KLF5 strengthens drug resistance of ovarian cancer stem-like cells by regulating survivin expression

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Abstract

Objectives: Ovarian cancer stem-like cells (CSCs), which can form non-adherent sphere cells in a stem-cell selection culture system, exhibit stemness and drug resistance to chemotherapeutics, which are properties not observed in differentiated cells. Recent studies have demonstrated that Kruppel-like factor 5 (KLF5) is involved in cell proliferation and mediates cell survival and tumourigenesis. Here, we investigated the role of KLF5 and its downstream target survivin, in strengthening drug resistance of ovarian CSCs.

Materials and methods: Ovarian cancer cell line SKOV3 was cultured under serum-free conditions and differentiating conditions to promote formation of sphere cells and differentiated cells, respectively. siRNA-KLF5 was used to knock down KLF5, and survivin expression vector was used to overexpress survivin. Cells were further analysed by qPCR, immunofluorescence staining and western blotting. Chromatin immunoprecipitation (ChIP) assay and electrophoretic mobility shift assay (EMSA) were performed to investigate the relationship between KLF5 and survivin expression. Drug resistance was examined by MTT and apoptosis assays.

Results: KLF5 was highly expressed in the ovarian cancer cell line SKOV3 sphere cells, accompanied by elevated survivin expression. Silencing KLF5 by small interfering RNA in sphere cells down-regulated survivin expression, which also sensitized the sphere cells to apoptosis induced by chemotherapeutic drugs (cisplatin or paclitaxel). Furthermore,

ChIP assay, survivin overexpression and EMSA results indicated that KLF5 controlled survivin expression by directly binding the survivin promoter in the cells.

Conclusions: The KLF5-mediated signalling pathway is a potential target for elimination of ovarian CSCs.

Introduction

Ovarian cancer is one of the leading causes of death from gynecological malignancies. Optimal cytoreductive surgery followed by systemic chemotherapy with paclitaxel and cisplatin is the current standard therapy for metastatic ovarian cancer upon diagnosis, with reported response rate of over 70%. However, overall 5-year survival has not been significantly improved by the current standard therapeutics (1,2). One of the most important causes of failure of ovarian cancer treatment is development of resistance to paclitaxel- and platinum-based chemotherapy (3). One emerging model for development of drug-resistant tumours involves a pool of self-renewing malignant progenitors known as cancer stem-like cells (CSCs) or cancer-initiating cells (CICs). According to CSC hypothesis, these cells are inherently resistant to chemotherapy due to their stem-cell properties, mainly their quiescence and their expression of drug membrane transporters (for example, ABCG2), two of the mechanisms by which they may survive therapy and regenerate a tumour (4,5). However, the means and pathways underlying this observation remain unclear.

Cancer stem-like cells have been identified in established ovarian cancer cell lines as well as in primary samples from ovarian cancer patients (6,7). We have previously obtained self-renewing and anchorage-independent spheroids by culturing patient-derived ovarian cancer cells or those of the SKOV3 line, maintained under stem cell-selective conditions. Spheroid cells display remarkable stem-cell properties, drug resistance and

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propagation of their original tumour phenotype, exhibiting behaviour expected of CSCs. We suggest that the sphere cell subpopulation may be a more reliable model than differentiated cells grown in the presence of serum (cells adhere to plates and form compact clusters, relatively uniform and cobble-like) for understanding the biology of ovarian cancer. As sphere cells can be frozen, stored and produced in consistently large numbers, they might prove to be a more reliable model system for research into CSCs, for screening new therapeutic agents and ultimately for designing clinical personalized tumour therapy (8,9).

Previously, a range of studies has demonstrated that Kruppel-like factor 5 (KLF5), a member of the Sp/KLF family, is involved in oncogenesis. Sp/KLF family has at least 20 members with highly related zinc finger proteins, important components of eukaryotic cell transcription machinery (10). Individual members of the Sp/KLF family have preferences for binding different DNA sequences of a target gene promoter and early studies have shown that KLF5 is a positive regulator of cell proliferation and mediates cell survival and tumorigenesis (11,12); in addition, its expression is inducible (13,14). In a recent investigation, Zhu *et al.* (15) examined expression of KLF5 in leukaemia bone marrow cells, from children with ALL (acute lymphoblastic leukaemia) as well as in a panel of leukaemia cell lines derived from paediatric ALL, and found that KLF5 was widely expressed in both ALL lines and fresh ALL samples. When analysing survivin promoter activity, they demonstrated that KLF5 promoted survivin expression and that tumour suppressor p53 could downregulate survivin expression by binding to KLF5. It is well known that survivin is a unique member of an inhibitor of apoptosis protein family and is highly expressed in almost all types of human cancer. High levels of survivin expression have been associated with cancer progression, drug resistance, poor prognosis and brief patient survival (16,17).

Although KLF5 is important in regulating cell proliferation, mediating cell survival and promoting tumorigenesis (11,12), its role on cancer stem-cell viability has not been explored. In the present study, ovarian cancer cell line SKOV3 sphere cells were used as potential model of CSCs and in them we examined expression of KLF5 and its downstream target survivin. We found that KLF5 was highly expressed and its expression was consistently associated with high levels of survivin; silencing of KLF5 by small interfering RNA downregulated survivin expression, which also sensitized the sphere cells to apoptosis induced by chemotherapeutic drugs. These findings identify a novel signalling mechanism of drug resistance in CSCs.

Materials and methods

Cell culture

SKOV3 ovarian cancer cell line was obtained from Shanghai Cell Bank of the Chinese Academy of Sciences; poorly differentiated ovarian serous adenocarcinoma cells, these had been derived originally from ascites fluid of a 64-year-old Caucasian female. SKOV3 differentiated cells were maintained as adherent cultures in McCoy's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% foetal bovine serum (FBS). Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO₂. SKOV3 non-adherent sphere cells were cultured on plates coated with poly (2-hydroxyethyl methacrylate) (Sigma, St Louis, MO, USA) and maintained under stem cell conditions in medium consisting of KO-DMEM with 10% KO serum replacement, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 0.1 mM 2-β-mercaptoethanol, 10 ng/ml bFGF, 12 ng/ml hLIF and penicillin (25 U/ml)–streptomycin (925 mg/ml) mixture as previously described (18).

RNA interference

siRNA-KLF5 (AAAGUAUAGACGAGACAGUGC) was used to knock down KLF5 expression in SKOV3 sphere or adherent cells, and scrambled siRNA was used as negative control (15). Cells were transfected with 1.0 nmol chemically synthesized siRNA using Lipofectamine™ Reagent (Invitrogen, Carlsbad, CA, USA) in 10 cm plates. Briefly, cells were seeded at 1×10^6 and cultured overnight. siRNA solution was then mixed with Lipofectamine™ Reagent in Opti-MEM media for 20 min following the manufacturer's protocol, then added to the cells. After 4–6 h incubation, culture medium was replaced and cells were maintained in culture for an additional 24 h.

RNA extraction and real-time PCR analysis

Total RNA was extracted from sphere cells and adherent cells using Trizol. Five hundred nanograms total RNA from each sample were utilized for reverse transcription (RT) using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). Real-time PCR was carried out on cDNA using IQ SYBR Green (Bio-Rad) with Mastercycler EP realplex (Hamburg, Germany). All reactions were performed in a volume of 20 µl; primers for the marker gene are shown in Table 1. Real-time PCR was performed by initial denaturation at 95 °C for 30 s, followed by 40 cycle two step reaction (5 s at 95 °C, 30 s at 60 °C). Specificity was verified by melting curve analysis and agarose gel electrophoresis. Threshold cycle

Table 1. DNA sequences for oligonucleotides used in this work

Oligonucleotides	Sequences (5'→3')
hKLF5-qRT-R	TCCCAGGTACACTGTATGGC
hKLF5-qRT-F	ACCCTGGTTGCACAAAAGTT
hSurvivin-qRT-R	ATCTGGCGGTTAATGGCGCG
hSurvivin-qRT-F	GATTACAGGCGTGAGCCACT
hOct4-qRT-R	ACCCAGCAGCCTCAAAATCCTCTC
hOct4-qRT-F	GGCCCGAAAGAGAAAGCGAACC
hNanog-qRT-R	TCTGCTGGAGGCTGAGGTAT
hNanog-qRT-F	TTCCTTCTCCATGGATCTG
h18s-qRT-R	GAATCGAACCCTGATTCCCCGTC
h18s-qRT-F	CGGCGACGACCCATTGCAAC
hSurvivin-CHIP-F	GATTACAGGCGTGAGCCACT
hSurvivin-CHIP-R	ATCTGGCGGTTAATGGCGCG
hSurvivin-EMSA-oligo	GCGGGGGGTGGACCGCTAA

(Ct) values of each sample were used in post-PCR data analysis. 18s RNA was used as internal control for mRNA level normalization.

Immunofluorescence staining

Sphere cells were cytopun on to glass slides, fixed in ice-cold 4% paraformaldehyde (4 °C, 30 min) and blocked for 30 min in 1% BSA. Adherent cells were directly fixed and blocked as above. An indirect immunofluorescent labelling technique was used to identify KLF5-expressing and survivin-expressing cells using primary antibodies: anti-KLF5 (1:200; Abcam, Cambridge, UK) and anti-survivin (1:300; BioVision, San Francisco, CA, USA) in PBS with 1% BSA (4 °C overnight). Slides were washed three times in PBS for 10 min then incubated in FITC-conjugated chicken anti-rat IgG (Invitrogen), in the dark at room temperature for 30 min. Fluorescent images were captured using a Leica MDI3000 (Wetzlar, HE, Germany) microscope; DAPI (Roche, Indianapolis, IN, USA) was used for nuclear staining, as reference. Positive control cells were stained for each antibody in parallel, and negative controls were included by substituting primary antibodies with PBS.

Western blotting

Total protein was determined in harvested cells using a BCA kit (Pierce, Gaithersburg, MD, USA). Fifty micrograms protein was separated using SDS-polyacrylamide gel, then electrophoresis was performed and protein transferred to PVDF membranes. Membranes were blocked with 5% non-fat dried milk, at room temperature for 2 h. Membranes were incubated with primary antibody against KLF5 (1:500; Abcam), survivin (1:500; BioVision) or β -actin (1:1000; Cell Signaling, Beverly, MA, USA) at 4 °C overnight. After thorough washing, membranes were incubated in peroxidase-linked goat

anti-rabbit-IgG (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) at room temperature for 1 h. Chemiluminescence was performed using the Western lightning ECL kit (Perkin-Elmer Life Science, Norwalk, CT, USA) and ChemImager imaging system (G: BOX SYNGENE; Gene Company Limited, Hong Kong).

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were carried out using antibodies to KLF5 (1:500; Abcam). Normal rabbit IgG (Upstate, Lake Placid, NY, USA) was used as negative control to verify immunoprecipitation specificity. Briefly, cells were fixed in 1% formaldehyde for 10 min at room temperature, sonicated on ice and subjected to immunoprecipitation by incubating cell lysates with antibodies at 4 °C overnight. Then, protein-DNA crosslinks were reversed and DNA was purified and analysed by PCR amplification under the following conditions: 95 °C for 10 min, 32 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, final extension 72 °C for 10 min. Primer sequences used for PCR are shown on Table 1.

Electrophoretic mobility shift assay (EMSA)

Non-radioactive EMSA was performed using Gel Shift Assay System (Promega, Madison, WI, USA) according to the manufacturer's instructions. Five micrograms nuclear extract of SKOV3 sphere cells or adherent cells were incubated with DIG-labelled mutated probes in reaction buffer for 20 min at room temperature. In the competition experiment, prior to mixing labelled probe with nuclear extracts, extracts were incubated with unlabelled oligonucleotides for 15 min at room temperature in the presence of 100-fold molar excess of labelled probe. Gel supershift assay was carried out by adding 2 μ g rabbit polyclonal anti-KLF5 antibody to DNA protein complex, for 30 min at room temperature. All reaction samples were analysed by electrophoresis in 4% non-denatured polyacrylamide gel and exposed to Kodak (Rochester, NY, USA) MS X-ray films at -80 °C. Oligonucleotides (sequences are shown in Table 1) were designed according to cloned survivin promoter.

Survivin expression vector

With reverse transcription product cDNA used as PCR template, CDS sequence of survivin was amplified and inserted into the expressing vector pCDH-CMV-MCS-EF1-copGFP through *Xba*I and *Bam*HI. Sequences of primers used are as follows: cds-survivin F. GCT CTA GAC GCC ATT AAC CGC CAG ATT T; cds-survivin R. CGG GAT CCA CCT CTG GTG CCA CTT TCA A.

Cell growth and drug resistance assessment

Sphere cells and adherent cells were seeded at 3×10^3 cells/well, in 96-well microtitre plates after being transfected with siRNA-KLF5, siRNA-mock or negative control (untransfected). Numbers of viable cells at 1, 2, 3, 4, 5 and 6 days were counted. For assessment of extent of drug resistance, total of 1×10^4 sphere cells and adherent cells (untransfected, transfected with siRNA-KLF5 or siRNA-mock) were plated in 96-well microtitre plates in culture medium at various concentrations (0, 12, 24 and 48 μM) of cisplatin or paclitaxel (Sigma-Aldrich) for 48 h. Cultures were set up in quintuplicate. Both cell population growth and survival were monitored by MTT assay and optical density (OD) reading at 490 nm. Growth curves were plotted for sphere and adherent cells exposed to different treatments. Percentage survival was determined as follows:

$$\left(\frac{\text{OD}_{490\text{sample}} - \text{OD}_{490\text{blank control}}}{\text{OD}_{490\text{control}} - \text{OD}_{490\text{blank control}}} \right) \times 100\%$$

Apoptosis assay

Apoptosis was quantified by annexin V-FITC and PI-double staining with the aid of a staining kit (Mbschem, Shanghai, China); phosphatidylserine was determined by flow cytometry. Briefly, cells were washed twice in PBS, and 400 μl binding buffer and 5 μl of annexin V-FITC were added. Following gentle mixing by vortex, the mixture was left in the cold (2–8 °C) in the dark for 15 min, then 10 μl PI was added. Following further gentle vortexing, samples were analysed using FC500 flow cytometry within a 1-h period.

Statistical analysis

Experimental data are expressed as mean $\pm$ SD and significance of differences was assessed with the Student's *t*-test. *P* values <0.05 were considered significant.

Results

KLF5 was highly expressed in SKOV3 sphere cells, its expression correlated with survivin expression

Recent studies have suggested that ovarian cancer is characterized by a pool of both differentiated cells (the majority), and a small population of cells expressing stem-cell surface markers, such as Oct-4, Nanog, CD113, or CD44, responsible for tumour initiation and maintenance (6,19). Previously, we have reported that non-adherent spheres

cells isolated from primary ovarian tumour samples, or SKOV3 cell line, display phenotypic/genotypic properties distinct from differentiated cells, as revealed by cDNA microarray results (8,9). Increasing evidence indicates that KLF5 seems to be important in cell survival and tumourigenesis. Thus, we wondered whether KLF5 would be involved in maintaining the stem-like property of ovarian CSCs. For the purpose of this study, we propagated ovarian cancer cell line SKOV3 sphere cells in serum-free conditions (Fig. 1a) and SKOV3-adherent cells in differentiating conditions (Fig. 1b), then examined expression of KLF5 mRNA and protein by real-time PCR and immunofluorescence. As we previously reported, stem cell-related genes *Oct-4* and *Nanog* were overexpressed in sphere cells. Unexpectedly, compared to differentiated cells, KLF5 mRNA was highly expressed in SKOV3 sphere cells; interestingly, expression of KLF5 there correlated with expression of survivin. Differentiated cells lacking KLF5 expression also expressed a lower level of survivin (Fig. 1c,d).

KLF5 directly regulated survivin gene expression by binding the survivin promoter in SKOV3 sphere cells

To further confirm association between KLF5 and survivin expression in sphere cells, we knocked down KLF5 expression in sphere cells and differentiated cells by siRNA and then determined expression of survivin. As shown in Figs 1c and 2a, transfection of siRNA-KLF5 into sphere cells downregulated KLF5 mRNA and protein expression, whereas siRNA-mock did not alter KLF5 expression pattern. Thus, siRNA specific to KLF5 could knock down KLF5 efficiently in sphere cells, and following KLF5 interference, expression of survivin was significantly reduced in sphere cells; mRNA expression levels of Oct4 and Nanog were also reduced in sphere cells (Figs 1,2a). However, both KLF5 and survivin expression levels were less in differentiated cells either before or after RNAi than in sphere cells (Fig. 1c,d), and protein expression from the genes could not be detected in differentiated cells by western blotting (Fig. 2a).

As it has been reported that KLF5 is recruited to the survivin promoter to mediate transcriptional activation in leukaemia bone marrow cells (15), we used ChIP assay to examine whether this reported function for KLF5 also applied to ovarian CSCs. In Fig. 2b, DNA segments corresponding to the survivin promoter region were amplified by PCR with extracted DNA samples as templates, before and after immunoprecipitation. As input control, genome DNA of sphere cells was used as template to amplify the survivin promoter region. After immunoprecipitation against KLF5, the survivin promoter region could be detected in KLF5-associated

Figure 1. SKOV3 cells exhibit distinct characteristics under different culture conditions. (a) SKOV3 cell form non-adherent, non-symmetric spheres under stem-cell culture conditions. (b) SKOV3 cells culture under differentiating conditions. (c) As shown by real-time PCR, KLF5 mRNA is overexpressed in SKOV3 sphere cells and can be knocked down by siRNA-KLF5. The mRNA levels of survivin, Oct4 and Nanog in SKOV3 sphere cells were also knocked down by siRNA-KLF5. 18S RNA was used as internal control. (d) Immunostaining of KLF5 and survivin in SKOV3 sphere cells and differentiated cells after siRNA-KLF5 transfection (untransfected and siRNA-Mock transfection as control). Nuclei were stained by DAPI. Original magnification, $\times 100$.

DNA by PCR in control sphere cells (including untransfection and siRNA-mock transfection). However, after KLF5 interference, amplification of the DNA segment corresponding to survivin promoter decreased. Amplification was lower in differentiated cells than in sphere cells. These results indicate that KLF5 regulated expression of survivin by binding to the survivin promoter in sphere cells.

We then performed EMSA to determine whether nuclear proteins, including transcription factor KLF5, would bind to the survivin promoter, as observed in the immunoprecipitation study. As shown in Fig. 2c, incubation of nuclear extracts from sphere cells (untransfection or siRNA-mock transfection), which contained KLF5 protein, resulted in a significant band shift of the survivin probe. After KLF5 interference, the band shift was not observed in sphere cells. In addition, differenti-

ated cells showed no positive binding with the nuclear extract. These results indicate that KLF5 could induce survivin promoter activity by directly binding to the promoter. To determine whether the nuclear extract–probe complexes contained KLF5 protein, we performed antibody supershift assay by treating the complexes with KLF5 antibody before gel electrophoresis. As shown in Fig. 2c, KLF5 antibody caused significant supershift compared to the sample without antibody. This finding further indicates that survivin is downstream of KLF5-mediated signalling in ovarian CSCs.

siRNA-KLF5 sensitized both sphere cells and differentiated cells to chemotherapeutic drugs

Our previous studies have reported that SKOV3 sphere cells exhibited higher resistance to chemotherapeutic

Figure 2. KLF5 directly regulates survivin expression by binding the survivin promoter in SKOV3 sphere cells. (a) KLF5 and survivin expression in SKOV3 sphere cells and differentiated cells before and after KLF5 knock down were examined using Western blot assay with β -actin as loading control. (b) Antibodies against KLF5 were used to immunoprecipitate KLF5-associated DNA. The promoter region of the survivin gene was amplified by PCR with specific primers. The PCR template of 'Input' was the genomic DNA of SKOV3 sphere cells extracted before immunoprecipitation. Normal rabbit IgG (IgG) was used as non-specific control. (c) EMSA was conducted using nuclear extracts from both SKOV3 sphere cells and differentiated cells. The presence and absence of protein extract, KLF5 antibody and the probe are shown by '+' and 'space', respectively. Band shifts are indicated at the right panel.

drugs than differentiated cells (8). High level of survivin expression confers resistance to apoptosis induced by chemotherapeutic drug doxorubicin in an acute lymphoblastic leukaemia cell line and thus might contribute to drug resistance (20). Here, we first examined effects of downregulation of KLF5 by siRNA-KLF5, on cell population growth. As shown in Fig. 3a, SKOV3 sphere cells proliferated faster than differentiated cells ($P < 0.05$). However, in either sphere cells or differentiated cells, transfection of siRNA-KLF5 did not inhibit cell population growth similar to controls (untransfection or transfection of siRNA-mock) ($P > 0.05$). From a morphological point of view, there was no difference in SKOV3 sphere formation after siRNA-KLF5 or siRNA-mock transfection, similar as observed in differentiated cells. However, we did notice that suspension spheres after KLF5 interference seemed to be relatively loose compared to spheres untransfected or transfected with siRNA-mock (Fig. 3b).

Furthermore, we examined effects of downregulation of KLF5 and survivin on sensitivity of sphere cells to cisplatin and paclitaxel, drugs commonly used to treat ovarian cancer patients. As shown in Fig. 4a and 4b, transfection of siRNA-KLF5 sensitized both sphere cells and differentiated cells to these chemotherapeutic drugs. Cell survival rates after 48 h cisplatin/paclitaxel treat-

ment was significantly less in siRNA-KLF5-expressing cells than in siRNA-mock-expressing cells, and survival varied in a cisplatin/paclitaxel concentration-dependent manner ($P < 0.05$). Consistent with these observations, 48 h post-treatment, flow cytometry revealed that sphere cells treated with cisplatin/paclitaxel exhibited higher percentages of annexin-V-positive cells when transfected with siRNA-KLF5 (from ~10 to 68%/~8 to 70%) than with siRNA-mock (from ~6 to 30%/~5 to 32%) (Fig. 4c,d).

We did not observe changes in mRNA and protein levels of KLF5 and survivin in SKOV3 differentiated cells (Figs 1c,2a), however, siRNA-KLF5 transfection sensitized differentiated cells to chemotherapeutic drugs (Fig. 4a,b,e,f). We then examined whether siRNA-KLF5 transfection could alter expression levels of KLF5 and survivin, which may be induced by chemotherapeutic drugs. Differentiated cells with or without siRNA-KLF5 transfection were cultured in 24 μ M cisplatin or paclitaxel for 48 h. Expression levels of KLF5 and survivin were analysed by qRT-PCR. As shown in Fig. 4g, expression levels of KLF5 and survivin in differentiated cells without siRNA-transfection were higher after chemotherapeutic drug treatment. SiRNA-KLF5 transfection reduced drug-induced increase in expression levels of KLF and survivin (Fig. 4h).

Figure 3. The effect of siRNA-KLF5 on cell growth and sphere formation. (a) The SKOV3 sphere and differentiated cells were transfected with siRNA-KLF5 and siRNA-Mock separately and then cultured in 96-well plates. The growth curves were analysed by MTT assay. (b) There is no difference in SKOV3 sphere formation after siRNA-KLF5 or siRNA-Mock transfection. Original magnification, $\times 100$.

Overexpression of survivin rescued siRNA-KLF5 phenotype in SKOV3 sphere cells

To further examine whether KLF5 functions through survivin, a survivin expression vector was transfected into sphere cells containing KLF5 siRNA. Both survivin and KLF5 expression were detected by qRT-PCR after 48-h transfection. Along with increase in survivin expression, expression of KLF5 did not change significantly (Fig. 5b). MTT analysis also revealed that upregulated survivin expression could promote drug resistance. Indeed, cells which had been sensitized to drugs by KLF5 RNA interference exhibited drug resistance after being transfected with survivin expression vector (Fig. 5c). Thus, KLF5 might mediate survivin expression, and this might account for drug resistance of ovarian CSCs.

Discussion

Increasing evidence suggests that CSCs are responsible for maintenance and growth of tumours (21,22). Although conventional treatments (surgery or chemotherapy) may cause tumours to shrink temporarily, small populations of CSCs may escape current regimens and

seed new tumours. Previous reports have demonstrated that CSCs can be enriched as sphere-like cell aggregates in serum-free medium *in vitro* (23,24). We have previously reported that SKOV3 sphere cells exhibit characteristics distinct from those of differentiated cells, and 3487 genes had more than 2-fold difference in expression level as revealed by cDNA microarray analysis (8). Among these, KLF5 mRNA expression was higher in sphere cells than in differentiated cells, prompting us to examine the potential of KLF5 as a therapeutic target.

Several results indicate that transcription factors belonging to the Kruppel-like family could have important roles in regulation of stem cells. Recently a combination of four transcription factors including KLF4, Oct4, Sox2 and c-Myc was shown to reverse differentiated cells into a pluripotent state (25–27). For maintenance of self-renewal and pluripotency of embryonic stem cells (ESCs), KLF4 was found to be dispensable, but KLF5 and KLF2 appeared to be required and were likely to regulate key pluripotency genes (28). Parisi *et al.* reported that KLF5 is expressed in mouse ESCs. KLF5 knockdown by RNA interference alters the molecular phenotype of ESCs and prevents their correct differentiation (29). These studies suggest that KLF5, a

Figure 4. The effect of siRNA-KLF5 on drug resistance and apoptosis induced by Cisplatin and Paclitaxel. (a, b) The SKOV3 cells in different culture systems were transfected with siRNA-KLF5 and treated with different concentrations of drugs as indicated. Cells were incubated for 48 h and the survival rate was determined by MTT assay. (c–f) The apoptosis was induced by cisplatin or paclitaxel in combination with either siRNA-KLF5 or siRNA-Mock transfection in SKOV3 sphere cells (c, d) and SKOV3-differentiated cells (e, f). The apoptotic cells were detected by annexin-V staining. Data are presented as the mean percentage of annexin-V-positive cells from three independent experiments. (g) SKOV3-differentiated cells were treated with cisplatin or paclitaxel. The expression levels of KLF5 and survivin were higher after drug treatment. (h) SKOV3-differentiated cells transfected with siRNA were treated with cisplatin or paclitaxel. SiRNA-KLF5 transfection inhibited the inducible expression of KLF and survivin (mean $\pm$ SD, * P < 0.05).

Figure 5. Overexpression of survivin increased drug resistance and resisted siRNA-KLF5 function. (a) The CDS sequence of survivin was amplified and inserted into the expressing vector pCDH-CMV-MCSEF1-copGFP through *XbaI* and *BamHI*. (b) Cells transfected with survivin expression vector were analysed by qRT-PCR for KLF5 and survivin expression. (c) RNA interference cells were transfected with survivin expression vector and drug resistance was analysed by MTT. SiRNA-KLF5 transfected cells recovered drug resistance by overexpressing of survivin.

zinc-finger transcription factor of the Kruppel-like family, plays a pro-proliferative role in stem/progenitor cells and could have an important role in regulation of stem cell self-renewal.

In this context, we examined the role of KLF5 in stem cell properties of ovarian CSCs. Here, we show that SKOV3 sphere cells expressed elevated levels of KLF5 and survivin compared to differentiated cells. After KLF5 RNA interference, variable downregulation of KLF5 and survivin occurred in sphere cells. However, transfection of siRNA-KLF5 did not inhibit cell population growth of sphere cells. We considered sphere cells to be a reliable model for enrichment of CSCs, but do not consider sphere cells to be equal to cancer stem cells. On the other hand, stem-cell markers, Oct-4 or Nanog, were also downregulated in sphere cells after siRNA-KLF5 transfection. CSCs may promote their lifespan from a dormant state to division stages, as downregulation of the stem cell property. KLF5 RNA interference did not inhibit cell population growth significantly. Previous studies have shown that KLF5 is required for formation of the trophectoderm and the

inner cell mass (ICM), and for repressing primitive endoderm development. KLF5(−/−) embryos fail to form an ICM/pluripotent colony and have very few Oct-4(+) cells (30). Recently, Jang reported that O-linked-N-acetylglucosamine (O-GlcNAc) modification in Oct-4 regulates Oct-4 transcriptional activity and is important for inducing many pluripotency-related genes, including *KLF5* in ESCs (31). Thus, KLF5 might play a specific role in maintenance of the pluripotent state. However, whether and how KLF5 is involved in maintaining stem cell characteristics of cancer stem cells needs to be further elucidated.

To test interaction of KLF5 with the survivin gene promoter, we employed ChIP assays, EMSA, survivin overexpression and KLF5 RNA interference, in sphere cells. As previously reported (15), we found that KLF5 directly regulated survivin gene expression through promoter-binding in SKOV3 sphere cells. Overexpression of survivin could increase drug resistance and resist siRNA-KLF5 function. These results indicate that survivin, a survival factor which confers resistance to apoptosis, is under the control of KLF5 in ovarian CSCs. Zhu

et al. found that KLF5 induced expression of survivin in acute lymphoblastic leukaemia. Indeed, downregulation of KLF5 by siRNA reduced expression of survivin. Mechanistically, KLF5 binds to p53 and abrogates p53-regulated repression of survivin (15). We were unable to detect p53 expression in either sphere or differentiated ovarian cancer cells (data not shown), and thus the interaction between KLF5 and p53 was not further explored.

Targeting CSCs therapeutically is likely to be challenging, as both bulk tumour cells and CSCs need to be eliminated, thereby requiring complex combination drug therapies. Since CSCs are molecularly distinct from bulk tumour cells, cells with different molecular characteristics within the same tumour may respond differently to anti-cancer therapeutics with some developing drug resistance (32). In our study, we did not observe any change in KLF5, survivin, Oct4 or Nanog expression levels in differentiated cells before or after KLF5 RNA interference. However, we did observe that KLF5 RNA interference could inhibit expression of KLF5 and survivin induced by drug treatment, in differentiated cells. These results indicate that KLF5 could target both the CSCs and the bulk tumour population effectively to attain the best patient response. Recent reports have indicated that emergence of CSCs occurs in part as a result of epithelial to mesenchymal transition (EMT) possibly *via* cues from tumour stromal components. EMT of tumour cells not only causes increased metastasis, but also contributes to drug resistance (33,34). EMT is characterized by increased expression of mesenchymal markers (vimentin, thrombospondin, N-cadherin, vitronectin), increased expression of extracellular matrix compounds (collagen IV and fibronectin), reduced expression of epithelial markers (E-cadherin, Occludin, Desmoplakin, and Mucin1), altered location of transcription factors (β -catenin, Snail, Slug, Twist, Sox 10, and NF κ B) and activation of kinases (ERK1, ERK2 and PI3K/AKT) (35). Witta *et al.* have reported that elevated E-cadherin expression is associated with sensitivity to EGFR kinase inhibitors, with drug-resistant cells becoming more mesenchymal-like (36). Some evidence has shown that epithelial-like cancers are initially more sensitive to targeted therapies, such as EGFR and HER2 antagonists, whereas mesenchymal cancers are more sensitive to DNA damaging agents such as doxorubicin (37).

In the present study, we found that KLF5-regulated survivin expression may play a role in regulating cell death induced by chemotherapeutic drugs. Interference of KLF5 by siRNA in both sphere and differentiated cells sensitized them to cisplatin or paclitaxel. Recently, Strauss *et al.* identified a subset of epithelial–mesenchymal hybrid (E/M) cells that express stem-cell markers in primary ovarian cancers. This subset of cells displays

features associated with cancer stem cells and can give rise to epithelial ovarian cancer cells. Trans-differentiation of E/M cells into mesenchymal or epithelial cells is associated with loss of stem-cell markers and tumourigenicity (38). These data suggest that pluripotency of CSC is intrinsically linked to occurrence of EMT and MET in ovarian cancer cells. We have found that EMT occurs when SKOV3 sphere cells differentiate (data not shown), thus we suggest that KLF5 expression might act as a positive regulator of ovarian CSCs and that KLF5-regulated survivin expression might contribute to drug resistance of ovarian cancer stem cells. However, whether KLF5 expression is associated with drug resistance due to EMT, needs to be further elucidated.

In conclusion, our results identify a novel mechanism of drug resistance in CSCs enriched from suspension cultivation and that KLF5-mediated signalling is a potential molecular target for reducing incidence of CSC-mediated resistance to cancer therapeutics.

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Competing interests

The authors declare that they have no financial or non-financial competing interests.

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