



# The Disruption of *Klf6*-Related Super-Enhancer Induces Growth Inhibition and Apoptosis in Human HepG2 Cells

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**ABSTRACT.** *Klf6* is a member of the Krüppel-like factor family, which is a group of zinc finger transcription factors involved in differentiation and development. In general, *Klf6* gene plays an important role in growth-related signal transduction pathways, cell proliferation, apoptosis, and angiogenesis. Recently, the *Klf6* gene is considered as one of tumor suppressor genes in several kinds of tumours, but there is also a conflict of experimental data. In this study, we investigated the influence of *Klf6*-related super-enhancer (SE) on tumor cell growth in human hepatoma (HepG2) cells. As a result, SE-deleted clones were obtained via the CRISPR/Cas9-mediated gene-editing technique. Moreover, it was found that some crucial properties associated with malignancy in those clones were equally affected by the disruption of *Klf6*-related super-enhancer: proliferation, migration, clonogenic potential and drug resistance. These results contribute to the deeper understanding on the potential roles of the *Klf6* related super enhancer in human hepatoma (HepG2) cells.

**Key words:** CRISPR/Cas9; *Klf6* gene; Super-enhancer; Apoptosis; Cancer

## INTRODUCTION

*Klf6* is a member of the Krüppel-like factor family, a group of zinc finger transcription factors involved in differentiation and development. It plays an important role in growth-related signal transduction pathways, cell proliferation, apoptosis, and angiogenesis [Jaya Sangodkar et al., 2009; Magali Humbert et al., 2011].

Although the exact mechanisms underlying the tumor suppressor roles of *Klf6* are unknown, a number of relevant pathways have been described: transactivation of P21 WAF1 in a P53- independent manner, reduction of cyclin D1/cdk4 complexes via interaction with cyclin D1, inhibition of c-Jun proto-oncoprotein activities,

decreased VEGF expression and induction of apoptosis [Benzeno S et al., 2004; SlavinDA et al., 2004; A.C. Racca et al., 2016].

*Klf6* has been postulated as a tumor suppressor since its gene is frequently inactivated by loss of heterozygosity (LOH), somatic mutations and/or decreased expression in human cancers, but there are also conflicting experimental data [A. Difeo et al., 2009; Analisa DiFeo et al., 2009; Ceshi Chen et al., 2003].

Several independent reports described apoptotic or anti-apoptotic functions for *Klf6*. siRNA-mediated *Klf6* knock down in hepatocarcinoma cell lines lead to a reduction in cell cycle progression and cells became more susceptible to DNA-damage induced apoptosis, indicating that *Klf6* function is required for cell proliferation and survival in these cells [D.S. D'Astolfo et al., 2008; E. Sirach et al., 2007]. As a result, the *Klf6* gene pathways are assumed to be very complex and diverse, and research urges clarification of the exact mechanism of *Klf6*, which plays an important role in tumorigenesis and development [Sigal Kremer-Tal et al., 2007; Ricardo C et al., 2011].

Recently, researchers have identified large non-coding enhancers, called super-enhancers, and attempted to identify their *in vivo* functions [Anna Vähärautio et al., 2014; Yanjun Wei et al., 2016]. Super-enhancers (SEs) shown to specifically regulate genes involved in cell identity and disease, including oncogenes [Adrienne R et al., 2015; Denes Hnisz et al., 2013; Denes Hnisz et al., 2015; Jakob Love'n et al., 2013; Marc R et al., 2014]. We used genome-editing techniques to identify *Klf6*-related super-enhancers in human HepG2 cells [Kumchol Ri et al., 2017; Cong L et al., 2013]. The results demonstrated that *Klf6*-related SE regulates the *Klf6* gene expression through long-range DNA looping [Kumchol Ri et al., 2017]. Recent studies from our laboratory found that inhibition of the *Klf6* gene expression by the CRISPR Technique inhibited proliferation and induced apoptosis in human hepatoma (HepG2) cells.

In this study, we obtained a clone of *Klf6*-related SE-deletion by using the CRISPR/Cas9-mediated genetic engineering technique [Kumchol Ri et al., 2017] and found that some crucial properties associated with malignancy in those clones, including proliferation, migration, clonogenic potential and drug resistance, were equally affected by the disruption of *Klf6*-related super-enhancer. These results suggest that the disruption of the *Klf6*-related SE induces growth inhibition and apoptosis in human HepG2 cells. These data lead us to explore and further define the potential role of the *Klf6* related super enhancer in human hepatoma (HepG2) cells.

## METHODS AND MATERIALS

### Cell culture and transfection experiments

Human HepG2 cells were purchased from the typical training content preservation committee cell bank, Chinese Academy of Sciences (China). The Cell culture and transfection experiment were performed as previously described (Kumchol Ri et al., 2017). The cells were passaged twice. For transfection, the cells were cultured with CRISPR plasmid and Lipofectamine 2000 (Addgene) for 6 hours (h).

After transfection, the cells were cultured for 48 h in fresh medium, 0.5 µg/mL of puromycin (Sigma) was supplemented to the cell culture medium for 3 days to select for the transfected cells. Cells were cultured for 24 h in a fresh medium, and clones were obtained by limiting dilution method, from which target clones were selected.

The target-specific CRISPR guide RNAs (sgRNAs) were designed using an online tool (<http://crispr.mit.edu/>). The designed oligonucleotides were inserted into pSpCas9 (BB)-2A-GFP (PX458) plasmid (Addgene) and used for cell transfection experiments.

### Real-time quantitative PCR and Western blot assay

Total RNA was isolated from the target clones using TRIzol (Life Tech) extraction method. Primer sequences for the *Klf6* genes and beta-actin are as follows: 5'-CGGACGCACAC AGGAGAAAA-3'/5'-CGGTGTGCTTTCGGAAGTG-3' (*Klf6*) and 5'-GTGTGGCGACATATG CAGCT-3'/5'-CAAGATCAGCAGTCTCATTTC-3' (beta-actin). A total of 40 cycles were performed, each including 95°C for 30 seconds, 54°C for 30 seconds, and 72°C for 30 seconds. Assays were done in triplicate and Relative levels of gene expression were normalized to beta-actin.

Western blot assays were performed as previously described [Kumchol Ri et al., 2017]. Cells were resuspended in lysis buffer. Proteins were separated on 10–15% SDS-PAGE gels. The following antibodies were used: rabbit polyclonal antibody to *Klf6* (R-173); anti-human E-cadherin (CD324) antibody; goat polyclonal to beta-actin (I-19).

### MTT assays and colony formation assay

MTT assay was performed as previously described [Sigal Kremer-Tal et al., 2007]. In brief, 200  $\mu$ L of MTT (Sigma) in phosphate buffered saline (2 mg/mL) was added to each well. After 4 h incubation at 37°C, the supernatant was discarded, and the precipitate was dissolved in 1 mL of dimethyl sulfoxide. Plates were then read on a microplate reader at 490 nm.

Colony formation assay was preceded as previously described [Jaya Sangodkar et al., 2009]. A total of 100 cells were seeded in 6-well plates, and colonies consisting of at least 50 cells were counted after 14 days.

### Migration assay

Migration ability was measured as previously described [Sigal Kremer-Tal et al., 2007]. Briefly, each clone cells was suspended at a concentration of  $5 \times 10^5$  cells/ $\mu$ L, and  $3 \times 10^4$  cells were placed in each well. The wells were removed gently after a 24 hour incubation. The width of the scratch was measured at the beginning and every 12 hours during cell migration and quantified the wound closure rate as previously described [Sigal Kremer-Tal et al., 2007].

### Cell cycle analysis

Each clone cells were trypsinized and collected, respectively. Cell pellets were fixed in 70% ethanol and stored at -20 °C overnight. Fixed cells were washed with PBS and resuspended in 50 mg/mL propidium iodide containing 125 U/mL RNase A. After 30 min incubation, DNA contents were analyzed by flow cytometry. The cells percentage in different phases of cell cycle was determined with the ModFit 2.0 computer program (Verity Software).

### Detection of apoptotic cells

Cells were trypsinized and resuspended in a solution containing Annexin-V APC (Invitrogen) and SYTOX green (Invitrogen). Labelling intensities were quantified using Cell Quest software on a FacScalibur station.

### Measurement of cell viability

Each clone cells were seeded at  $5 \times 10^3$  cells/well of a 96-well dish. After 48h incubation, cells were treated with *Gemcitabine* at the different concentrations. After 48 h incubation, cell viability was measured using Cell titer96<sup>®</sup> Aqueous One Solution Cell Proliferation assay (MTS) (Promega).

### Statistical analysis

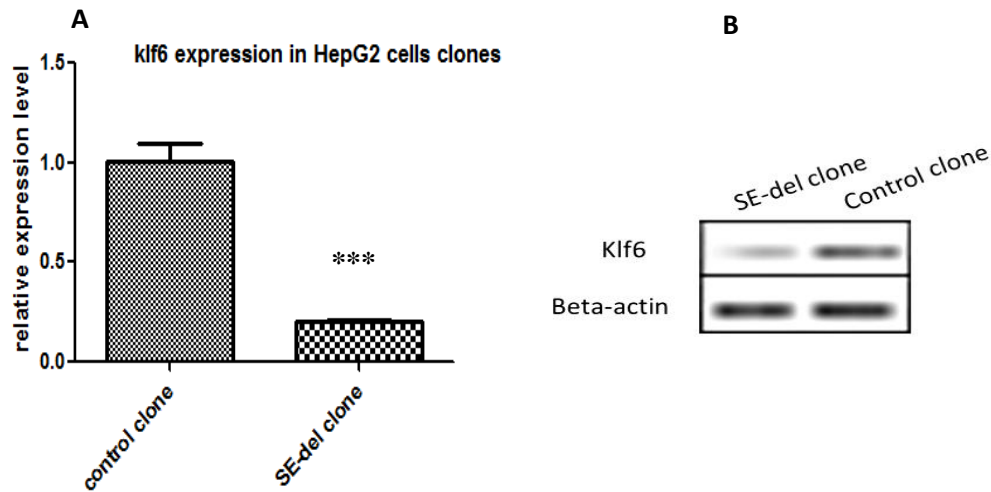
Statistical analyses were performed using GraphPad Prism (GraphPad Software). Data represent the mean  $\pm$  SEM or SD, as indicated. Differences between the means were analyzed using the 2-tailed unpaired Student's t test or ANOVA or the Mann-Whitney U or Kruskal-Wallis test. A P value of less than 0.05 was considered significant.

## RESULTS

### Disruption of *Klf6*-related SE regulates *Klf6* expression in HepG2 cells

We first generated a clone of *Klf6*-related SE-deletion (SE-del) in in HepG2 cells using the CRISPR/Cas9 system to evaluate the functions of *Klf6* gene (described in detail [Kumchol Ri et al., 2017]). RT-qPCR analysis was performed to examine the gene expression of *Klf6* on the obtained SE-del clone (Figure 1A). As shown in the figure, the expression of *Klf6* in the SE-del clone is reduced by 80% or more compared to the control.

To further confirm the expression characteristics of *Klf6*, the *Klf6* protein expression was tested using western blot analysis (Figure 1B). The *Klf6* Protein expression also showed a significant decrease in SE-del clone compared to the control clone.

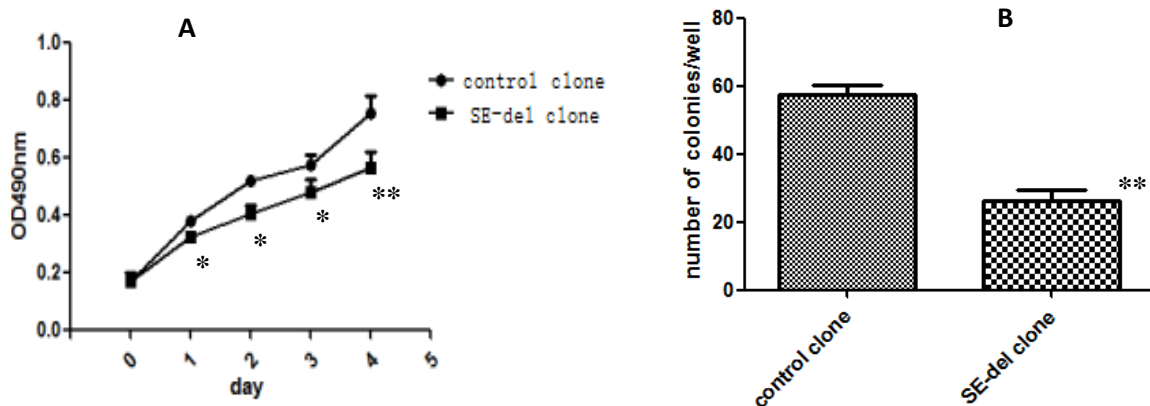


**Figure 1.** *Klf6*-related SE regulates *Klf6* expression in HepG2 cells. (A) Comparison of RT-qPCR analysis in the SE deletion clones and control clone. Results are the mean of three different amplifications, \*\*\*  $p < 0.0001$ . (B) Western blot analysis of *Klf6* expression in SE deletion clone and control clone. Beta-actin blotting were used as loading controls.

The results show that the *Klf6*-related SE plays an important role in the regulation of the expression of the *Klf6* gene and that the disruption of the *Klf6*-related SE decreases the expression of the *Klf6* gene by more than 80%. We next conducted an experiment to determine whether the inhibition of *Klf6* gene expression had any biological relationship with some tumor properties.

### Disruption of *Klf6*-related SE decreases clonogenic potential of HepG2 cells

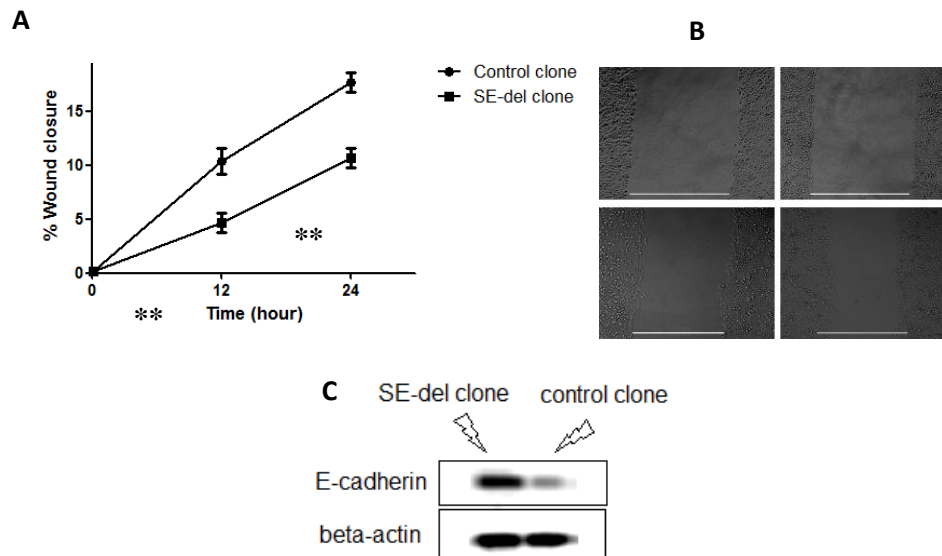
We have evaluated the effect of disruption of the *Klf6*-related ES on cell growth and clonogenic potential of the HepG2 cells, starting from the fact that malignant tumor cells exhibit a high clonogenic potential (Figures 2A and 2B). The results revealed strong inhibition of cell growth, but also remarkable inhibition of clonogenic potential in SE-del clone, compared with the control clone. Therefore, it concluded that disruption of *Klf6*-related SE decreases clonogenic potential of HepG2 cells.



**Figure 2.** Disruption of *Klf6*-related SE decreases clonogenic potential. (A) Effect of *Klf6* related SE on cell growth. Data represent mean $\pm$ SD of three experiments performed in triplicate, \*  $p < 0.05$ , \*\*  $p < 0.001$ . (B) Colony formation assay. The total cell number was counted under microscopy at the times indicated. Data represent mean $\pm$ SD of three experiments performed in triplicate, \*\*  $p < 0.001$ .

### Disruption of *Klf6*-related SE decreases migratory potential

In general, tumor-associated genes can control cell migration in cancer. Therefore, wound-healing assays were performed to examine the effect of *Klf6* related SE on HepG2 cells migration.

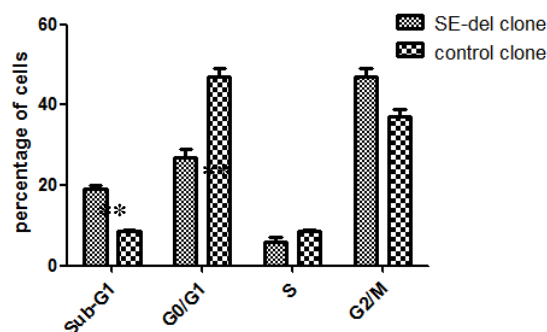


**Figure 3.** Disruption of *Klf6*-related SE decreases migratory potential. Error bars indicate means  $\pm$  SE. \*\*  $p < 0.001$ . (B) Scale bars represent the size at the initial starting point,  $500\mu\text{m}$ . (C) E-cadherin expression levels in the indicated cell clones. E-cadherin were analyzed by Western blot. Beta-actin were used as loading controls.

As shown in the Figures 3A and 3B, migration was decreased by 40-50% in SE-del clone, at 24h, compared with the control clone. The reduction of E-cadherin expression in cancer cells leads to epithelial- mesenchymal transition (EMT) [Hao L et al., 2012]. It also found that SE-del clone exhibited increased E-cadherin expression (Figure 3C). These results indicate that *Klf6*-related SE is involved in the regulation of migration capacity in HepG2 cells.

### Disruption of *Klf6*-related SE induces changes in cell-cycle distribution

To determine the mechanisms of the reduced rate of cellular proliferation, we then conducted cell cycle analysis of SE-del clone and control clone using flow cytometry.



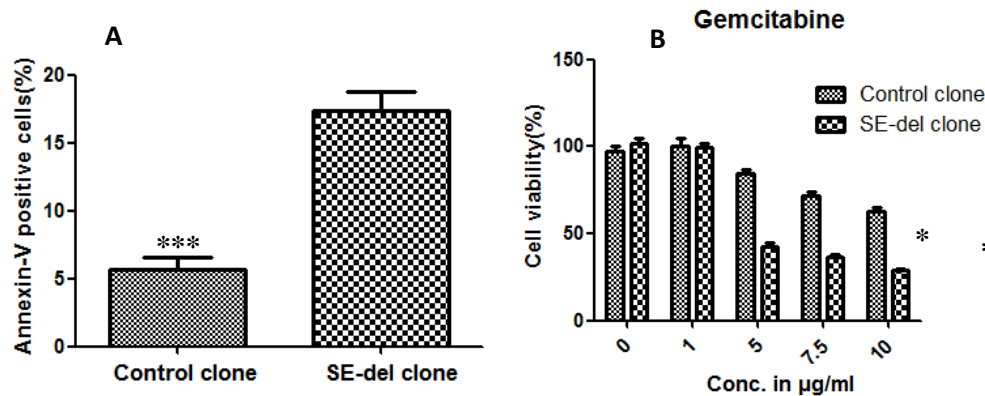
**Figure 4.** Disruption of *Klf6*-related SE induces changes in cell-cycle distribution. Phase of the cell cycle analyzed by flow cytometry. Each panel is representative of three analyses. The graph represents the average percentage of cells in the different phases of the cell cycle obtained from three analyses, \*\*  $p < 0.001$ .

As shown in Figure 4, this reduced proliferation was accompanied by an increased proportion of sub-G1 phase and a lower percentage in Go/G1 phase in SE-del clone. SE-del clone increased the percentage of the total cell population in the sub-G1 phase, decreased the percentage of cells in the Go/G1 phase, but, had no consistent effect on the percentage of cells in the S phase, compared to the controls. Significantly, these data clearly demonstrated that disruption of *Klf6*-related SE increased the sub-G1 apoptotic cell population and decreased

the Go/G1 cell population. These results increase the possibility that inhibition of cell proliferation induced by disruption of *Klf6*-related SE is associated with cell apoptosis.

### Disruption of *Klf6*-related SE induces apoptosis and increases sensitivity to Gemcitabine

To determine the reduced rate of cellular proliferation, we also performed apoptosis analysis of SE-del clone and control clone using flow cytometry (Figure 5A). The increase of SE-del clone suggested a higher apoptotic rate, which was confirmed by Annexin V measurements. Altogether, these findings are in favour of the anti-proliferative and apoptotic effects of the SE-del clone.



**Figure 5.** Disruption of *Klf6*-related SE induces apoptosis and increases sensitivity to Gemcitabine (A) Percentage of apoptotic cells. The percentage of apoptotic cells was determined by cytofluorimetric analysis using Annexin-V labelling. Data represent mean±SD of three experiments performed in triplicate, \*\*\* p < 0.0001. (B) Sensitivity of SE-del clone to Gemcitabine. Data represent mean±SD of three experiments performed in triplicate, \* p < 0.05.

The SE-del clone and control clone were grown in the presence of gemcitabine. After 48 h, cell viability was measured using an MTS assay. The graphs represent the average of three experiments in triplicate and are expressed as the percentage of viable cells relative to this percentage in the corresponding untreated cells (100%). The results observed that SE-del clone increased sensitivity to gemcitabine treatment (Figure 5B).

Although the molecular mechanisms remain to be elucidated, these findings demonstrate that *Klf6*-related SE increased sensitivity to the apoptotic and cytotoxic agent, gemcitabine.

## DISCUSSION

Clarifying the interactions between transcription factors and cancer properties is of great interest in understanding how cancer cells promote formation, development and metastasis through regulation of gene expression. *Klf6* is a transcription factors involved in growth-related signal transduction pathways, cell proliferation, apoptosis, and angiogenesis. *Klf6* has been postulated as a tumor suppressor since its gene is frequently inactivated by loss of heterozygosity (LOH), somatic mutations and/or decreased expression in human cancers [A. Difeo et al., 2009]. But several reports described apoptotic or anti-apoptotic functions for *Klf6*. siRNA-mediated *Klf6* knock down in hepatocarcinoma cell lines lead to a reduction in cell cycle progression and cells became more susceptible to DNA-damage induced apoptosis, indicating that *Klf6* function is required for cell proliferation and survival in these cells [D.S. D'Astolfo et al., 2008; E. Sirach et al., 2007]. Therefore, the exact functions of *Klf6* gene remain controversial. This study used the CRISPR/Cas9 system to establish SE-del clone of *Klf6*-related super-enhancer and examined the function of *Klf6*-related SE in HepG2 cells. We previously used the CRISPR/Cas9 system to identify a transcription factor, *Klf6*-related SE, in HepG2 cells [Kumchol Ri et al., 2017]. During these experiments, it found that disruption of *Klf6*-related SE inhibits cell proliferation and induces apoptosis in in HepG2 cells.

The SE-del clones exhibited decreased *Klf6* gene expression and endogenous *Klf6* protein expression compared to the control clones (Figure 1). These results demonstrate that the *Klf6*-related SE regulates the expression of *Klf6* gene in in HepG2 cells.

In our study, disruption of the *Klf6*-related SE alters cell growth, but also significantly decreased malignant potential, including colony formation (Figure 2), migration (Figure 3), changes in cell-cycle distribution (Figure 4) and drug resistance (Figure 5). These results suggest that the *Klf6*-related SE activates cancer cell properties.

These data are consistent with literatures [D.S. D'Astolfo et al., 2008; E. Sirach et al., 2007] described apoptotic or anti-apoptotic functions for *Klf6*.

Our results demonstrated that the *Klf6*-related SE activates migration capacity via E-cadherin expression. E-cadherin promotes epithelial cell-cell adhesions, and decreased E-cadherin expression is important for EMT [Hao L et al., 2012].

Our data indicate that the *Klf6*-related SE is relevant to drug resistance. These results indicate that the *Klf6*-related SE is involved in the tumorigenic potential of HepG2 cells which is consistent with these previous studies [Benzeno S et al., 2004; E. Sirach et al., 2007; D.S. D'Astolfo et al., 2008].

## CONCLUSION

To sum up, we established the SE-del clone, in which the *Klf6*-related super-enhancer was deleted via the CRISPR/Cas9 system. Our results indicate that the *Klf6*-related super-enhancer activates many properties associated with malignancy in HepG2 cells, including colony formation capacity, migration capability, and drug resistance. However, it is necessary to further explore and define the potential role of the *Klf6*-related super-enhancer transcriptional network in the regulation of *Klf6* signaling in human hepatoma (HepG2) cells.

## ACKNOWLEDGEMENTS

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## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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