Suppression of OCT4B Enhances Sensitivity of Lung Adenocarcinoma A549 Cells to Cisplatin *via* Increased Apoptosis

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Abstract. *Background: Resistance to chemotherapy in lung adenocarcinoma remains a major obstacle. We examined the potential role of Octamer-binding transcription factor-4B (OCT4B) in enhancing sensitivity of lung adenocarcinoma cells to cisplatin. Materials and Methods: RNAi interference was used to examine the role of OCT4B in cisplatin-treated A549 cells. Cells were transfected with OCT4B siRNA prior to a 48-h cisplatin treatment. Propidium iodide (PI) and caspase-3 staining were used to determine cell viability and apoptosis. Cell-cycle analysis was performed to evaluate alterations in phase distribution. Results: OCT4B suppression in cells increased the number of non-viable, PI^+, and apoptotic, caspase-3^+ cells in the presence and absence of cisplatin treatment. Importantly, cisplatin treatment of OCT4B-suppressed cells resulted in a marked transition of cells from G_0/G_1 to G_2/M phase. Conclusion: Silencing of OCT4B confers sensitivity to cisplatin treatment in A549 cells via cell-cycle regulation, increased proliferation and enhancement of cisplatin-induced apoptosis. OCT4B clearly protects A549 cells from apoptosis.*

Lung cancer is the leading cause of cancer mortality in the world. Lung adenocarcinoma (LAC), categorized as a non-small lung cancer (NSCLC), constitutes approximately 40% of this histological type. It is associated with increasing incidence with fewer than 15% of patients surviving five years (1). *Cis*-diammine-dichloroplatinum(II) (cisplatin)-based chemotherapy treatment is a main component of the standard therapy for LAC. The efficacy of this treatment, however, is insufficient as a result of inherent drug-resistance of LAC cells to chemotherapeutic agents (2, 3). It is, therefore, imperative to identify molecular targets responsible for chemoresistance as well as to develop new methods to enhance the sensitivity of LAC cells to chemotherapeutic agents.

The transcription factor OCT4 (OCT4; POU5F1; also known as OCT3 and OCT3/4) primarily functions to maintain pluripotency and self-renewal of embryonic stem cells. By alternative splicing, the human *OCT4* gene can generate OCT4A (variant 1 NM_002701), OCT4B (variant 2, NM_203289) and OCT4B1 (variant 3, GenBank EU518650) (4-7). OCT4A has been proposed to have an essential role in the tumorigenesis of solid tumours such as those of bladder, stomach, prostate and lung (8, 9). We showed that both *OCT4A* and *OCT4B* are up-regulated in lung adenocarcinoma (10). Moreover, we have recently demonstrated an anti-apoptotic role for the OCT4B1 isoform in gastric adenocarcinoma (11). In spite of increasing reports on the characterization of OCT4B isoform and its functions, little is known about its role in cancer. Hence, further assessment of the biological behaviour of OCT4B may reveal a potential role in LAC.

Classically, the OCT4B isoform is localized in the cytoplasm and is neither a stemness factor nor a transcriptional activator (5, 12). A single *OCT4B* mRNA can generate at least three protein isoforms; OCT4B-164, OCT4B-190, and OCT4B-265, through alternative translation (13). It has been demonstrated that OCT4B-190 is up-regulated under stress conditions, and may provide cells with protection against apoptosis (14). Increased OCT4B-265 expression has also been recorded in stem cells under genotoxic stress and may be implicated in stress

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response *via* the p53 pathway (15). Furthermore, overexpression of OCT4B-190 in HeLa cells increased resistance to apoptosis induced by heat shock (14). Taken together, it is apparent that OCT4B has a likely role in cell protection during stress or other forms of cellular insult.

In the present study, we aimed to examine the protective role of OCT4B, in particular in response to cisplatin, a genotoxic stress agent, using RNA interference. We hypothesized that silencing *OCT4B* in the A549 cell line may reveal essential functions in apoptosis and the cell cycle, perhaps inducing cellular events leading to sensitivity to standard chemotherapy for LAC.

Materials and Methods

Cell lines and culture. The NCI-A549 non-small cell lung cancer (NSCLC) cell line (A549) (LGC Promochem, Sarl, France) and NTERA embryonic carcinoma cell line (clone D1; European Collection of Cell Cultures, UK) were cultured in RPMI (Invitrogen, Basel, Switzerland) medium supplemented with 10% fetal bovine serum, (FBS; PAA, Austria) and 2% antibiotic/antimycotic (Invitrogen) solution. The human fibroblast cell line (CCD-16LU, hFB16Lu) (ATCC; www.atcc.org) was maintained in MEMα with 10% FBS and 1% antibiotic/antimycotic. Human lung mesenchymal stromal cells (hLMSC) were harvested as previously described (16) and maintained in MCDB-201 supplemented with insulin- transferrin-selenium, epidermal growth factor (Invitrogen) and 1% FBS.

Transfection and gene silencing. For suppression of *OCT4B*, the following siRNA was designed by the siRNA Selection Program (Whitehead Institute for Biomedical Resarch; http://jura.wi.mit.edu/) and synthesized by Applied Biosystems (Applied Biosystems, Rotkreuz, Switzerland). The sequences of the siRNAs were as follows: target sequence: AAG ATG CCT TGA GCT CCC TCT, sense: (GAU GCU UUG AGC UCC CUC U)dT dT, antisense: (AGA GGG AGC UCA AAG CAU C)dT dT.

Twenty-four hours prior to siRNA transfection, 1×10^5 cells per well (30-50% confluency at time of transfection) were cultured on six-well plates in growth media without antibiotics. Cells were transfected using the LipofectamineTM RNAiMAX Transfection Reagent (Invitrogen). Briefly, 5 μl of siRNA (20 μM) solution and 4.5 μl RNAi-MAX reagent were diluted in 250 μl Opti-MEM (Invitrogen) and incubated for 15 min at room temperature. The mixture was then added to the cells in a final volume of 2.5 ml per well. Cells were further incubated for 72 h at 37˚C in an incubator with 5% CO_2.

Drug sensitivity assays. For the determination of the half-maximal inhibitory concentration (IC_{50}), a dilution series of two-fold increments of cisplatin (0-100 μM; Bristol Myers Squibb, Basel, Switzerland) was prepared to test the drug sensitivity of A549 cells. Cells at 5×10^3 cells/100 μl/well in 96-well plates were incubated in medium with or without the addition of cisplatin. Following a 48-h incubation period, the media were aspirated and replenished with 2,3-*bis*-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide (XTT) cell proliferation assay (Roche Chemicals, Basel, Switzerland) reagents. After a 30-min incubation at 37˚C, formazan production was measured spectrophotometrically at 450 nm. Three independent experiments in triplicate were performed independently.

For cisplatin treatments, cells were cultured in six-well plate culture dishes 24 h prior to treatment (approximately 80% confluency) after which they were treated with the genotoxic drug cisplatin at a concentration of 15 μM in RPMI containing 10% FBS and 1% antibiotic/antimytotic solution. The specified concentration corresponds to the previously determined IC_{50} value for the A549 cell line. Following the 48-h treatment at 37˚C, media were replenished with growth media in the absence of cisplatin, and cells were allowed to recover for an additional 24 h.

Cell viability, apoptosis and cell-cycle analysis. To analyze the cell viability, cells were harvested by trypsinization, washed once with PBS and resuspended in 100 μl propidium iodide (PI) solution (1 μg/μl) for 15 min on ice. Cells were then washed twice with PBS and resuspended in 300 μl of fluorescence activated cell sorting (FACS) stain buffer prior to analysis using LSR II flow cytometer (Becton Dickinson, Basel, Switzerland).

To measure apoptosis, cells were washed, harvested by trypsinization and stained for activated caspase-3 using the Casp Glow Fluorescein Activity Caspase-3 staining kit (Lubio Science, Lucerne, Switzerland). Briefly, cells were harvested by trypsinization, adjusted to 2.5×10^5 cells in 300 μl of staining buffer and incubated with 1 μl of fluorescein isothiocyanate caspase-3 inhibitor fluoromethylketone (FITC-DEVD-FMK) for 45 min at 37˚C in an incubator. Cells were then washed twice and resuspended in FACS staining buffer for flow cytometric analysis.

To analyze the cell-cycle distribution, cells were harvested by trypsinization, adjusted to 1×10^6/ml and washed with cold PBS. Cells were then fixed in 70% ice-cold ethanol overnight at 4˚C, then washed once with PBS before the addition of 400 μl of PI (50 μg; Sigma) and RNase (40 μg; Invitrogen) solution. After a 30 to 60-min incubation on ice, cells were immediately analysed using an LSR II flow cytometer. All data analyses were performed using Flow Jo software (Treestar, Olten, Switzerland).

RNA extraction and real-time quantitative reverse transcription polymerase chain reaction (RT-PCR). Cell cultures were collected in RNA Protect^R Cell Reagent (Qiagen, Hombrechtikon, Switzerland) followed by total RNA extraction (RNeasy Kit; Qiagen) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using the High-capacity cDNA reverse transcription kit (Applied Biosystems, Rotkreuz, Switzerland) as per the manufacturer's protocol. The mRNA transcript levels of the housekeeping gene *β2*-microglobulin, *B2M*, and target gene *OCT4B*, were evaluated with commercially available TaqMan Assay on Demand primer/probes (Hs_99999903_m1, *B2M*; *OCT4B – Hs00742896.S1 pouF1, OCT4B*) (Applied Biosystems). Twenty-five nanograms of resulting cDNAs were subjected to quantitative RT-PCR, in a 10 μl final reaction volume and analyzed in triplicate. Gene expression was detected using the ABI 7500 Fast sequence detection system. All target gene *C*t values in each parameter were normalized by those of the reference gene, *B2M*, *C*t value to determine the ΔCt value (target gene *C*t-reference gene *C*t). Baseline and threshold for Ct calculation were set automatically with the ABI Prism SDS 2.1 software. The quantitative RT-PCR data represent the relative quantity of the target gene mRNA (target gene mRNA/*B2M* mRNA ratio) in comparison to that of human embryonic carcinoma cell line, NTERA, used as the calibrator and with expression set at 1.

Preparation of protein samples. Cells (in T75 flasks) were rinsed once with ice-cold PBS, and scraped off in 3 ml of ice-cold PBS containing complete protease inhibitor cocktail (Roche Diagnostics GmBH, Germany). Cells were centrifuged at 500× *g* for 5 minute at 4˚C. The resulting pellet was resuspended in a suitable amount of lysis buffer (20 mM HEPES, 0.12 M NaCl, 0.2 M EDTA, 1% Triton X-100) containing protease inhibitor cocktail and transferred into an Eppendorf tube before subjecting to ultrasound homogenization for 2×16 sec at 16 cycles (10×) each on ice. Resulting homogenates were centrifuged at 13000 ×g for 15 minute to remove the cell debris. Protein concentrations were determined by Micro BCA™ protein assay reagent kit (Pierce Biotechnology, Bonn, Germany) according to the manufacturer's instructions.

Immunoblotting. Forty micrograms of protein samples were loaded onto a mini 10% pre-cast gel (Bio-Rad, Munich, Germany) and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) under reduced conditions. Separated proteins were blotted onto nitrocellulose membranes (GE Healthcare, Dassel, Germany). After blocking with 2% ECL advanced blocking reagent (GE Healthcare) in TBS-T (10 mM Tris base, 250 mM NaCl, 0.1% tween 20) for one hour at room temperature. Blots were then incubated overnight at 4˚C with anti-OCT 3/4 (R&D Systems, MAB1759) or anti-GAPDH clone 2D4A7 used as loading control. Anti-rat IgG, horseradish peroxidase (HRP)-conjugated antibody (Cell Signalling, Frankfurt, Germany) or anti-mouse IgG (Santa Cruz, Biotech, CA, USA) was used as secondary antibody with one hour of incubation at 37˚C. For chemiluminescence detection of OCT3/4 and GAPDH immunoreactive bands, blots were treated with Amersham ECL advance™ Western blotting detection reagents (GE Healthcare) according to the manufacturer's instructions. Images were acquired using Versa Doc Imaging Systems (Bio-Rad).

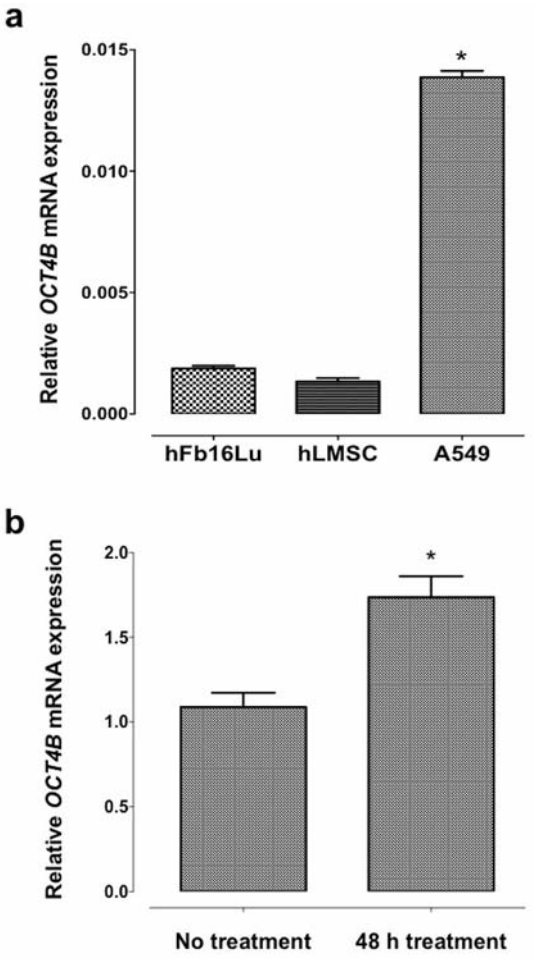

Figure 1. *Comparative mRNA expression of octamer binding transcription factor-4B (OCT4B). a: Adenocarcinoma cell line A549 exhibits greater OCT4B expression (*p=0.009) compared to non-malignant lung cells, human normal lung fibroblasts (hFB16Lu) and human lung mesenchymal stem cells (hLMSC). b: Significant amplification (*p=0.03) of OCT4B expression after a 48-h cisplatin treatment of parental A549 cells (A549^{Parental}). Values represent the mean±SD of three independent experiments using the embryonic carcinoma cell line, NTERA-2, as a reference control.*

Results

Expression of OCT4B in the lung adenocarcinoma cell line, A549. We previously reported an increase of *OCT4B* mRNA expression in LAC tumour tissues compared to their normal tissue counterparts (10). To verify this expression pattern, we compared the OCT4B amplification signals using an isoform-specific primer/probe in A549, a LAC cell line, human normal lung fibroblasts (hFB16Lu), and human lung mesenchymal stem cells (hLMSC) (Figure 1a). We measured a significantly higher level of *OCT4B* in A549 (p=0.0009) compared to hFB16Lu and hLMSC cells.

To determine whether *OCT4B* has a potential role in sensitivity to cisplatin, A549 cells were treated with 15 μM cisplatin (previously obtained IC_{50}) for 48 h followed by evaluation of mRNA expression by quantitative RT-PCR. We found significantly higher (p=0.03) *OCT4B* expression in the treated A549 cells relative to non-treated cells (Figure 1b) suggesting a potential pro-survival/anti-apoptotic role of OCT4B in A549 cells.

Suppression of OCT4B by Lipofectamine™-mediated RNAi in A549 cells. To further investigate the involvement of *OCT4B* in cisplatin-sensitivity, we employed RNA interference, to suppress the expression of this gene in lung adenocarcinoma A549 cells [adapted from our previous studies investigating *OCT4B1* (11)]. Messenger RNA and protein lysates were extracted from A549 cells transfected with scrambled siRNA and A549 cells transfected with *OCT4B* siRNA for verification of changes in *OCT4B* expression by quantitative RT-PCR and western blotting. As shown in Figure 2a, *OCT4B* siRNA transfection significantly reduced *OCT4B* mRNA expression levels in comparison to scrambled siRNA transfection, used as a negative control (20.3±6.8% *versus* 81.7±5.5%). We obtained approximately 75% silencing of *OCT4B* relative to the A549 cells with

scrambled siRNA. Similarly, at the protein level, silencing of *OCT4B* was depicted by a faint band at 30-34 kDa compared to that of the siRNA scrambled-treated cells (Figure 2b). These observations confirmed the suppression of *OCT4B* in A549 at the gene and protein levels.

Suppression of OCT4B increases apoptosis of A549 cells. To assess the effect of *OCT4B* suppression on A549 sensitivity to cisplatin and cisplatin-induced apoptosis, we determined the IC_{50} of $A549^{Scrambled}$ and $A549^{OCT4B-}$ cells. In comparison to the $A549^{Scrambled}$ cells, $A549^{OCT4B-}$ cells showed increased sensitivity to cisplatin with a lower IC_{50} ($A549^{Scrambled}$, 15 μM±0.84 *versus* $A549^{OCT4B-}$, 6.5 μM±1.5; p=0.02) (Figure 3a). Additionally, we performed both flow cytometry-based PI and caspase-3 assays to identify the presence of necrotic and apoptotic cells. Our data revealed a significant increase of PI^+ cells (7.3±2.2% *vs*. 16.3±2.2%, p=0.0002) (Figure 3b) and likewise, an enhancement of activated caspase-3^+ cells (5.4±2.2% *vs*. 16.8±4.0%, p=0.04) in $A549^{OCT4B-}$ compared to $A549^{Scrambled}$ control cells (Figure 3c). Apoptosis results were confirmed through measurement of the sub-G_1 DNA content, as well as DNA condensation with Hoechst 33258 staining (data not shown). Our results demonstrated that silencing of *OCT4B* in A549 cells increases the percentage of non-viable and apoptotic cells, clearly indicating an anti-apoptotic role of *OCT4B* in A549 cells.

Suppression of OCT4B enhanced cisplatin-mediated apoptosis of A549 cells. To evaluate whether OCT4B has an anti-apoptotic role under conditions inducing cellular stress, we treated parental A549, $A549^{Scrambled}$ and $A549^{OCT4B-}$ with 15 μM cisplatin for 48 h. We also evaluated the non-treated parental A549 cells as an additional control. As expected, cisplatin treatment at this concentration resulted in a significant increase in the number of non-viable and apoptotic cells (Figure 4) in parental A549 cells. Suppression of *OCT4B* in A549 cells resulted in an even stronger decrease in cell viability represented by the higher number of PI^+ cells (Figure 4a) compared to $A549^{Scrambled}$ cells (p=0.01). Moreover, the number of cells with activated caspase-3 (Figure 4b) was markedly enhanced in comparison to the $A549^{Scrambled}$ cells (p=0.04). No significant differences were observed between the parental A549 and $A549^{Scrambled}$ cells in either assay. As expected, there were few apoptotic/dead non-treated parental A549 cells. These results show that suppression of *OCT4B* sensitizes A549 cells to cisplatin-induced apoptosis.

Suppression of OCT4B in A549 cells induced changes in cell-cycle phase distribution following cisplatin treatment. To probe whether the suppression of OCT4B was associated with alterations in cell-cycle progression, A549 cells were transfected with scrambled siRNA and *OCT4B* siRNA respectively, followed by cell-cycle distribution analysis by flow cytometry. Transfection with *OCT4B*-specific siRNA resulted in a slight decrease in the proportion of cell in the G_0/G_1 phases compared to parental A549 and $A549^{Scrambled}$ cells (Figure 5a). Furthermore, suppression of *OCT4B*, reduced the proportions of cells in the S and G_2/M phases by 32.8±15.5% and 46.6±17.9% respectively in comparison to $A549^{Scrambled}$ (Figure 5a).

Treatment of parental A549, $A549^{Scrambled}$ and $A549^{OCT4B-}$ cells with cisplatin for 48 h resulted in a strong decrease in the percentage of cells in the G_0/G_1, and an increased number of cells in the S and G_2/M phases compared to non-treated cells (Figure 5a *vs*. Figure 5b). As expected, cisplatin treatment in parental A549 cells, reduced the percentage of cells in the G_0/G_1 phase by 60.8±3.8% (compared to non-treated cells), and resulted in an increase of 27.4±5.5% and 26.7±3.6% of cells in the S phase and G_2/M phases respectively. This trend was similarly observed in the cisplatin-treated $A549^{Scrambled}$ cells, with a decrease of 58.0±2.9% in the G_0/G_1 phase and an increase of 30.4±4.3% and 24.4±3.8% in the S and G_2/M phases, respectively (Figure 5b).

Interestingly, cisplatin treatment had a dramatically different effect on the cell cycle of $A549^{OCT4B-}$ cells. In addition to a reduction in the G_0/G_1 phase (53.3±7.1%) in comparison to non-treated $A549^{OCT4B-}$ cells, an increase of 9.8±1.7% of cells in the S phase and a dramatic increase of 56.1±3.5% cells in G_2/M were also measured. Notably, a significant decrease in the S phase proportion (p<0.0001) was observed in $A549^{OCT4B-}$, as well as a striking increase in the G_2/M phase proportion compared to $A549^{Scrambled}$ (p<0.0001). These findings indicate that silencing of *OCT4B* induces cell proliferation after cisplatin treatment, highly suggestive of a direct involvement of *OCT4B* in conferring sensitivity to cisplatin, as proliferating cells are deemed more susceptible to chemotherapy.

Discussion

Increasing sensitivity of LAC to standard cisplatin therapy remains a primary objective in therapeutic modalities. In this study, we report evidence that silencing of *OCT4B* confers sensitivity to cisplatin treatment in LAC *via* regulation of cell cycle, increased cell proliferation and enhancement of cisplatin-induced apoptosis. Suppression of *OCT4B* in A549 cells increased the number of non-viable, PI^+, and apoptotic, caspase-3^+, cells both in the presence and absence of cisplatin treatment, indicating its crucial role in apoptosis. More importantly, cisplatin treatment of *OCT4B*-suppressed A549 cells resulted in a dramatic transition of cells from G_0/G_1 to G_2/M, signifying enhanced cell proliferation, generating a higher apoptotic index compared to A549 cells transfected with control siRNA. Our results show the direct participation of *OCT4B* in augmenting cell proliferation

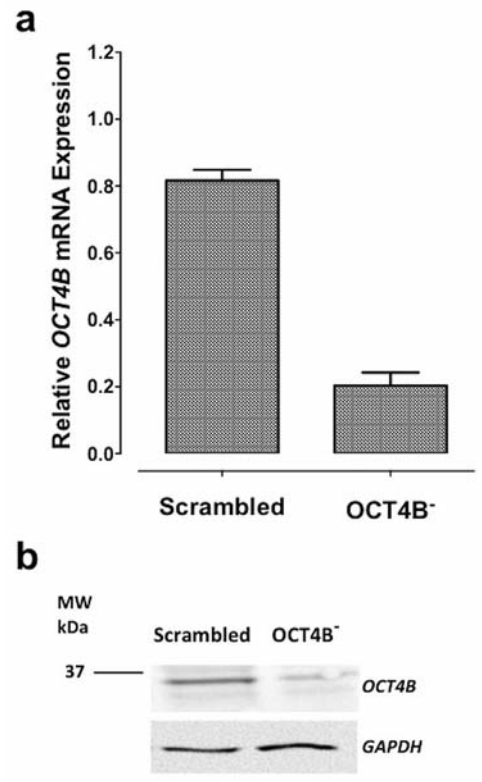

Figure 2. *Efficiency of octamer binding transcription factor-4B (OCT4B) suppression at the transcriptional and translational levels in A549 cells. Cells were transfected with OCT4B-specific siRNA or control/scrambled siRNA for 72 h. a: OCT4B mRNA expression was determined using quantitative real time reverse transcriptase polymerase chain reaction to evaluate the efficiency of silencing. b: Protein lysates were isolated from transfected A549 cells, separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoreactivity to OCT4B antibody at 30-33 kDa was detected by western blotting. GAPDH was used as loading control.*

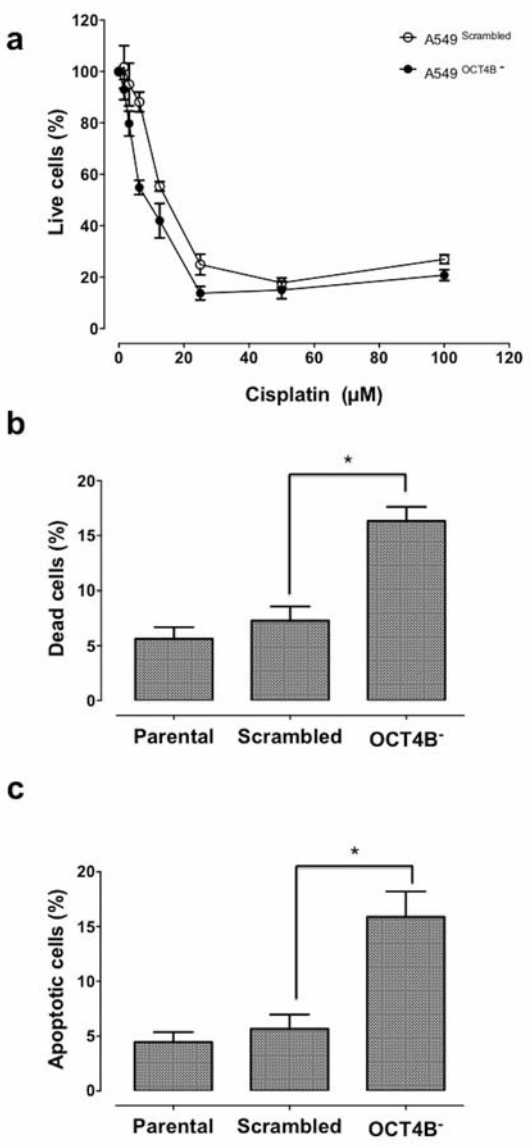

Figure 3. *Effect of suppression of octamer binding transcription factor 4B (OCT4B) on chemoresistance, cell viability and apoptosis. Partial suppression of OCT4B in A549 cells significantly increased sensitivity to cisplatin, cell death and apoptosis. a: Down-regulation of OCT4B markedly increased the sensitivity of A549 cells ($A549^{OCT4B-}$) to cisplatin (*p=0.02) in comparison to cells transfected with scrambled siRNA ($A549^{Scrambled}$). $A549^{OCT4B-}$ cells showed a significant increase (*p=0.0002) in the percentage of propidium iodide $(PI)^+$ cells (b), and activated caspase 3^+ cells (*p=0.04) (c) compared to the $A549^{Scrambled}$ cells. Suppression of OCT4B by siRNA generated a higher percentage of apoptotic cells, as assessed by flow cytometry. Values are the mean±SD of three independent experiments.*

under genotoxic stress, supporting the notion of sensitization to chemotherapy *via* cell proliferation.

Operationally, the *OCT4B* isoform is unique compared to *OCT4A* as the former cannot sustain self-renewal in embryonic stem cells and has no transactivation properties (17); no doubt *OCT4A* and *OCT4B* have different spatial and temporal expression patterns in normal and malignant tissues. Using an isoform-specific primer/probe, we found significantly higher *OCT4B* mRNA levels in A549 cells compared to human normal lung fibroblasts (hFB16Lu) and human lung mesenchymal stem cells (hLMSCs). These data reflect our previous findings of an increased *OCT4B* expression in primary LAC tumour tissues compared to normal counterparts (10). Others have also shown the expression of *OCT4B* in prostate cancer and benign prostate hyperplasia (18); and by immunohistochemisty, Atlasi *et al*. demonstrated greater cytoplasmic staining of OCT4B in cancerous cells in comparison to normal somatic cells (7). Although the presence of OCT4B has been demonstrated in the malignant setting, little is known about its oncogenic role, and whether is it involved in chemoresistance to standard

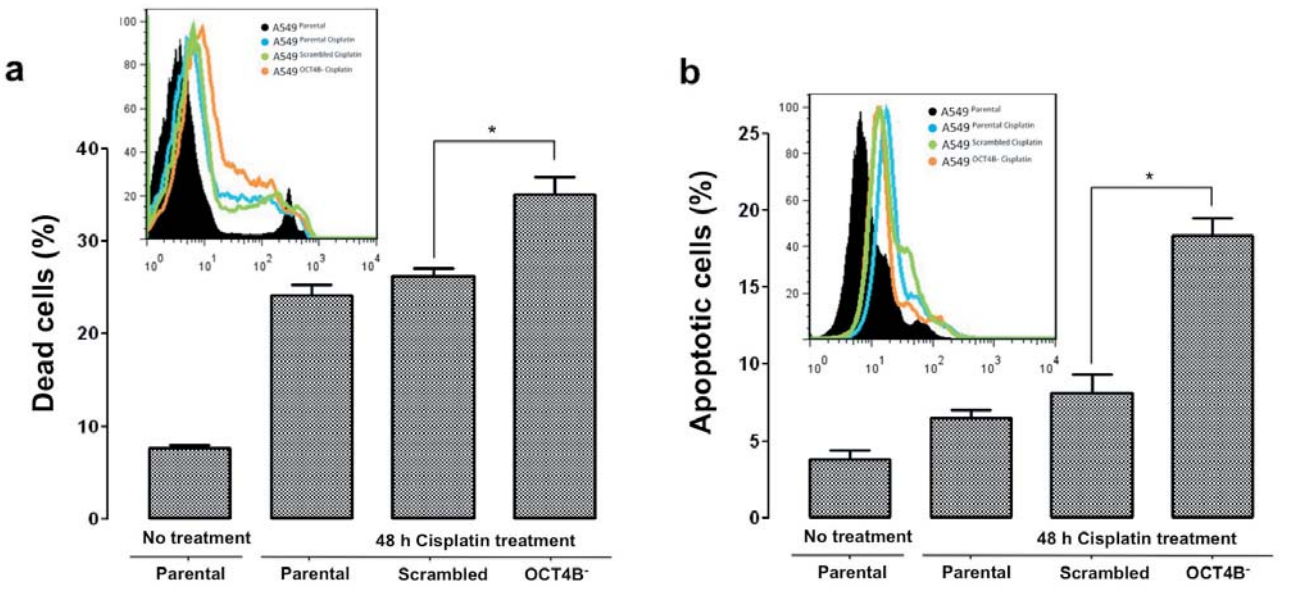

Figure 4. *Effect of partial suppression of octamer binding transcription factor-4B (OCT4B) on propidium iodide (PI) and activated caspase-3 staining after cisplatin treatment. The percentage of dead cells after a 48-h cisplatin treatment of parental A549, A549^{Scrambled} and A549^{OCT4B-} cells was determined by flow cytometry-based PI and caspase-3 staining. a: Suppression of OCT4B in A549 cells resulted in a significant increase of PI^{+} cells (*p=0.01) after a 15 μM cisplatin treatment compared to cisplatin-treated A549^{Scrambled} cells. A marginal percentage of non-treated cells was PI^{+}. b: Suppression of OCT4B in A549 cells resulted in a significant increase of caspase-3+ cells (*p=0.04) after 15 μM cisplatin treatment compared to the cisplatin-treated A549^{Scrambled} cells. Insets show histograms representative of a single experiment. The means±SDs represent data from at least three independent experiments.*

chemotherapeutic agents, such as cisplatin. Here, we illustrate a significant up-regulation of *OCT4B* in A549 cells after short-term exposure (48-h) to cisplatin. To further evaluate the potential protective function of *OCT4B*, we assessed cisplatin sensitivity in *OCT4B*-suppressed A549 cells.

The loss-of-function experiment offers a useful approach to study for specific gene functions. We obtained approximately 70-80% suppression of *OCT4B* in the A549 cell line, which was confirmed at the transcriptional and translational levels. Silencing of *OCT4B* increased the sensitivity of A459 cells to cisplatin, manifested by a lower IC_{50} compared to parental A549 cells. We found that the increased sensitivity was a result of the enhancement of cisplatin-induced apoptosis. Most apoptotic signaling is accompanied by the activation of caspases, a family of cysteine proteases normally expressed in cells as inactive zymogens, and are converted into their active form at the onset of apoptosis (19). Caspase-3 is one of the effector caspases in apoptosis which may play a crucial role in post-target resistance to cisplatin (20), and is an appropriate gauge for evaluating apoptotic response to cisplatin treatments. We observed an increasing PI^{+} and caspase-3^{+} A549^{OCTB-} cells compared to A549^{Scrambled} cells clearly indicating an essential role of *OCT4B* in apoptosis. This was consistently observed after a 48-h cisplatin treatment, highlighting this pro-apoptotic function under genotoxic stress. Genotoxic substances are chemical compounds *e.g.* cisplatin that have the capacity to covalently modify DNA molecules (21). Cisplatin exerts its anticancer effect by the generation of DNA lesions, activation of DNA damage response, and induction of mitochondrial apoptosis (20).

Cellular responses to genotoxic stress are a complicated network, including the activation of transcription factors which regulate the expression of genes involved in DNA repair, cell-cycle arrest and apoptosis (22). At least two OCT4B isoforms respond to cellular stress. OCT4B-190 was reported to be up-regulated and antagonized cell apoptosis after heat shock and oxidative stress, whereas OCT4B-265 did not respond to this non-genotoxic stress. However, OCT4B-265 is up-regulated under genotoxic stress (mitomycin, doxorubicin or UV irradiation) in stem cells, promoting apoptosis which serves as a protective mechanism for the cells (13, 15). Our findings show that silencing *OCT4B* enhances cell apoptosis under genotoxic stress, which is a potentially valuable tool in the development of an effective therapy. Notably, these data also demonstrate that silencing of *OCT4B* is an effective approach to reverse its anti-apoptotic property under genotoxic-dependent cellular stress.

Cisplatin exerts its cytotoxic effect by inhibition of DNA synthesis and cell-cycle arrest at G_1/S and early S

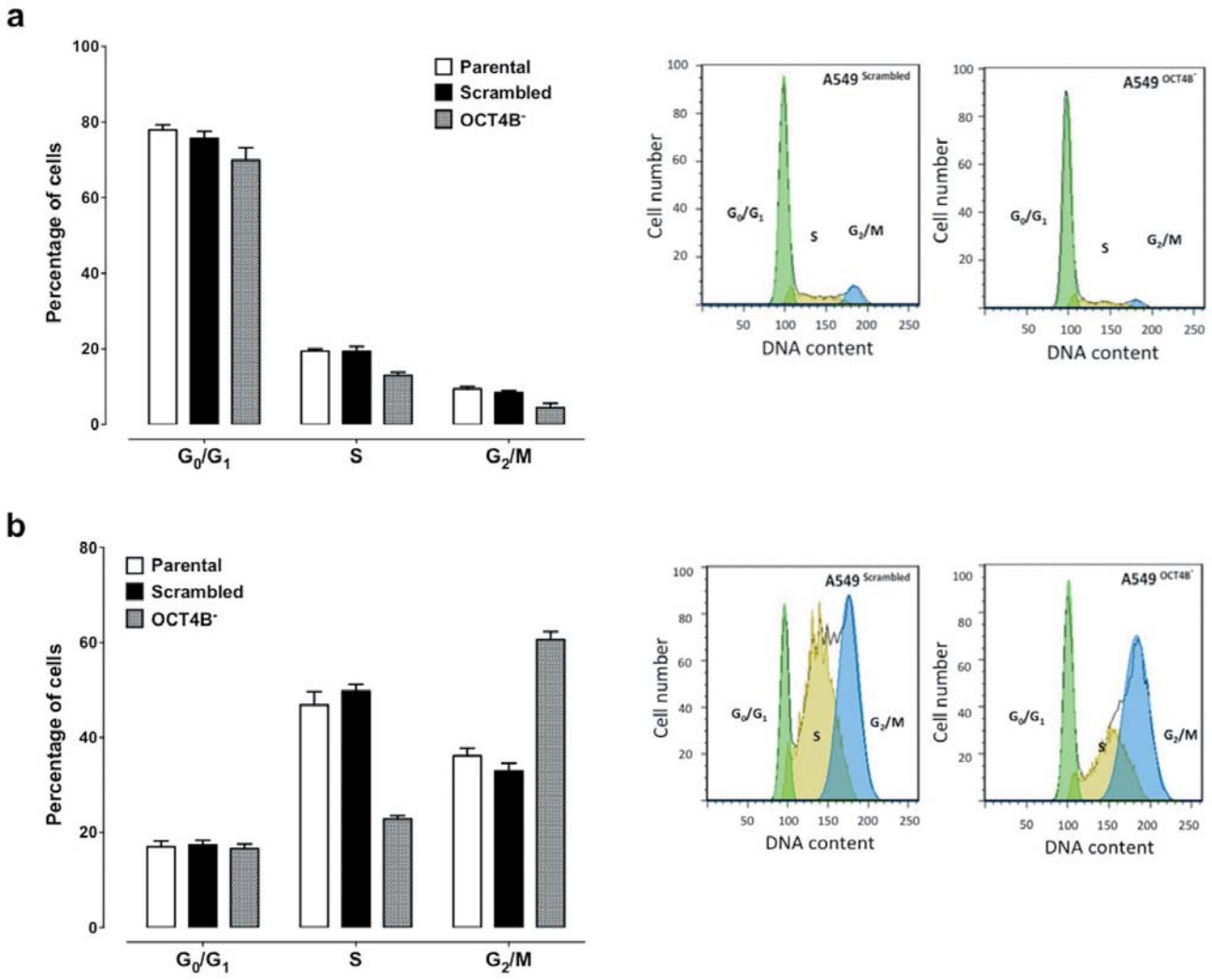

Figure 5. *Effect of partial suppression of octamer binding transcription factor-4B (OCT4B) on the cell cycle of A549 cells after cisplatin treatment. Cell-cycle analyses of A549^{Parental}, A549^{Scrambled} and A549^{OCT4B-} with and without cisplatin treatment were assessed by flow cytometry. The basal cell cycle of non-cisplatin treated parental, scrambled siRNA-treated and OCT4B-suppressed A549 cells exhibited a profile of high G_0/G_1 and low G_2/M proportions. a: The insets show representative flow cytometric profiles of the cell-cycle distribution in A549^{Scrambled} and A549^{OCT4B-} cells, respectively. A 48-h cisplatin treatment of A549^{Parental} and A549^{Scrambled} cells strongly induced cell transition from G_0/G_1 to the S and G_2/M phases of the cell cycle (b). In contrast, cisplatin treatment of OCT4B-suppressed A549 cells resulted in marked transition from the G_0/G_1 to G_2/M phase but not to the S phase. The insets show representative flow cytometric profiles of the cell cycle distribution in A549^{Scrambled} and A549^{OCT4B-} cells, respectively, following cisplatin treatment. The mean±SD represent data from at least three independent experiments.*

phases during which the cells repair the cisplatin-induced damage (23). As expected, and like other cancer cell lines, cell-cycle analysis of non-treated A549^{OCTB-} cells showed a relatively high proportion of G_0/G_1 cells and reduced cell fractions in the S and G_2/M phases, suggesting slow cell proliferation or cells in a state of quiescence. Moreover, in accordance with the aforementioned studies, cisplatin treatment of A549 cells resulted in a reduction of cells in the G_0/G_1 cells and cell-cycle arrest at the S phase. Importantly, cisplatin treatment of *OCT4B*-suppressed A549 cells led to a significant reduction in G_0/G_1 cells and a marked transition of cells from G_0/G_1 to G_2/M, not observed in the cisplatin-treated parental, and cisplatin-treated A549 scrambled cells. Although these results clearly reveal that silencing of *OCT4B* has a direct role on cell-cycle transitions, the mechanism by which this occurs is not yet known. We suppose that A549^{OCT4B-} cells and not parental A549 cells comprise an activatable cell population in the G_0/G_1 and S phases which initially escaped or survived the damaging effect of cisplatin. These

cells potentially traversed the S phase and proceeded directly to the G_2/M phase, resulting in an increase of proliferating cells which were then more susceptible to cisplatin treatment. This assumption was strengthened by an increased apoptotic index after cisplatin treatment of $A549^{OCT4B-}$ compared to $A549^{Scrambled}$ and parental A549 cells. We believe that these findings support sensitization *via* augmentation of cell proliferation to cisplatin in A549 cells in which OCT4B is silenced.

The regulation of non-cycling to cycling cells in solid cancer is mostly aimed to target cancer stem cell populations *via* manipulation of specific signaling events, and is comprehensively studied in the hematopoietic system. In the context of stem cell biology, it is postulated that conventional chemo- and radio-therapies target proliferating cells and require cells to be actively cycling for induction of apoptosis. Studies in human hematopoietic stem cells (HSCs) suggest that the state of dormancy protects them from chemotherapy-induced killing, *in vivo*. The activation of quiescent/dormant HSCs to enter the cell cycle by application of cytokines, such as granulocyte colony-stimulating factor (G-CSF) and interferon-alpha (IFNα) has been correlated with increased sensitivity to chemotherapy (24). We speculate that silencing of *OCT4B* may have an endogenous property to directly drive non-cycling G_0/G_1 cells to re-enter G_2/M under genotoxic exposure, thus making them sensitive to cisplatin treatment. This hypothetical sensitization machinery leads to the observed high number of apoptotic $A549^{OCTB-}$ compared to $A549^{Scrambled}$ cells after cisplatin treatment. If cell quiescence functions as a safeguard mechanism against conventional chemotherapy, and increased cell proliferation is a pre-requisite of effective treatment, then our presumption may hold true. Further studies, in particular, on the role of *OCT4B* in regulation of cell-cycle circuits are mandatory to exploit this mechanism.

To the best of our knowledge, this is the first study to investigate a possible involvement of *OCT4B* in chemoresistance to cisplatin in LAC, and the first to show that silencing of *OCT4B* in A549 cells enhances their sensitivity to cisplatin. By direct activation of quiescent/dormant cells to actively-proliferating cells, cisplatin treatment resulted in a higher number of apoptotic cells. If activation of dormant/quiescent cells is crucial to cisplatin treatment, then this may be used as a combinatorial protocol in the treatment of LAC. In this context, we highlight the potential of *OCT4B* suppression in combination with cisplatin treatment as a therapeutic approach to enhance chemosensitivity in cisplatin-based treatment for LAC. These findings contribute to knowledge on the biology of OCT4, particularly, OCT4B, and also present a challenging new concept for increasing sensitivity to cisplatin by specific silencing of the latter.

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