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KLF4 sensitizes the colon cancer cell HCT-15 to cisplatin by altering the expression of HMGB1 and hTERT

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ABSTRACT

Aims: Insensitivity of cancer cells to therapeutic drugs is the most daunting challenge in cancer treatment. The mechanism of developing chemo-resistance is only partly understood to date. In continuation of some earlier reports, we hypothesize that KLF4, a key transcription factors that also has a crucial role in maintaining the stemness in cancer cells, may offer a basis for chemo-resistance.

Main methods: Sensitivity of cells to cisplatin was analyzed by cell proliferation, colony formation, and cell growth assay. Cell cycle analysis and immunophenotyping were used to measure cell cycle arrest and level of reactive oxygen species respectively. Immunoblotting was used to analyze the change in expression hTERT and HMGB1 involved in KLF4 mediated cisplatin resistance.

Key findings: We found that KLF4 expression sensitizes cancer cell to cisplatin cytotoxicity. Further, KLF4 promotes the cisplatin-mediated G2/M cell cycle arrest while KLF4 knocked down induces cisplatin-mediated S-phase arrest compared to control. Decreased level of reactive oxygen species (ROS) in cisplatin-treated and KLF4 knocked down HCT-15 cells compared to vector control, accounting for increased cell survival. Immuno-blotting showed that KLF4 positively regulates expression of the survival proteins hTERT and HMGB1 while in presence of cisplatin, expression of HMGB1 and hTERT is negatively regulated by KLF4.

Significance: This study suggests the involvement of KLF4-HMGB1/hTERT signaling in offering the basis for chemo-resistance in colon cancer cells and KLF4 overexpression as a probable strategy for sensitizing drug-resistant cancer cells to chemotherapy. The present study opens up new avenues for cancer research and therapeutics.

1. Introduction

Colorectal cancer (CRC) is the fourth leading cause of mortality among cancer patients globally with the 5-year relative survival rate of only 8% [1]. Development of drug resistance in cancer cells is a great challenge for cancer therapeutics. It is believed that only a small fraction of cancer cell population is able to drive tumor initiation and metastases [2–4] and this biologically distinct population of cancer cells called cancer stem cells have been identified in most solid tumors including colon cancer [5–7]. Colorectal cancer stem cells present in heterogeneous cancer cell population exhibit resistance to the most promising chemotherapeutic agents like 5-fluorouracil (5-FU), and oxaliplatin possibly due to altered survival signaling and alterations in DNA damage repair mechanisms [8,9]. 38% of patients treated with 5-FU-based adjuvant chemotherapy following resection of stage II and III colon cancer experience either recurrence or die within 8 years of follow-up [10]. Up to 50% of patients with metastatic colorectal cancer are resistant to 5-FU-based chemotherapy [11,12]. Despite an enormous effort spent in the advancement of diagnostic and therapeutic methods and tools, drug resistance, tumor recurrence, and metastases are critical patient survival-influencing factors of colorectal cancer. It has been observed that some cancer cells, such as breast and colon cancer acquire the characteristics of cancer stem cells through the epithelial-mesenchymal transition (EMT) [13–15]. Therefore, a better understanding of the molecular mechanism of resistance is essential to develop an effective therapeutic approach to deal with the chemo-resistance in patients receiving systemic therapy to combat colon cancer progression and metastases.

In the present study, we chose cisplatin as a model antitumor drug as the emergence of cisplatin resistance in cell populations is well-

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known, and together with other derivatives, it is the most effective anticancer drug used to treat several types of malignancies including colon cancer [16]. Platinum-based neoadjuvant chemotherapy together with radiotherapy is suggested before surgery in colon cancer of advanced stage [17], and it is recommended to be continued after tumor resection to prevent the high risk of relapse.

KLF4 is a gut-enriched zinc finger-containing transcription factor that regulates a diverse array of cellular processes including development, differentiation, and carcinogenesis. KLF4 functions as a transcriptional activator or repressor depending on the interaction partner and cell type [18]. Recently, an association of KLF4 with chemotherapy resistance has been reported in several malignancies [19–23]. It also has been reported that KLF4 is a bona fide tumor suppressor for both gastric and colorectal carcinoma [24,25]. It is also involved in transcriptional regulation of hTERT in cancer cells [26]. Enhanced expression of KLF4 activated expression of human telomerase reverse transcriptase (hTERT) in fibroblast cells while its down-regulation reduces expression of hTERT in cancer and stem cells, which normally exhibit high expression of hTERT [26]. Our previous report on microarray analysis in HeLa cells showed that knockdown of hTERT upregulates the expression of KLF4 [27]. In the present study, we asked whether KLF4 contributed to the cisplatin resistance in colon cancer cell line HCT-15, and which proteins might mediate its molecular mechanism.

2. Materials and methods

2.1. Cell line and reagents

Colon cancer cell line HCT-15 was used for the experiments and the HEK-293T cells were used as packaging cell line for the lentivirus preparation. The cell line was maintained in DMEM with 10% FBS and 1% antibiotic under standard culture conditions (5% CO2, 37 °C temperature and 95% humidity). Anti-human KLF4 (St John's Laboratory Ltd., UK), anti-human hTERT (Santa Cruz Biotechnology, USA), anti-human HMGB1 (Abcam, UK) were used for Western blotting. HCT-15 cell line stably knocked down for KLF4 expression (KLF4 shRNA) were obtained by transducing anti KLF4 shRNA through lentivirus vector and selecting it in puromycin (2 μg/ml) DMEM for 10 days through three passages. Cisplatin was purchased from Sigma-Aldrich (#P4394) and dissolved in 0.9% sodium chloride solution as per instruction of supplier. All experiments were done in triplicates.

2.2. Preparation of KLF4 Over expression stable cell line

To prepare KLF4 Over expression stable cell full length KLF4 over-expression vector FL-KLF4 pBABE was purchased from Addgene (#34589) [28]. KLF4 over-expressing HCT-15 stable cell line (KLF4 OE) was prepared by the transfecting FL-KLF4 pBABE vector using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, USA) and selecting it in puromycin (2 μg/ml) DMEM for 10 days through three passages. Level of over expression in stable cell line was checked by real time PCR and western blotting.

2.3. shRNA cloning and preparation of stable cell line

For the knocked down of KLF4 in HCT-15 cells, shRNA (sequence oligo1: 5′CGCCACCCACACTTGTGATTACTCGA GTAATCACAAGTGTG GGTGGCGTTTTTG3′; oligo2: 5′CAAAAACGCCACCCAC ACTTGTGATT ACTCGAGTAATCACAAGTGTGGGTGGCG3′) targeting the KLF4 transcript was cloned in pLKO.1 vector and co-transfected with lentiviral packaging vectors (psPAX2 and pMD2.G) in HEK-293T cells using Lipofectamine 3000 Reagent (Thermo Fisher Scientific, USA). After 12 h of incubation in optimum culture condition (5% CO_2, 37 °C temperature, and 95% humidity), the media was replaced by fresh media with 5% serum. After 24 h of incubation media (having lentiviral particle targeting KLF4) was harvested two times at 12 h interval and was either used immediately or stored at −80 °C for future use.

2.4. MTT and cell growth assay

To assess the respective cell proliferation HCT-15 cells were seeded in 96 well plate (5000 cells/well) and incubated with varied concentration of cisplatin for 48 h. MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) solution was added and incubated for 2 h. Formazan crystals formed were dissolved in DMSO and color produced was measured at 570 nm wavelength. IC-50 for cisplatin in HCT-15 cells was found to be 20 μM. For cell growth assay, cells were seeded at 40000 cells/well in 12 well plate with or without cisplatin and incubated for 72 h. The cells were washed with PBS, fixed with methanol and stained with 0.1% crystal violet solution for 10 min. Stained cells were washed with PBS three-time and 1 ml 10% acetic acid was added to each well and incubated for 20 min with shaking. 300 μl of the acetic acid solution with stain was taken from each well, diluted four times and color was measured at 590 nm wavelength.

2.5. FACS analysis

For cell cycle analysis, cells were treated with different concentrations of cisplatin, harvested, washed with PBS and fixed with methanol for overnight at −20 °C. Next day after RNase treatment at 37 °C for 1 h, cells were stained with propidium iodide and analyzed by BD FACSCalibur. For the detection of cellular ROS level respective cells treated with cisplatin were harvested, washed with PBS and incubated with DCFDA (2′,7′–dichlorofluorescein diacetate) dye at the optimum condition in DMEM. After 30 min, cells were washed and histogram analysis was done using BD FACSCalibur.

2.6. Immunoblotting

Whole cell proteins were extracted by lysing cells in RIPA buffer [50 mM Tris-Cl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1% NP-40 and 0.1% SDS] containing protease inhibitor cocktail (Sigma, USA) on ice. Total protein concentration was estimated by Bradford assay. An equal amount of protein (40 μg) was separated on 10% SDS-PAGE and electro-blotted to the PVDF membrane (Millipore Corporation, Billerica, MA, USA). The membrane was blocked with 5% non-fat milk in TBST buffer (Tris-Cl, NaCl, Tween-20) for 1 h at room temperature and probed with the respective primary antibody at 4 °C for overnight followed by enzyme (alkaline phosphatase or horseradish peroxidase) tagged secondary antibody for 1 h at room temperature. After washing with TBST buffer, the blots were developed with NBT-BCIP (Ameresco, USA) solution in case of alkaline phosphatase and with DAB-Peroxidase Substrate Solution luminata (Millipore, US) in case of horseradish peroxidase tagged secondary antibody.

3. Statistical analyses

Data represent means ± SEM. Significance was set at $p < 0.05$. BD FACS Calibur (USA) Kolmogorov-Smirnov (KS) statistical analysis was done to show a significant horizontal shift of histogram.

4. Results

4.1. KLF4 over-expressing Cells are sensitized towards cisplatin

Lentiviral-mediated knocking down and overexpression of KLF4 in HCT-15 cells were confirmed by both western blotting and quantitative real-time PCR. We observed > 50% down-regulation of KLF4 at transcription ($p = 0.002$) and protein level after stable transfection with shRNA (KLF4 shRNA), while overexpression of KLF4 by over-expression (OE) vector showed eight and two fold increase in transcription

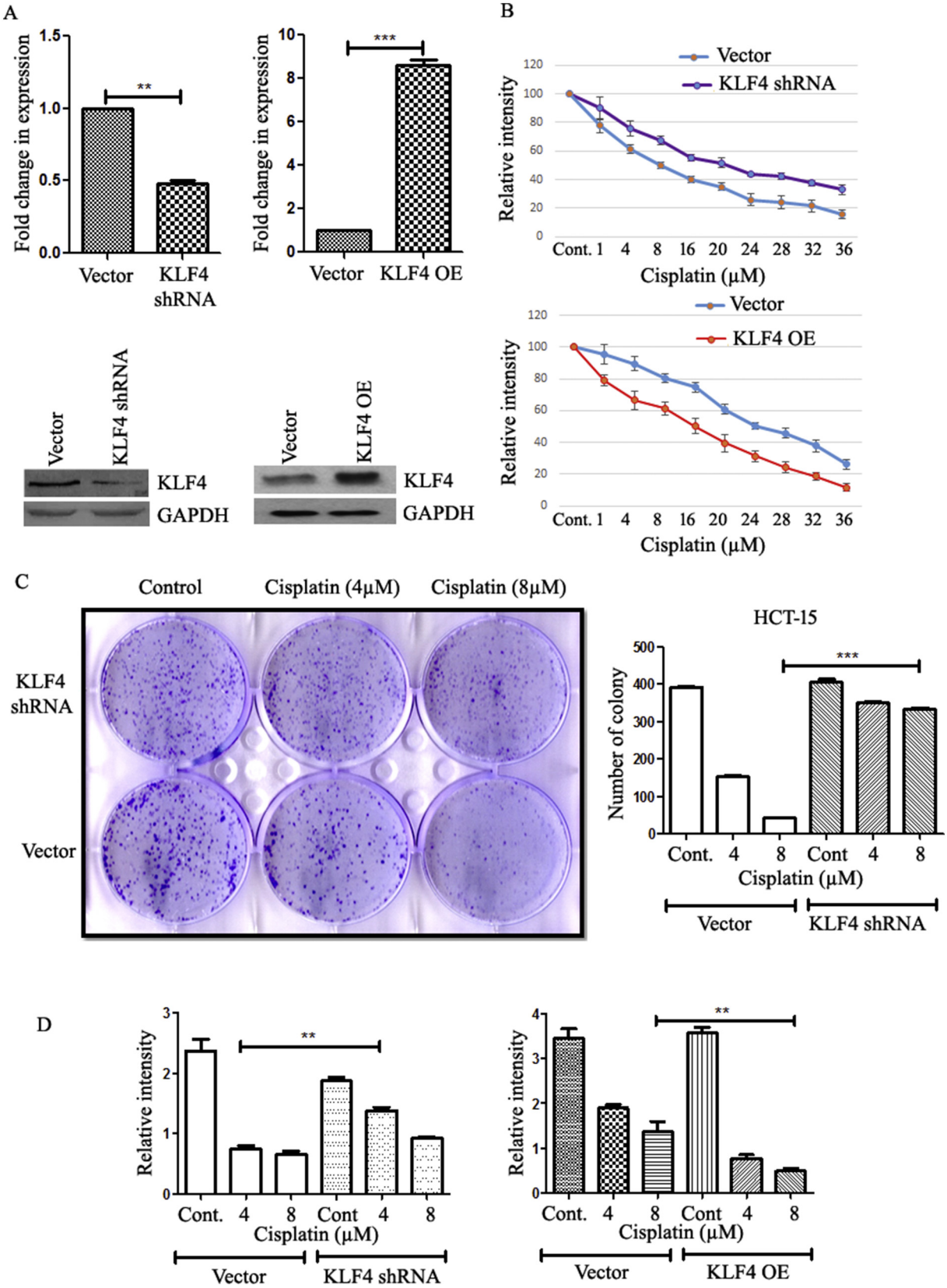

Fig. 1. KLF4 mediated cisplatin sensitivity of HCT-15. A. KLF4 Knocking down (by KLF4 shRNA) and over-expression of KLF4 (KLF4 OE) in HCT-15 assayed by quantitative real-time PCR (upper panel) and Western Blotting (lower panel). Y-axis represents the fold change in mRNA expression level compared to vector control. B. MTT assay showing the effect of cisplatin on the survival of HCT-15 cells following Knocking down (upper panel) and overexpression (lower panel) of KLF4. Y-axis represents the color intensity corresponding to a number of viable cells. C. Colony formation assay showing the increased survival of cisplatin-treated cells knocked down for KLF4 expression (left panel). Bar diagram showing a change in a number of colonies formed in cisplatin-treated KLF4 knocked down cells compared to cisplatin-treated vector control (right panel) D. Effect of cisplatin on cell growth of KLF4 knocked down (left panel) and over-expressing (right panel) HCT-15 cells. Y-axis represents an average number of colonies per well. **, $p \leq 0.003$; ***, $p \leq 0.0001$.

($p = 0.0001$) and protein expression respectively (Fig. 1A). Difference in level of transcription and protein after KLF4 overexpression may be due to high turnover of KLF4 protein as Gamper et al. suggest that KLF4 is kept at cellular steady state levels by maintaining the balance between synthesis and ubiquitin/proteasome-mediated turnover in all tested cells including colon cancer cells [29]. We observed the IC-50 of cisplatin for PLKO.1 vector and KLF4 shRNA transfected cells as 16.59 and 20.43 μM respectively. Increased IC50 inKLF4 shRNA transfected cells suggest the development of chemo-resistance for cisplatin after KLF4 down-regulation. Similarly, decreased IC50 (16.11 μM) in KLF4 over-expressing cells compared to vector control (19.30) suggests the increased sensitivity to cisplatin after over-expression of KLF4 although, we chose much lower dose i.e. 4 μM and 8 μM for further experiments. MTT assay (cell proliferation assay) showed that KLF4 knocked down cells (KLF4 shRNA) were less sensitive to cisplatin (4 μM and 8 μM) compared to vector transfected control (Fig. 1B upper panel), while KLF4 over-expressing cells (KLF4 OE) were more sensitive to cisplatin compared to vector transfected control (Fig. 1B lower panel). Colony formation assay showed increased clonogenic efficiency and growth in cisplatin-treated KLF4 knocked down cells compared to vector control (Fig. 1C, D) while cisplatin-treated KLF4 overexpressing cells showed reduced crystal violet staining (cell growth) compared to vector control (Fig. 1D). These results suggest that KLF4 knocking down makes cancer cells resistant to cisplatin, while overexpression of KLF4 sensitizes them.

(2) Cisplatin-treated KLF4 knocked down HCT-15 cells showed S-phase cell cycle arrest while KLF4 overexpressing cells showed G2/M arrest: To Analyze the KLF 4 mediated cisplatin-induced cell cycle arrest we performed FACS analysis of cisplatin-treated KLF4 knocked down and over-expressing cells. In cells knocked down for KLF4 expression, we found a larger number of cells (35–40%) in S-phase compared to vector control (25% and 32.53%) (Fig. 2A and B). On the other hand, cisplatin-treated KLF4 overexpressing cells showed cell cycle arrest in the G2/M phase (51–53%) compared to vector control (< 39.75%).

4.2. Cisplatin-treated KLF4 knocked down HCT-15 cells show a lower level of ROS

Level of ROS may be a predictor of cancer cell survival. We hypothesize that cisplatin-treated KLF4 knocked down cells may show a reduced level of ROS resulting in increased cell survival. Estimation of cellular ROS by FACS histogram analysis based on DCFDA staining showed that KLF4 knocked down HCT-15 cells treated with 8 μM cisplatin had a lower level of ROS (D = 0.38; $p \leq 0.001$) compared to vector control (D = 0.05; $p \leq 0.001$) (Fig. 3A and E), although there was very little change in cells treated with 4 μM cisplatin (D = 0.14; $p \leq 0.001$) (Fig. 3A and D). KLF4 knocking down alone does not show the difference in the level of ROS (Fig. 3A and C). KLF4 over expressing cells showed reduced ROS level compared to vector control (D = 0.39; $p \leq 0.001$) (Fig. 3B). Cisplatin treated KLF4 over expressing cells showed more prominent increase in ROS level compared to both vector control (D = 0.41; $p \leq 0.001$) and cisplatin treated vector control (D = 0.40) (Fig. 3B). Here D value in Kolmogorov-Smirnov (KS) statistical analysis represents the horizontal shift of histogram corresponding to the level of ROS.

4.3. Cisplatin-treated cells show a negative association between KLF4 and expression of HMGB1 and hTERT

Western blotting showed that KLF4 knocking down results in decreased expression of both HMGB1 and hTERT in colon cancer cell HCT-15 (first and fourth lane/bar of Fig. 4A; $p = 0.003$, $p = 0.008$), while overexpression of KLF4 induces the expression of these proteins (first and fourth lane/bar of Fig. 4B; $p = 0.004$, $p = 0.001$) suggesting a positive association. To test how cisplatin impacted on this KLF4 mediated positive regulation of expression of these two known cell survival proteins, we treated the KLF4 knocked down and over-expressing cells with cisplatin and assayed the expression level of HMGB1 and hTERT. Cisplatin treatment on KLF4 knocked down cell induces the expression of both HMGB1 and hTERT compared to cisplatin treated cells transfected with vector only (Fig. 4A; lane/bar 5 and 6 compared to lane/bar 3, $p < 0.05$) while, cisplatin-treated KLF4 overexpressing cells showed reduced expression of both HMGB1 and hTERT compared to cisplatin treated cells transfected with vector only (Fig. 4B; lane/bar 5 and 6 compared to lane/bar 3, $p < 0.05$). Since increased expression of both HMGB1 and hTERT are associated with increased cell survival, we can speculate that increased cell survival in cisplatin-treated KLF4 knocked down cell is due to the increased level of HMGB1 and hTERT. Cisplatin treatment in PLKO.1 vector only transfected cells reduces the expression of cell survival protein HMGB1 and hTERT. However cisplatin treatment in pBABE vector only transfected cells do not show significant effect, probably the concentration of cisplatin used is not sufficient to affect expression of these proteins.

5. Discussion

Colorectal cancer is the third most common cancer worldwide and the fourth leading cause of death accounting for over half a million deaths every year [30]. The most daunting aspect of cancer therapeutics is the emergence of drug-resistant cells. Dissecting the molecular mechanism and improving the specificity and effectiveness of chemotherapeutic drugs may offer alternative cancer therapeutics. As we have discussed previously, KLF4 functions as a transcriptional activator or repressor depending on the interaction partner and sequences contextual of the binding sites. Involvement of both KLF4 and hTERT in chemoresistance has been recently reported in lung cancer and Osteosarcoma cells [31,32]. The present study investigated the sensitivity of colon cancer cell HCT-15 to cisplatin in the excessive or limiting level of KLF4. Observation of sensitivity towards cisplatin based on MTT (cell proliferation assay), crystal violet assay (growth assay) and colony formation assay confirm that KLF4 makes cells more sensitive towards cisplatin. Increased/decreased IC50 after KLF4 knocking down/over-expression suggests the key role of KLF4 in development of chemo-resistance. In a similar study, it was demonstrated that inhibition of ATR expression selectively enhanced cisplatin sensitivity in colorectal cancer cells HCT 116 with inactivated p53 [33]. It has been reported that KLF4 induces the expression of p53 [34]. Upon DNA damage ATM/ATR activate the p53 leading to cell cycle arrest and cell death [35]. p53 also blocks colon cancer cells HCT116 at the G2M checkpoint involving inhibition of Cdc2 [36]. Similarly in this study it can be speculated that KLF4 induces expression and/or activation of DNA damage induced p53 leading to G2M arrest probably involving inhibition of Cdc2 after cisplatin treatment in KLF4 overexpressing cells. In contrast to our findings, Huang et al. observed that Knocking down of KLF4 sensitizes osteosarcoma cells to cisplatin [37]. This discrepancy may be due to different genetic backgrounds and different interacting partners of the KLF4 in the two types of cancer cells [38]. Further, prolonged S-phase in KLF4 knocked down cells allowing more time for DNA repair leads to increased survival of the cells and it is in conformity with our result of MTT and growth assay. It is evident that transmission of error-free genetic material to subsequent generations is critical to survival at both the cellular and organismal levels. Previously, Lau et al. (2009) also have reported similar findings in non-transformed diploid cells as depletion of the pre-replicative complex protein Cdc6 causes nonlethal S-phase arrest, due to the activation of an ATR-dependent S-phase checkpoint, inhibiting replication fork progression [39]. ATM/ATR are the major checkpoint molecules in S-phase cell cycle arrest and cisplatin-induced stalled replication forks activate ATR-Chk1 signaling pathway [40]. The present study suggests the potential role of KLF4 in cisplatin-mediated cell cycle arrest and survival/proliferation. On the other hand G2M cell arrest in cisplatin-treated KLF4 over-expressing cells results in decreased cell proliferation in a combination of cisplatin

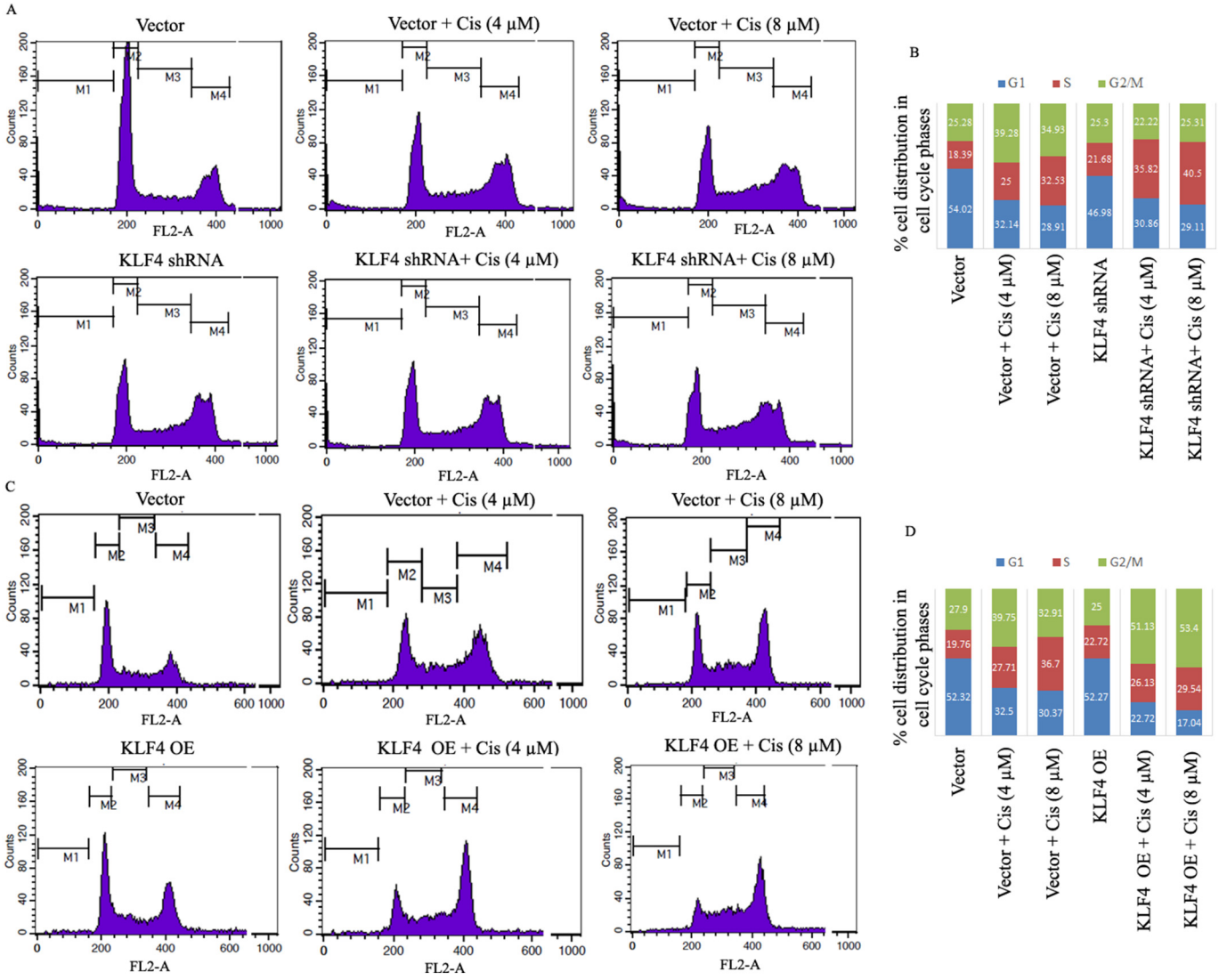

Fig. 2. Flow cytometric profiling of cells showing KLF4 mediated cisplatin-induced cell cycle arrest. A. Cell cycle analysis showing the distribution of cell population in different phases (G1, S, and G2/M) of the cell cycle in cisplatin-treated (4 μM and 8 μM) vector control and KLF4 knocked down cells (KLF4 shRNA) (Left panel). B. Quantitative representation of 'A', showing more number of cells arrested in S-phase (35.82% and 40.5%) in cisplatin-treated KLF4 knocked down cells compared to vector control (25% and 32.53%). C. Cell cycle analysis showing the distribution of cell population in different stages (G1, S, and G2/M) of the cell cycle in cisplatin-treated (4 μM and 8 μM) vector control and KLF4 over-expressing cells (KLF4 OE) (left panel). D. Quantitative representation of 'C', showing more number of cells arrested in G2M-phase (51.13% and 53.4%) in cisplatin-treated KLF4 overexpressing cells compared to vector control (39.75% and 32.91%).

and KLF4 as compared to cisplatin alone substantiating a role of KLF4 in sensitizing the cells towards cisplatin. G2M cell cycle arrest has been known to lead to apoptosis including in colon cancer cells [41,42]. Robert S. DiPaola in 2002 argued that both approaches to enhance G2M cell cycle arrest or abrogate G2M arrest have been seen to improve cytotoxicity of known chemo-therapeutics [43]. Further, it is also evident that low doses of reactive oxygen species stimulate cell proliferation in a wide variety of cancer cell types [44,45]. FACS analysis showed a decreased level of ROS after cisplatin treatment in KLF4 knocked down cells, which may result in reduced sensitivity of KLF4 knocked down cells towards cisplatin. Our observations are in line with Zhang et al. (2017) who demonstrate that hTERT inhibits cisplatin-induced apoptosis by alleviating intracellular ROS in osteosarcoma cells [32]. On the other hand increased level of ROS in cisplatin treated KLF4 overexpressing cells compared to cisplatin treated vector control potentiates the findings. Liu et. Al have shown that KLF4 knock out mouse embryonic cells showed decreased expression of the antioxidant gene, Gsta4 and downregulation of the Gsta4 correlates with ROS accumulation [46]. We can speculate as one of the possible mediator responsible for the decreased level of ROS after over expression of KLF4. Many other reports also show the involvement of hTERT, as targeting hTERT by miR-1182 induces sensitivity to cisplatin [47], and inhibition of hTERT enhances chemosensitivity in head and neck cancers [48]. Apart from hTERT, several other molecules are also involved in chemo-resistance in cancer cells, including a danger associated molecule HMGB1 (high mobility group box 1) which is increasingly being noticed. Suppression of HMGB1 mediated autophagy renders tumor cells more sensitive to cisplatin in ovarian cancer [49] and, induction of HMGB1 mediated autophagy induces chemo-resistance in Hepatocellular carcinoma [50], neuroblastoma [51] and leukemia cells [52].

As our results suggest, KLF4 sensitizes the colon cancer cells HCT-15 towards cisplatin. To reflect on molecules mediating this process, we assayed the expression of hTERT and HMGB1 by western blotting. Induction of KLF4 expression by cisplatin in a dose-dependent manner corroborates earlier reports [37]. Expression of hTERT and HMGB1 has a positive association with the expression of KLF4 (Fig. 4A and B) in non-treated cells. Interestingly we observed a negative correlation of expression of HMGB1 and hTERT with KLF4 in cisplatin-treated cells

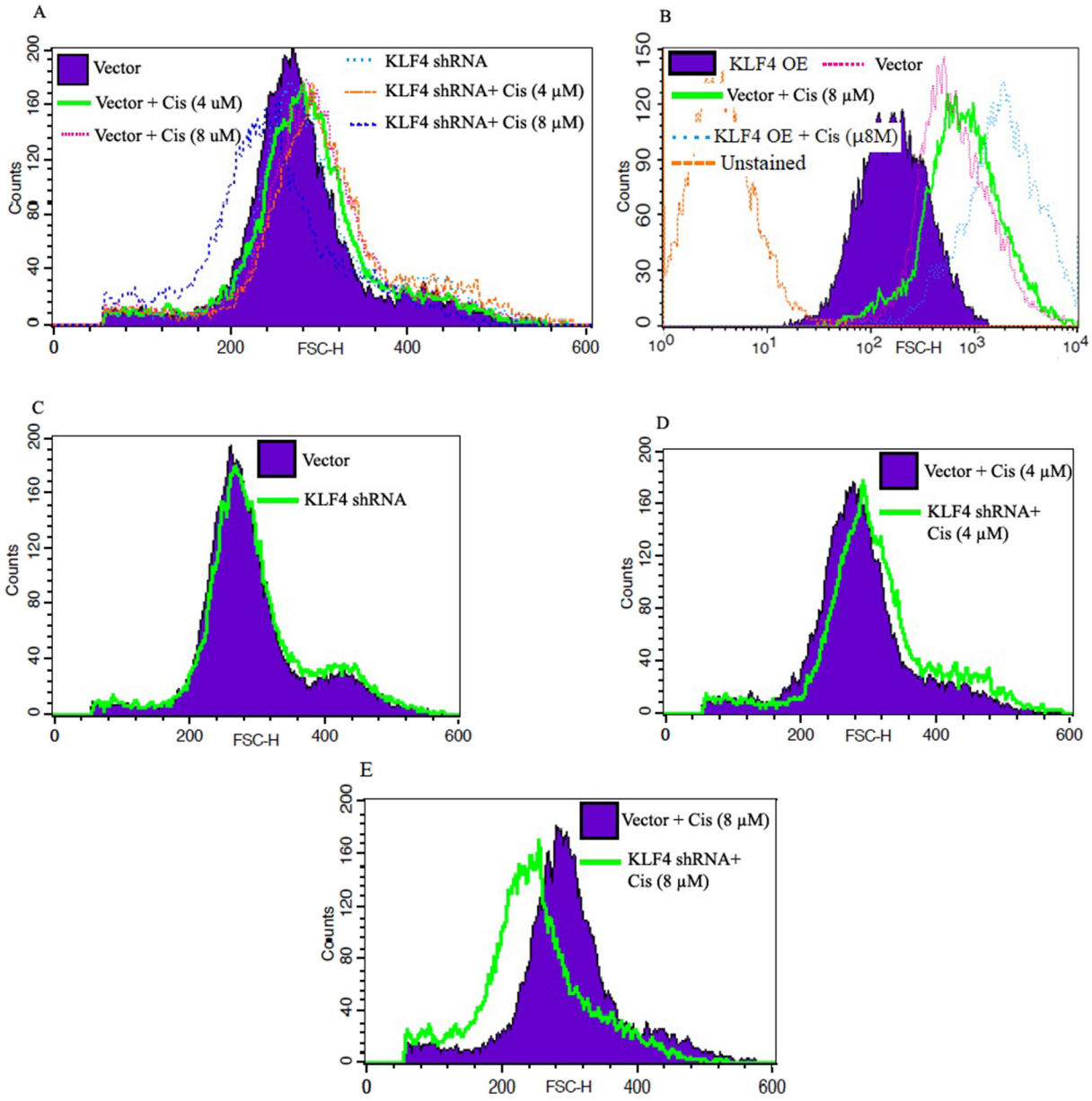

Fig. 3. Level of ROS in KLF4 knocked down cisplatin-treated cells. A. Histogram showing the level of reactive oxygen species (ROS) in cisplatin (4 μM and 8 μM) treated vector control and KLF4 knocked down (KLF4 shRNA) HCT-15 cells. B. Histogram showing the level of reactive oxygen species (ROS) in cisplatin (8 μM) treated vector control and KLF4 over expression (KLF4 OE) HCT-15 cells. C. Comparison of ROS level in vector control and KLF4 knocked down HCT-15 cells. No horizontal shift between two histograms showing no change in the level of ROS. D. Comparison of ROS level in cisplatin treated vector control and cisplatin-treated (4 μM) KLF4 knocked down HCT-15 cells. No significant change in the horizontal shift between two histograms showing negligible change in the level of ROS E. Comparison of ROS level in cisplatin treated vector control and cisplatin-treated (8 μM) KLF4 knocked down HCT-15 cells. The shift of histogram towards left represents the decreased level of ROS in a cisplatin-treated KLF4 knocked down cell compared to vector control.

(Fig. 4A and B). This observation suggests a key role of hTERT and HMGB1 in KLF4 mediated cisplatin sensitivity in at least one colon cancer cell HCT-15. It can be speculated that differential overexpression of hTERT in cisplatin-treated cells knocked down for KLF4 expression, may counteract cisplatin-induced cell death as recently reported in osteosarcoma cells [32] and increase cell survival. With reference to HMGB1, there are two possibilities viz., 1) Increased expression of HMGB1 in KLF4 knocked down cisplatin-treated cells may induce the hTERT activity leading to inhibition of cisplatin-induced cell death. Ke et al. (2015) reported that knockdown of HMGB1 inhibited telomerase activity and cell proliferation while increasing the extent of apoptosis in MCF-7 cells [53]. 2) HMGB1 itself is an indicator of autophagy leading to increased cell survival [54]. Thus, as seen in the Western blots, we can argue that increased/decreased level of HMGB1 in KLF4 knocked down or overexpressing cisplatin-treated cells may enhance/reduce cell survival leading to decreased or increased sensitivity towards cisplatin. Our western blot experiment showed that cisplatin treatment induces the expression of KLF4, which is also reported earlier, irrespective of shRNA/overexpression vector treatment. Since we used the stable cell line for KLF4 knockdown and overexpression, it suggests that hTERT and HMGB1 mediated signaling after a KLF4 knockdown is an earlier event compared to cisplatin-induced KLF4 expression.

6. Conclusion

Our results demonstrate the significance of KLF4 in the sensitivity of cancer cells to the chemotherapeutic drug cisplatin. Further studies using in-vivo model will strengthen the finding of this work. At this

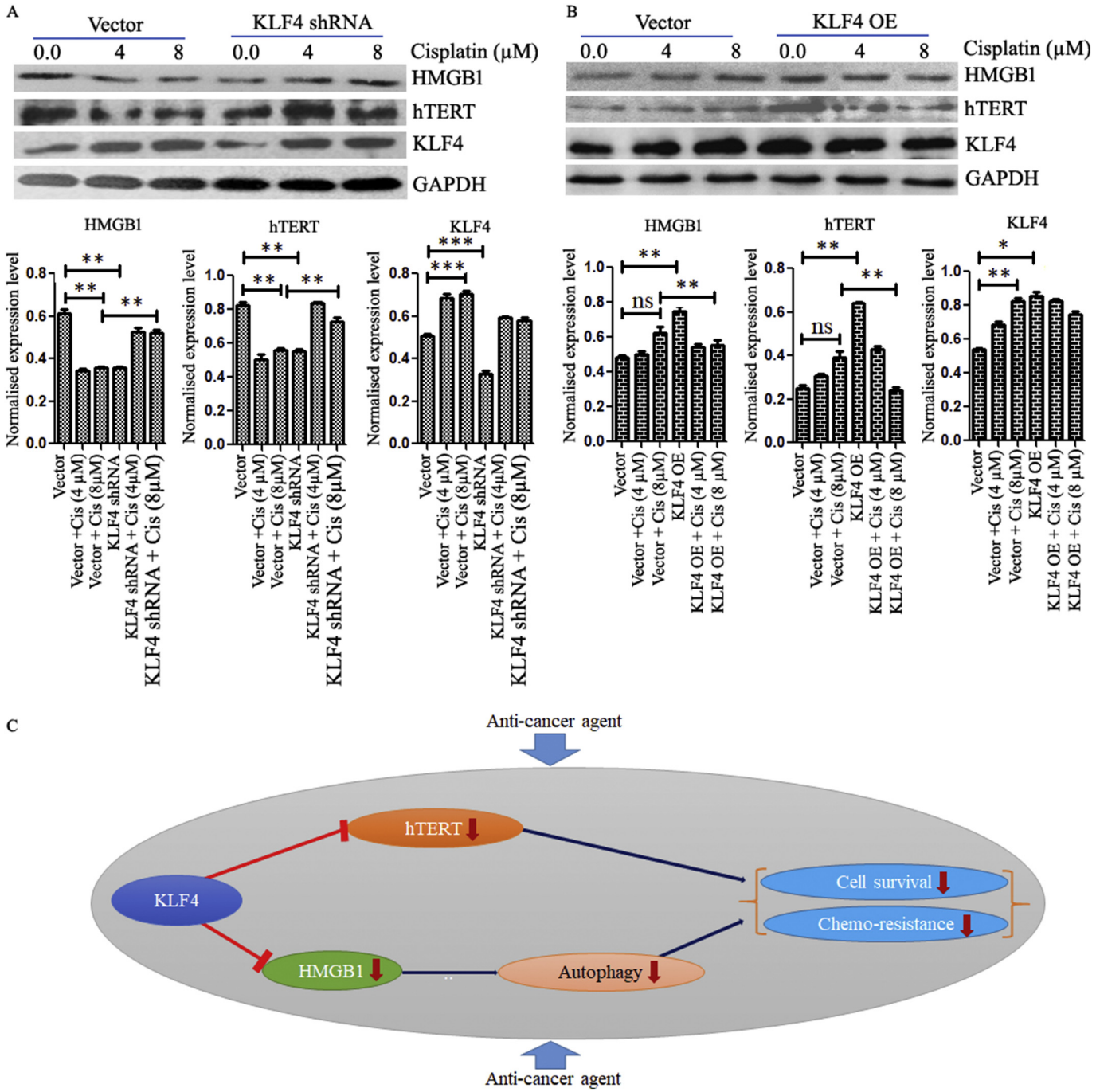

Fig. 4. Expression of KLF4, HMGB1, and hTERT in KLF4 knocked down/overexpressing cisplatin-treated cells. A. Western blots showing the expression of HMGB1, hTERT, and KLF4 in cisplatin-treated vector control and KLF4 knocked down (KLF4 shRNA) HCT-15 cells (upper panel). Normalized quantitative representation of the expression of the different protein shown in the gel (lower panel) B. Western blot gel showing expression of HMGB1, hTERT, KLF4 and internal control GAPDH in cisplatin-treated vector control and KLF4 over-expressed (KLF4 OE) HCT-15 cells (upper panel). Normalized quantitative representation of the expression of the different proteins shown in the gel (lower panel). C. A schematic representation of 1) in the presence of anti-cancer agent KLF4 inhibits the expression of hTERT resulting in reduced cell survival and chemo-resistance, 2) in the presence of anti-cancer agent KLF4 inhibits the expression of HMGB1 and as a consequence autophagy, resulting in reduced cell survival and chemo-resistance. *, $p \leq 0.05$; **, $p \leq 0.003$; ***, $p \leq 0.0001$; ns, non-significant.

stage, it indicates that modulating the level of KLF4 and its signaling in chemo-resistance and carcinogenesis may aid in developing a more effective therapeutic strategy.

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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