Identification of Klf6-Related Super Enhancer in Human Hepatoma (HepG2) Cells by CRISPR Technique

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Genet. Mol. Res. 16 (4): gmr16039841

Received September 11, 2017

Accepted November 08, 2017

Published November 29, 2017

DOI http://dx.doi.org/10.4238/gmr16039841

ABSTRACT. The Klf6 gene is a tumor suppressor gene belonging to the family of the Klf gene and closely associated with tumor formation. Recently, super enhancers(SEs) have been shown to play a particularly important role in regulating cell identity and are attempting to evaluate the in vivo function of SEs. But direct functional evidence of SE associated with Klf6 is lacking. Using genomic editing technology, we have attempted to identify super enhancer associated with the expression of Klf6 and clarify its function in human hepatoma (HepG2) cells. As a result, it identified the Klf6 related SE and demonstrated that the Klf6-related SE is responsible for more than 80% of Klf6 gene expression. It also revealed the hierarchical structure of the Klf-6 related SE and the function of individual enhancers. Our results provide the functional significance of the super enhancer in understanding the transcriptional regulation mechanism of Klf6.

KEY WORDS: Super Enhancer, Klf6, Klf6 related SE, SE

INTRODUCTION

The Klf6 gene is a tumor suppressor gene belonging to the family of the Klf. It is involved in the physiological processes such as cell differentiation and development, cell growth signal transduction, cell proliferation, apoptosis, and angiogenesis [Michaela et al,2006; A.C. Racca et al,2016; Magali et al,2011]. Inhibition of Klf6 gene expression in tumor cells is closely related to tumor formation and development. The Klf6 gene is expressed in several tumours, but its degree of expression is mutually independent [Ricado et al, 2011].

Recently, mutation of the Klf6 gene has been observed in various tumours including liver cancer, stomach cancer, and colon cancer [Ceshi et al, 2003; Magali et al,2011; Sigal et al, 2007]. As a result of mutation, it has been

shown to induce the tumor. This suggests that identifying the transcriptional regulatory mechanism of the klf6 gene is crucial in understanding tumorigenesis [Jaya et al, 2009; Jaya et al, 2009; Analisa et al, 2009].

Enhancer plays a very important role in regulating gene transcription in multicellular animals. Recent genome-wide analyses have revealed that enhancer is very abundant in the genome, and these represent the cell identity in a way that regulates their distinctive transcriptional programs in individual cells [Jonathan et al, 2014; Andrienne et al, 2015; Creyghton et al, 2010]. Recently it has been mentioned as a direct functional evidence that super enhancers play a particularly important role in regulating cell identity and disease [Anna et al, 2014; Marc et al, 2014; Denes et al, 2015; Sophia et al, 2016; Denes et al, 2013].

It has been confirmed that the recently developed crispr/cas9 system can be used as a tool for evaluating enhancer functions in vivo with highly effective genome editing technology [Jialiang et al, 2016; Mali et al, 2013; Cong et al, 2013]. Recent studies have used the CRISPR technique to assess the importance of a SE near GATA2 gene in chronic myeloid leukemia, suggesting that SE is responsible for 80% of GATA2 gene expression [Stefan et al, 2014]. It is also shown that a SE of Sox2 in the mouse gemone is ~100 kb away from the Sox2 gene and is responsible for 90% of the Sox2 gene expression [Yan et al, 2014]. Experimental studies of the super enhancer are now being extensively undertaken, while direct functional evidence of SE associated with Klf6 is lacking.

Based on these developments, we attempted to find an SE associated with the expression of Klf6. It identified a super enhancer that are marked by activated histone markers in regions near Klf6 gene of human hepatoma (HepG2) cells. It demonstrated that the Klf6-related SE is responsible for more than 80% of Klf6 gene expression in hepatoma (HepG2) cells using a double- CRISPR excision method.

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 Cells were cultured as described previously [Michaela et al, 2006]. Importantly, the cells were passaged twice. The cells were transfected for 24 h before transfection. The cells were cultured with CRISPR plasmid and Lipofectamine 2000 (Addgene) for 6 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.

Design of CRISPR constructs

The target-specific CRISPR guide RNAs (sgRNAs) were designed using an online tool (http://crispr.mit.edu/). sgRNAs were designed to optimize uniqueness and to have limited off-target. The designed oligonucleotides were inserted into pSpCas9 (BB)-2A-GFP (PX458) plasmid (Addgene) and used for cell transfection experiments. The characteristics of the designed sgRNA-specifying oligo sequences are shown in Table 1.

Table 1. sgRNA localization, deletion size, chromosome, genomic coordinates (GRCh37/hg19), and sequence for each sgRNA pairs

				SgRNA-A		SgRNA-B	
№		Deletion(kb)	Chr	Genomic Coordinates	Sequence	Genomic Coordinates	Sequence
1	E	29	10	3,873,065-3,873,082	CATATGCTACATACCAGC	3,844,061-3,844,078	CATTTGACCAGCCGCCCG
2	E1	6.5	10	3,850,540-3,850,557	AAGGGCAGGCTCCACGCC	3,844,061-3,844,078	CATTTGACCAGCCGCCCG
3	E2	6.7	10	3,850,540-3,850,557	AAGGGCAGGCTCCACGCC	3,857,219-3,857,236	CGTGCAACGAGTGAATGG
4	E3	6.6	10	3,873,065-3,873,082	CATATGCTACATACCAGC	3,866,418-3,866,435	GGATGCGACAGGGTCCTT
5	E1-2	13.2	10	3,857,219-3,857,236	CGTGCAACGAGTGAATGG	3,844,061-3,844,078	CATTTGACCAGCCGCCCG

PCR and Sanger sequencing for Super enhancer deletion clones

Genomic DNA was isolated from the super enhancer deletion (SE-del) clones using DNeasy Blood & Tissue Kit (Qiagen). To select the deleted clones, the primer flanking the outside of CRISPR sgRNAs were designed and amplified a ~ 29kb region. The size of the amplified product is ~ 920 bp and the primer is 5'-

GCCTGAGATGAGAGTTACAG-3'(forward) and 5'-CTGTACGTTTCGGAAAGT TG-3' (reverse). The PCR condition: after initial incubation at 95 ° C for 5 minutes, a total of 35 cycles were performed, each including 94 ° C for 30 seconds, 55 ° C for 40 seconds, and 72 ° C for 1 min.

In order to evaluate the biallelic or monoallelic deletion characteristics of the target clones, two pairs of primers were designed to flank the inside of the CRISPR sgRNAs and amplified ~ 564 bp and ~ 617 bp products. It was verified by sanger sequencing.

Real-time quantitative PCR

Total RNA was isolated from the target clones using TRIzol (Life Tech) extraction method. A total of 1 μg RNA was used as a template for cDNA synthesis. cDNA (100ng) was used as a template for PCR amplification and SYBR green-based QRT-PCR was performed on an ABI PRISM 7900HT Sequence Detection System.

Primer sequences for the Klf6 genes and beta-actin are as follows: 5′-CGGACGCA CACAGGAGAAAA-3′/5′-CGGTGTGCTTTCGGAAGTG-3′ (KLF6) and 5′-GAAATCGTGCGTG ACATTAAG-3′/5′-CTAGAAGCATTTGCGGTGGACGATGAGGGGCC-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 assay

Western blot assays were performed as previously described [Michaela et al, 2006]. The following antibodies were used: rabbit polyclonal antibody to Klf6 (R-173): goat polyclonal to beta-actin (I-19). Visualisation was performed by the enhanced chemoluminescent method (Amersham International, Freiburg, Germany) and quantified with the BIOQUANT NOVA imaging system (BIOQUANT NOVA PRIME Measurement Software).

Chromosome Conformation Capture

Chromosome conformation capture were performed as previously described [N. naumova et al, 2012]. The cells were cross-linked with 1% formaldehyde in the presence of 10% FBS for 10 min at room temperature and lysed on ice. Collected nuclei were digested with 400 U of HindIII restriction enzyme over night at 37 °C with shaking. Ligation was performed for 4 h at 16 °C and 30min at room temp with 100 U of T4 DNA ligase. DNA was ethanol precipitated and analysed by PCR using primers designed to amplify across junctions of Klf6 promoter and each enhancer (E1, E2, E3). As a control (normal hepatocyte), genomic DNA regions covering restriction sites of interest was purified and analysed by PCR using the same primers.

RESULTS

Identification of Klf6-related super enhancers in various human cancer cell lines

Using CHIP-seq data, it confirmed that a super-enhancer is commonly present in upstream of the Klf6 gene of 16 cancer cell lines [Yanjun et al, 2016]. As a result, it observed a super enhancer conservatively present in 16 cancer cell lines, approximately ~26 kb away from the Klf6 gene (Fig. 1). As shown in Fig. 1, the super enhancer was not observed in adipose, pancreas, spleen, and thymus tissues, unlike in selected cancer cell lines. For example, panc1 has SE but does not exist in normal pancreas. These results show that the super enhancer can be formed by the changes in upstream regions of Klf6 before and after tumor formation. This hypothesis is supported by the literature [Jonathan et al,2014]. This contains a relatively large ~29kb sequence.

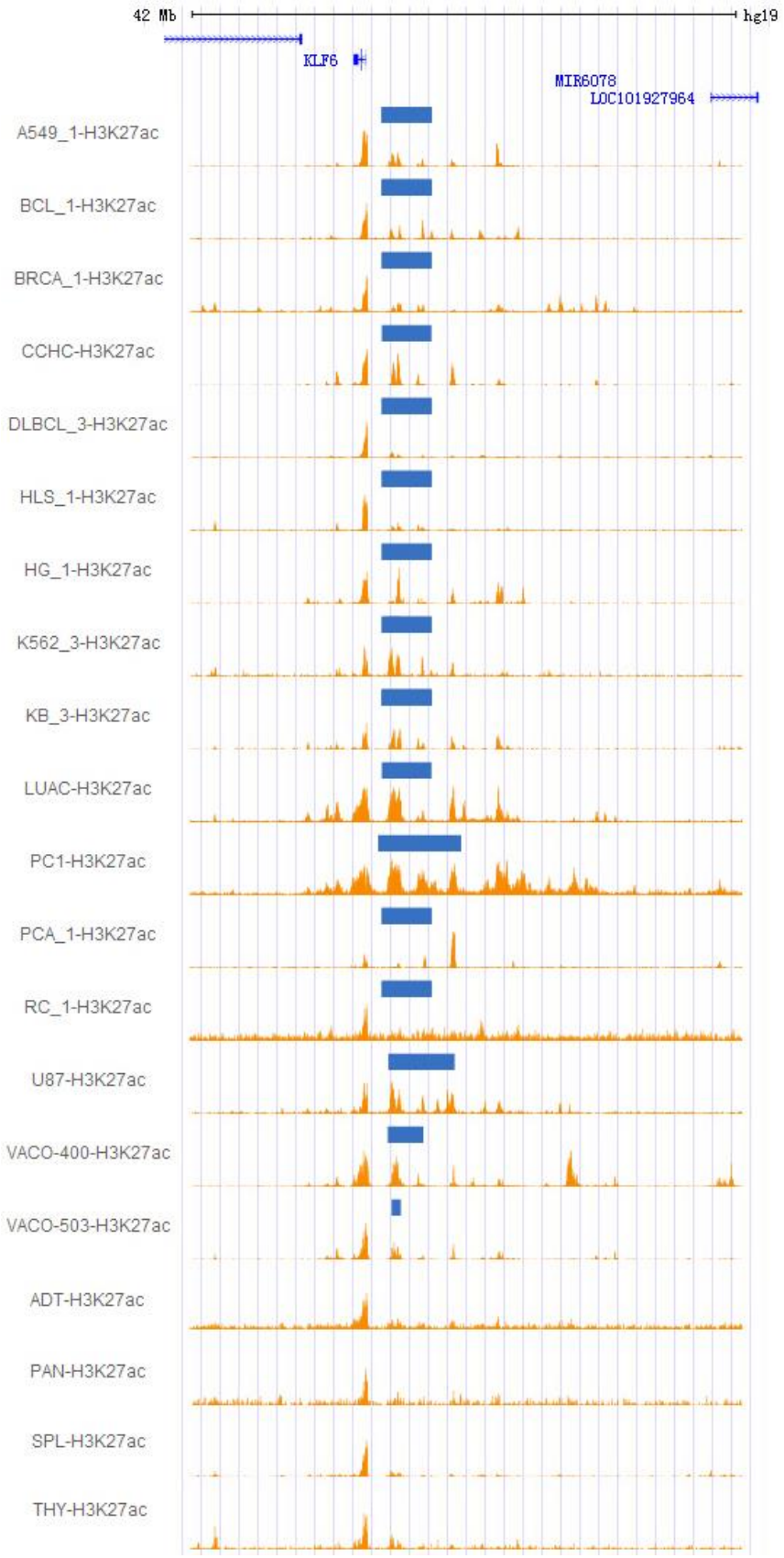


Figure 1. Identification of the conservative super enhancer near Klf6 gene

The figure shows 20 tracks of H3k27ac. The upper 16 tracks show an conservative super enhancer on various cancer cell lines, and the lower 4 tracks have not super enhancer as contrast. The blue bands represent the super

enhancer. These data were obtained from Super Enhancer Archive (SEA, http://sea.edbc.org). Based on this, it selected human HepG2 cells among 16 human cancer cell lines and verified their functional characteristics, assuming that this super enhancer is associated with the expression of Klf6 gene in human HepG2 cells.

CRISPR/Cas9 mediated deletion analysis of the Klf6-related super enhancer

To elucidate the functional properties of the Klf6-related super enhancer, it selected a human HepG2 cells and used CRISPR/Cas9 technology to eliminate the enhancer sequence predicted as a super enhancer. Recently, the CRISPR/Cas9 genome editing technology has been regarded as one of the simplest and most effective methods to delete intron as well as exon. We used this editing technique to obtain 10 HepG2 cell clones. One of 10 clones were a monoallelic clone and 3clones were biallelic clones, as confirmed by PCR analysis and sanger sequencing (Fig. 2). As shown in Fig 2.C and Fig 2.D, our experimental results show that double CRISPR/Cas9 genome editing technology can eliminate large genome sequences with very high efficiency.

RT-qPCR analysis of the Klf6-related SE

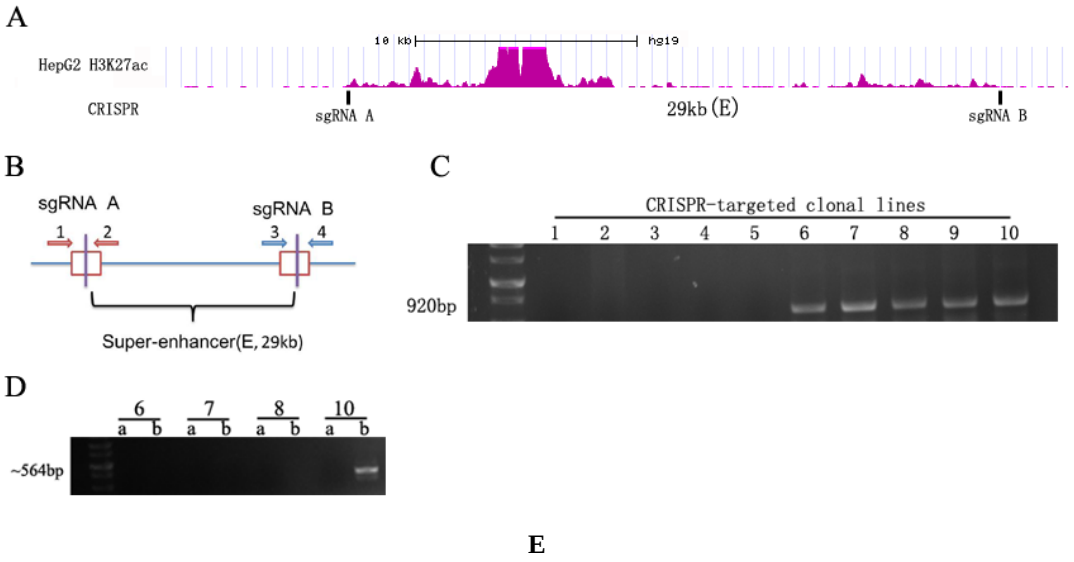
It performed allele-specific RT-qPCR analysis on HepG2 cell clones to quantify the effect of the Klf-related SE on Klf6 gene transcriptions (Fig. 3). In monoallelic SE deletion clone, Klk6 expression decreased to approximately 50% of total Klf6 expression, while in biallelic SE deletion clones, Klf6 expression level decreased by more than 80%. These results show that a single SE regulates transcription of the Klf6 gene in cis. It performed RT-qPCR analysis on a total of 30 upstream and downstream genes, each 3 Mb apart from the Klf6 gene, to further assess the cis effect of SE deletion on transcription. These results also emphasized that the Klf6-related SE is required for the transcription of the Klf6 gene. Importantly, there is no effect on the expression of genes present on the same chromosomes (~6 Mb region) after deletion of the Klf6 related SE. This suggests that the Klf6 gene may be a target gene for the Klf6 related SE.

It next performed western blot analysis to assess the expression level of Klf6 on SE deletion clone (Fig3.C). Western blot analysis was also significantly lower than that of control in the SE-del clone as in RT-qPCR analysis. These results show that the expression of Klf6 is regulated by the Klf6-related super enhancer.

The hierarchical structure of the Klf6-related super enhancer and RT-qPCR analysis

Based on the H3K4me1 and H3K27ac ChIP-seq it divided the super enhancer sequence into three enhancers and designed sgRNAs that flank the individual enhancers (see method). The Figure 4 show the hierarchical structure of the Klf6-related super enhancer and deletion schematic for three enhancers in HepG2 cells. Based on this results, it obtained clones containing biallelic deletion of each enhancer (E1, E2, E3, E1-2).

Next, we isolated RNA from clones and used RT-qPCR analysis to evaluate the effect of individual enhancers on the expression of Klf6 (Fig 5.A). As shown in the figure, the deletion of the individual enhancers surprisingly had a large effect on the expression of Klf6. Among them, the expression of Klf6 was greatly reduced in the E1-del clone and the expression of the E3-del clone was the smallest.



E

Deletion allele clone 5′TGATCTTTGCCATTTGACCAGCCGCCCGAGGGCAGACATGTTC--**29kb**-- ACAGTAATCATGTCATATGCTACATACCAGCAGGGAACAGAG 3′

Biallelic deletion 6 5′TGATCTTTGCCATTTGACCAGCCG ---------- CAGCAGGGAACAGAG 3′

Clone 7 5′TGATCTTTGCCATTTGACCAGCCGC ---------- CAGCAGGGAACAGAG 3′

8 5′ 26bp deletion ---------- 20bp deletion 3′

10 5′TGATCTTTGCCATTTGACCAGCCGCC---------- GCAGGGAACAGAG 3′

Figure 2. The Klf6-related super enhancer deletion by CRISPR/Cas9 system

(A) schematic of CRISPR-mediated SE deletion in HepG2 cells. This figure shows two guide RNAs flanking ~29kb Klf6-related SE. (B) SE deletion verification by PCR primers flanking sgRNA A and sgRNA B. (C) primers flanking 29kb Klf6- related SE were used to amplify genomic DNA isolated from 10 clones. Successful deletion (monoallelic or biallelic) results in a ~ 920 bp PCR product. (D) Two pairs of primers (a, b) flanking inside of sgRNA A and sgRNA B were used to amplify genomic DNA isolated from five clones. Biallelic does not produce ~ 564 bp and ~ 617 bp products, but monoallelic produces at least ~ 564 bp or ~ 617 bp products. Clone 9 was excluded from the experiment due to contamination during operation. (E) Sequencing of the deletion allele in biallelic deletion clones. SgRNAs are shown in blue and PAM sequences in red. Deletion sequences are shown by dash marks and highlighted in green.

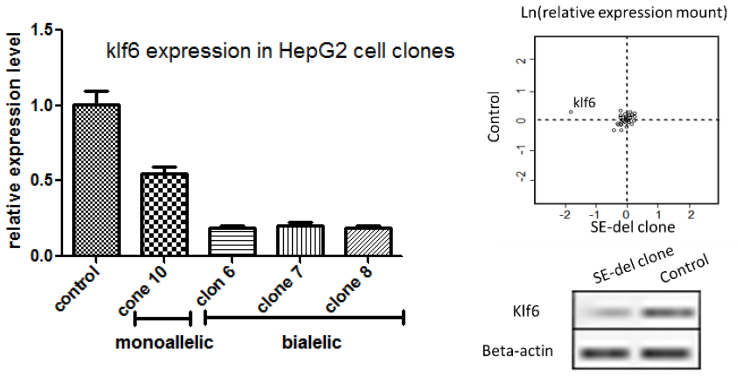


Figure 3. Klf6-related SE regulates Klf6 expression in cis.

(A) allele specific RT-qPCR analysis in HepG2 cell clones. (B) comparison of allele specific RT-qPCR analysis in the SE deletion clones and control clone. Allele features were defined as the Ln value of relative expression amount. 31 genes on chromosome 10, including the Klf6 gene, were plotted. Only the Klf6 gene is shown to be affected by SE, which ultimately emphasizes the direct regulation of SEs associated with Klf6. (C) western blot analysis of Klf6 expression in SE deletion clone and control clone. Beta-action blotting were used as loading controls.

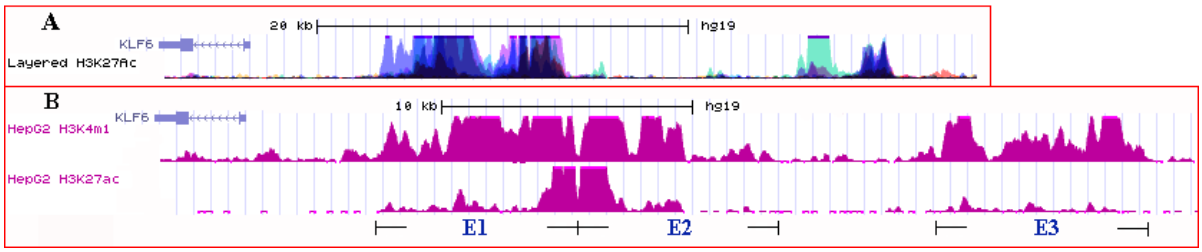


Figure 4. Schematic of the hierarchical structure of the Klf6-related super enhancer

(A) The layered H3K27ac ChIP-seq of 16 cancer cell lines. It shows that there are three enhancers in the Super enhancer region. Based on this, a design for deleting the individual enhancers was conducted. (B) Deletion schematic for three enhancers in HepG2 cells. These data were obtained from UCSC Browser (http://genome.ucsc.edu)

These results suggest that the enhancers far from the Klf6 gene are less likely to regulate the expression of the Klf6 gene. Eventually, the E1 enhancer shows that it is functionally stronger than its neighboring enhancers in regulating transcriptional activation.

Thus, our enhancer deletion assays demonstrate that the Klf6-related SE is composed of a functional hierarchy containing components that modulate the transcriptional activation of Klf6.

Next, it performed chromosome conformation capture assay to elucidate the physical interaction between the enhancers and the Klf6 promoter (Fig 5.B). This result highlight that the Klf6-related SE regulates Klf6 gene through long-range DNA looping.

DISCUSSION

We identified and the Klf6-related super enhancer that regulates Klf6 expression in human HepG2 cells. The Klf6-related super enhancer we identified is a H3K27ac-rich intronic region (~ 29kb) that fits the recently defined super enhancer criteria. Recently, several studies have suggested that super enhancers play an important role in maintaining and regulating cell identity in various tumor cells and stem cells, and have also attempted to establish methods of drug development and treatment targeting these SEs [Jakob et al, 2013]. Our experimental results are merely functional study data of SE supporting the experimental direction of super enhancers.

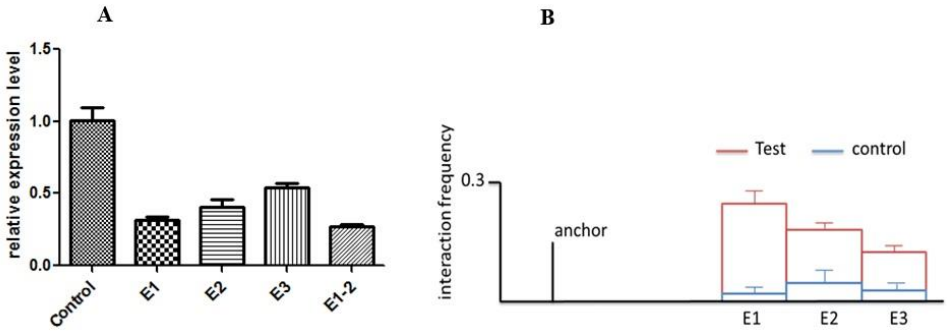


Figure 5. RT-qPCR analysis and 3C assay of enhancers(A) RT-qPCR analysis of enhancers. (B) chromosome conformation capture assays(n=3) of enhancers. The 3C results were shown as interaction frequency ± SEM(n=3). The primers target the Klf6 promoter and the enhancer regions.

It observed that an Klf6-related super enhancer present in 16 cancer cells was not present in normal cells (Fig. 1). This suggests that the Klf6-related super enhancer can be formed in the posterior and posterior processes of tumor formation in normal cells. Some researchers [Emily et al, 2015] have found evidence of an epigenetic basis for the transition between WDLPS (well differentiated LPS) and DDLPS (dedifferentiated LPS) in Liposarcoma (LPS). They observed elevated H3K9me3 levels in DDLPS tumours and found that increased H3K9me3 may mediate through Klf6 and these increased H3K9me3 sites are associated with super enhancers as the upstream region of Klf6. These experimental data support our hypothesis on the formation of the Klf6-related super enhancer [Jonathan et al, 2014]. Based on these studies, it would be very interesting to further study the process of tumor formation with the super enhancer.

Next, it successfully deleted ~29 kb non-coding region using the CRISPR/Cas9-mediated genomic deletion method. It found that the deletion of the Klf6-related super enhancer reduced by more than 80% of the Klf6 expression and did not affect the expression of other neighboring genes (Fig.3). This suggests that the Klf6-related super enhancer modulates the expression of Klf6 in cis.

In addition, with the remarkable effectiveness of the CRISPR/Cas9 system, it could further elaborate the hierarchy of the Klf6-related super enhancer. Based on H3K4me1 and H3K27ac ChIP-seq, it identified 3 enhancers in the Klf6-related super enhancer and found that these genes also functioned to regulate the expression of Klf6. As a result, it was confirmed that the Klf6-related super enhancer was composed of 3 enhancers and regulated the expression of Klf6 (Figure 5).

In addition, chromosome conformation capture assay (3C) analysis revealed that Klf6-related SE regulates the Klf6 gene through long-range DNA looping. finally, it believes that the CRISPR / Cas9 system can be a powerful tool in studying in vivo super enhancer function. There are several problems to be solved the function of the Klf6-related super enhancer in the future. What are the transcription factors involved in the expression of Klf6, and what are its functional interactions? Resolving these problems will be very robust in understanding the function of super enhancer and applying it to future applications.

ACKNOWLEDGEMENTS

We thank Ph.D. wuqiong for guidance in this search. This work was supported by the Program for Chang jiang Scholars and Innovative Research Team in University (IRT1220) and the National Natural Science Foundation of China (Grant No. 54289562).

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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