Kevin, 2011. Metformin decreases the dose of chemotherapy for prolonging tumor remission in mouse xenografts involving multiple Cancer cell types. Cancer Res. 71 (9), 3196–3201. https://doi.org/10.1158/0008-5472.can-10-3471.

Janzer, A., German, N.J., Gonzalez-Herrera, K.N., Asara, J.M., Haigis, M.C., Struhl, K., 2014. Metformin and phenformin deplete tricarboxylic acid cycle and glycolytic intermediates during cell transformation and NTPs in cancer stem cells. Proc. Natl. Acad. Sci. U. S. A. 111 (29), 10574–10579. https://doi.org/10.1073/pnas.1409844111.

Klemba, A., Purzycka-Olewiecka, J.K., Wcislo, G., Czarnecka, A.M., Lewicki, S., Lesyng, B., Szczylik, C., Kieda, C., 2018. Surface markers of cancer stem-like cells of ovarian cancer and their clinical relevance. Contemp. Oncol. (Pozn) 22 (1A), 48–55. https://doi.org/10.5114/wo.2018.73885.

Kumar, S., Meuter, A., Thapa, P., Langstraat, C., Giri, S., Chien, J., Rattan, R., Cliby, W., Shridhar, V., 2013. Metformin intake is associated with better survival in ovarian cancer: a case-control study. Cancer 119 (3), 555–562. https://doi.org/10.1002/cncr.27706.

Kwon, Mi, Shin, Young, 2013. Regulation of ovarian cancer stem cells or tumor-initiating cells. Int. J. Mol. Sci. 14 (4), 6624.

Latifi, A., Luwor, R.B., Bilandzic, M., Nazaretian, S., Stenvers, K., Pyman, J., Zhu, H., Thompson, E.W., Quinn, M.A., Findlay, J.K., Ahmed, N., 2012. Isolation and characterization of tumor cells from the ascites of ovarian cancer patients: molecular phenotype of chemoresistant ovarian tumors. PLoS One 7 (10). https://doi.org/10.1371/journal.pone.0046858. e46858.

Lauri, I., Savorani, F., Iaccarino, N., Zizza, P., Pavone, L.M., Novellino, E., Engelsen, S.B., Randazzo, A., 2016. Development of an optimized protocol for NMR metabolomics studies of human colon cancer cell lines and first insight from testing of the protocol using DNA G-Quadruplex ligands as novel anti-cancer drugs. Metabolites 6 (1). https://doi.org/10.3390/metabo6010004.

Lengyel, E., Litchfield, L.M., Mitra, A.K., Nieman, K.M., Mukherjee, A., Zhang, Y., Johnson, A., Bradaric, M., Lee, W., Romero, I.L., 2015. Metformin inhibits ovarian cancer growth and increases sensitivity to paclitaxel in mouse models. Am. J. Obstet. Gynecol. 212 (4), 479. https://doi.org/10.1016/j.ajog.2014.10.026. e1-479 e10.

Li, X.W., Gao, H.Y., Liu, J., 2017. The role of taurine in improving neural stem cells proliferation and differentiation. Nutr. Neurosci. 20 (7), 409–415. https://doi.org/10.1080/1028415X.2016.1152004.

Liao, J., Qian, F., Tchabo, N., Mhawech-Fauceglia, P., Beck, A., Qian, Z., Wang, X., Huss, W.J., Lele, S.B., Morrison, C.D., Odunsi, K., 2014. Ovarian cancer spheroid cells with stem cell-like properties contribute to tumor generation, metastasis and chemotherapy resistance through hypoxia-resistant metabolism. PLoS One 9 (1). https://doi.org/10.1371/journal.pone.0084941. e84941.

Liu, X., Romero, I.L., Litchfield, L.M., Lengyel, E., Locasale, J.W., 2016. Metformin targets central carbon metabolism and reveals mitochondrial requirements in human cancers. Cell Metab. 24 (5), 728–739. https://doi.org/10.1016/j.cmet.2016.09.005.

Lombardini, J.B., 1991. Taurine: retinal function. Brain Res. Brain Res. Rev. 16 (2), 151–169.

Lu, Q., Zhang, Y., Elisseeff, J.H., 2015. Carnitine and acetylcarnitine modulate mesenchymal differentiation of adult stem cells. J. Tissue Eng. Regen. Med. 9 (12), 1352–1362. https://doi.org/10.1002/term.1747.

Menzie, J., Pan, C., Prentice, H., Wu, J.Y., 2014. Taurine and central nervous system disorders. Amino Acids 46 (1), 31–46. https://doi.org/10.1007/s00726-012-1382-z.

Qin, X.Y., Wei, F., Tanokura, M., Ishibashi, N., Shimizu, M., Moriwaki, H., Kojima, S., 2013. The effect of acyclic retinoid on the metabolomic profiles of hepatocytes and hepatocellular carcinoma cells. PLoS One 8 (12). https://doi.org/10.1371/journal.pone.0082860. e82860.

Rattan, R., Graham, R.P., Maguire, J.L., Giri, S., Shridhar, V., 2011. Metformin suppresses ovarian cancer growth and metastasis with enhancement of cisplatin cytotoxicity in

vivo. Neoplasia 13 (5), 483–491.
Renehan, A.G., Tyson, M., Egger, M., Heller, R.F., Zwahlen, M., 2008. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. Lancet 371 (9612), 569–578. https://doi.org/10.1016/S0140-6736(08)60269-X.
Roth, V. 2006. Available from http://www.doubling-time.com/compute.php.
Shackelford, D.B., Abt, E., Gerken, L., Vasquez, D.S., Seki, A., Leblanc, M., Wei, L., Fishbein, M.C., Czernin, J., Mischel, P.S., Shaw, R.J., 2013. LKB1 inactivation dictates therapeutic response of non-small cell lung cancer to the metabolism drug phenformin. Cancer Cell 23 (2), 143–158. https://doi.org/10.1016/j.ccr.2012.12.008.
Shafiee, M.N., Khan, G., Ariffin, R., Abu, J., Chapman, C., Deen, S., Nunns, D., Barrett, D.A., Seedhouse, C., Atiomo, W., 2014. Preventing endometrial cancer risk in polycystic ovarian syndrome (PCOS) women: could metformin help? Gynecol. Oncol. 132 (1), 248–253. https://doi.org/10.1016/j.ygyno.2013.10.028.
Shibue, T., Weinberg, R.A., 2017. EMT, CSCs, and drug resistance: the mechanistic link and clinical implications. Nat. Rev. Clin. Oncol. 14 (10), 611–629. https://doi.org/10.1038/nrclinonc.2017.44.
Shiraki, N., Shiraki, Y., Tsuyama, T., Obata, F., Miura, M., Nagae, G., Aburatani, H., Kume, K., Endo, F., Kume, S., 2014. Methionine metabolism regulates maintenance and differentiation of human pluripotent stem cells. Cell Metab. 19 (5), 780–794. https://doi.org/10.1016/j.cmet.2014.03.017.
Singh, R.K., Gaikwad, S.M., Jinager, A., Chaudhury, S., Maheshwari, A., Ray, P., 2014. IGF-1R inhibition potentiates cytotoxic effects of chemotherapeutic agents in early stages of chemoresistant ovarian cancer cells. Cancer Lett. 354 (2), 254–262. https://doi.org/10.1016/j.canlet.2014.08.023.
Singh, Ram K., Dhadve, Ajit, Sakpal, Asmita, De, Abhijit, Pritha, Ray., 2016. An active IGF-1R-AKT signaling imparts functional heterogeneity in ovarian CSC population. Sci. Rep. 6, 36612. https://doi.org/10.1038/srep36612. https://www.nature.com/articles/srep36612#supplementary-information.
Steenland, K., Nowlin, S., Palu, S., 1995. Cancer incidence in the National Health and Nutrition Survey I. Follow-up data: diabetes, cholesterol, pulse and physical activity. Cancer Epidemiol. Biomark. Prev. 4 (8), 807–811.
Subramani, E., Rameshbabu, A.P., Jothiramajayam, M., Subramanian, B., Chakravorty, D., Bose, G., Joshi, M., Ray, C.D., Lodh, I., Chattopadhyay, R., Saha, S., Mukherjee, A., Dhara, S., Chakravarty, B., Chaudhury, K., 2017. Mycobacterial heat shock protein 65 mediated metabolic shift in decidualization of human endometrial stromal cells. Sci. Rep. 7 (1), 3942. https://doi.org/10.1038/s41598-017-04024-w.
Tang, D.G., 2012. Understanding cancer stem cell heterogeneity and plasticity. Cell Res. 22 (3), 457–472. https://doi.org/10.1038/cr.2012.13.
Thakur, B., Ray, P., 2017. Cisplatin triggers cancer stem cell enrichment in platinum-resistant cells through NF-kappaB-TNFalpha-PIK3CA loop. J. Exp. Clin. Cancer Res. 36 (1), 164. https://doi.org/10.1186/s13046-017-0636-8.
Touil, Y., Zuliani, T., Wolowczuk, I., Kuranda, K., Prochazkova, J., Andrieux, J., Le Roy, H., Mortier, L., Vandomme, J., Jouy, N., Masselot, B., Segard, P., Quesnel, B., Formstecher, P., Polakowska, R., 2013. The PI3K/AKT signaling pathway controls the quiescence of the low-Rhodamine123-retention cell compartment enriched for melanoma stem cell activity. Stem Cells 31 (4), 641–651. https://doi.org/10.1002/stem.1333.
Tu, S., Zhang, X.L., Wan, H.F., Xia, Y.Q., Liu, Z.Q., Yang, X.H., Wan, F.S., 2018. Effect of taurine on cell proliferation and apoptosis human lung cancer A549 cells. Oncol. Lett. 15 (4), 5473–5480. https://doi.org/10.3892/ol.2018.8036.
Valeriote, F., van Putten, L., 1975. Proliferation-dependent cytotoxicity of anticancer agents: a review. Cancer Res. 35 (10), 2619–2630.
Varum, S., Rodrigues, A.S., Moura, M.B., Momcilovic, O., th Easley, C.A., Ramalho-Santos, J., Van Houten, B., Schatten, G., 2011. Energy metabolism in human pluripotent stem cells and their differentiated counterparts. PLoS One 6 (6). https://doi.org/10.1371/journal.pone.0020914. e20914.
Vorkas, P.A., Isaac, G., Anwar, M.A., Davies, A.H., Want, E.J., Nicholson, J.K., Holmes, E., 2015. Untargeted UPLC-MS profiling pipeline to expand tissue metabolome coverage: application to cardiovascular disease. Anal. Chem. 87 (8), 4184–4193. https://doi.org/10.1021/ac503775m.
Wu, B., Li, S., Sheng, L., Zhu, J., Gu, L., Shen, H., La, D., Hambly, B.D., Bao, S., Di, W., 2012. Metformin inhibits the development and metastasis of ovarian cancer. Oncol. Rep. 28 (3), 903–908. https://doi.org/10.3892/or.2012.1890.
Xia, J., Mandal, R., Sinelnikov, I.V., Broadhurst, D., Wishart, D.S., 2012. MetaboAnalyst 2.0–a comprehensive server for metabolomic data analysis. Nucleic Acids Res. 40 (Web Server issue), W127–33. https://doi.org/10.1093/nar/gks374.
Xintaropoulou, C., Ward, C., Wise, A., Marston, H., Turnbull, A., Langdon, S.P., 2015. A comparative analysis of inhibitors of the glycolysis pathway in breast and ovarian cancer cell line models. Oncotarget 6 (28), 25677–25695. https://doi.org/10.18632/oncotarget.4499.
Xu, S., Yang, Z., Jin, P., Yang, X., Li, X., Wei, X., Wang, Y., Long, S., Zhang, T., Chen, G., Sun, C., Ma, D., Gao, Q., 2018. Metformin suppresses tumor progression by inactivating stromal fibroblasts in ovarian Cancer. Mol. Cancer Ther. 17 (6), 1291–1302. https://doi.org/10.1158/1535-7163.MCT-17-0927.
Yamamoto, T., Ebisuya, M., Ashida, F., Okamoto, K., Yonehara, S., Nishida, E., 2006. Continuous ERK activation downregulates antiproliferative genes throughout G1 phase to allow cell-cycle progression. Curr. Biol. 16 (12), 1171–1182. https://doi.org/10.1016/j.cub.2006.04.044.
Zhang, D., Wang, Y., Dong, L., Huang, Y., Yuan, J., Ben, W., Yang, Y., Ning, N., Lu, M., Guan, Y., 2013. Therapeutic role of EF24 targeting glucose transporter 1-mediated metabolism and metastasis in ovarian cancer cells. Cancer Sci. 104 (12), 1690–1696. https://doi.org/10.1111/cas.12293.
Zhang, X., Tu, S., Wang, Y., Xu, B., Wan, F., 2014. Mechanism of taurine-induced apoptosis in human colon cancer cells. Acta Biochim. Biophys. Sin. (Shanghai) 46 (4), 261–272. https://doi.org/10.1093/abbs/gmu004.
Zhou, C., Zhang, X., Xu, L., Wu, T., Cui, L., Xu, D., 2014. Taurine promotes human mesenchymal stem cells to differentiate into osteoblast through the ERK pathway. Amino Acids 46 (7), 1673–1680. https://doi.org/10.1007/s00726-014-1729-8.
Zordoky, B.N., Bark, D., Soltys, C.L., Sung, M.M., Dyck, J.R., 2014. The anti-proliferative effect of metformin in triple-negative MDA-MB-231 breast cancer cells is highly dependent on glucose concentration: implications for cancer therapy and prevention. Biochim. Biophys. Acta 1840 (6), 1943–1957. https://doi.org/10.1016/j.bbagen.2014.01.023.