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Research paper

Minimal essential region for *krüppel-like factor 5* expression and the regulation by specificity protein 3-GC box binding

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ABSTRACT

Krüppel-like factor 5 (KLF5) transcriptionally controls the proliferation-differentiation balance of epithelium and is overexpressed in carcinomas. Although genomic region modifying *KLF5* expression is widespread in different types of cells, the region that commonly regulates basal expression of the genes across cell-types is uncertain. In this study we determined the minimal essential region for the expression and its regulatory transcription factors using oral carcinoma cells. A reporter assay defined a 186 bp region downstream of the transcription start site and a cluster of six GC boxes (GC1-GC6) as the minimal essential region. Mutation in the GC1 or GC6 regions but not other GC boxes significantly decreased the reporter expression. The decrease by the GC1 mutation was reproduced in the 2 kbp full-length promoter, but not by the GC6 mutation. Additionally, specificity proteins (Sp) that can be expressed in epithelial cells and bind GC box, Sp3 co-localized with KLF5 in oral epithelium and carcinomas and chromatin immunoprecipitation analyses showed Sp3 as the prime GC1-binding protein. Inhibition of Sp-GC box binding by mithramycin A and knockdown of Sp3 by the short interfering RNA decreased expression of the reporter gene and endogenous KLF5. These data demonstrate that a 186 bp region is the minimal essential region and that Sp3-GC1 binding is essential to the basal expression of *KLF5*.

1. Introduction

Development and maintenance of squamous epithelium requires strict regulation and appropriate expression of epithelial genes including regulatory transcription factors. Aberrations in the program cause severe developmental failures associated with progression of carcinomas (Hanahan and Weinberg, 2011). Numerous transcription factors involved in epithelial development have been reported, including krüppel-like factors (KLFs), three C_2H_2-zinc finger transcription factors consisting of 17 homologues in mammals. KLFs regulate target gene transcription through binding the CACCC box and GC box in the promoters. Squamous epithelial cells preferentially express KLF4 and KLF5 in their developmental process, KLF4 in the differentiating suprabasal cells and KLF5 in the proliferative basal cells. KLF5 down-regulation decelerates epithelial cell proliferation and stimulates differentiation (McConnell and Yang, 2010). Mice overexpressing KLF4 and KLF5 in the basal cells exaggerate keratinization and proliferation of the skin epithelial cells, respectively (Jaubert et al., 2003; Sur et al., 2006). Although $KLF5^{-/-}$ mice are embryonic lethal, $KLF5^{+/-}$ mice and the conditional homozygous deletion in the intestine exhibit aberrant structures and homeostasis of the intestinal epithelium (Nandan et al., 2014; McConnell and Yang, 2010). $KLF5^{+/-}$ mice rescue tumor formation by Apc^{Min} mutation (McConnell et al., 2009). These emphasize an important role of *KLF5* in epithelial tissues and the carcinogenesis.

Squamous cell carcinomas of the oral cavity are the most frequently observed malignant tumors of the head and neck, but the patients' prognoses have not been sufficiently improved (Choi and Myers, 2008; Rivera, 2015). Expression of KLF4 and KLF5 is unbalanced in the carcinomas. Specifically, KLF5 overexpression disturbs desmosome formation (Shibata et al., 2015). Moreover, KLF5 stimulates proliferation, dedifferentiation and migration of carcinoma cells (McConnell and Yang, 2010). Therefore KLFs have been identified as potential therapeutic targets for patients with malignant tumors (Tetreault et al., 2013; Farrugia et al., 2016). These facts suggest that understanding the basic mechanism of *KLF5* expression may contribute to development of a novel strategy to treat the patients.

Spatio-temporal gene expression is largely affected by far distal elements wide-spread in the genome depending on cell-types. The region that controls the basal expression is commonly located in a narrow range near the transcription start site. It is referred to the minimal

Abbreviations: KLF, Krüppel-like factor; Sp, specificity proteins; MER, minimal essential region; PCR, polymerase chain reaction; Luc, luciferase; siRNA, short interfering RNA; RLA, relative Luc activity; ChIP, chromatin immunoprecipitation.

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essential region (MER). In TATA-less genes such as *KLF5*, the MER is preferentially present in GC-rich region (Kim and Shiekhattar, 2015; Danino et al., 2015). Defining the MER and the transcription factor involved in the process provides crucial information to understanding the mechanism of gene expression and function. Despite the significance in pathophysiology of the epithelium, a mechanism for *KLF5* basal expression is largely unknown. Previous studies documented that the regulatory regions are scattered in genome depending on cell-types (Oishi et al., 2010; Federzoni et al., 2014; Chen et al., 2004), but the MER has not been identified. In the present study, we describe a 186 bp region juxtaposed to the transcription start site is the MER, and examine the regulation by transcription factors.

2. Materials and methods

2.1. Cells and constructs

Immortalized cell lines of oral carcinomas (Ca9.22, Ho-1-u-1, HOC313, HSC2, HSC3, KOSC2, OSC19, and TSU), and MCF7, DLD1, T24, HEK293 and HaCaT cells were cultured in the presence or absence of 100 nM mithramycin A (Merck Millipore). A human bacterial artificial chromosome clone RP11-138K4 was used to amplify a 2 kbp region encompassing a transcription start site of *KLF5* gene (−1576 to +424; transcription start site was designated to as +1) by polymerase chain reaction (PCR) using KOD-FX DNA polymerase (TOYOBO). The position of bases was determined by comparison to a sequence deposited in GenBank database under GenBank Accession Number NM_001730. Eight truncated fragments starting from different upstream sites and stopping at +424 were generated. PCR primers were designed to have *Kpn* I- and *Xho* I-susceptible sites at the 5′ end of forward and reverse primers, respectively (Table 1). After digestion of PCR amplicons with *Kpn* I and *Xho* I, they were ligated to upstream of Luciferase (Luc)-reporter genes in pGL4.10 plasmid (Promega). We referred plasmids with the full-length region to clone #1 and the truncated fragments to clones #2–9 (Fig. 1A). Site directed mutagenesis at GC boxes was carried out by PCR using primer sets listed in Table 1. All constructs were confirmed to have intended DNA sequence by sequence analysis. *KLF4* and *KLF5* expressing plasmids were generated in a previous study (Shibata et al., 2015). Short interfering RNA (siRNA) for *specificity protein 1 (Sp1*, VHS40867 form Invitrogen), *KLF4* (s17793) or *KLF5* (s2116 from Applied Biosystems), negative control siRNA (siGENOME Non-Targeting siRNA #1 from Dharmacon), and a custom siRNA for *Sp3* (5′-GCGGCAGGUGGAGCCUUCACUdTdT-3′; Chadalapaka et al., 2008) were transfected into cells and used for subsequent experiments 48 h after the transfection.

2.2. Reporter assay

Cells were transfected with Luc-reporter and renilla control plasmids, and harvested after 24 h, and standardized by renilla reporter expression for relative Luc activity (RLA) using Dual-Luciferase Reporter Assay System (Promega). To analyze fold changes of RLA, a Luc-reporter assay was performed 24 h after *KLF* cDNA transfections or 48 h after siRNA transfection. Mithramycin A was added into the culture medium 20 h before reporter plasmid transfection according to a previous study (Lee et al., 2006).

2.3. Western blot

Total cell proteins were separated by size with SDS-PAGE electrophoresis and electrotransferred to polyvinylidene difluoride membranes. The membranes were probed with antibodies to KLF5 (sc-22797), Sp1 (sc-59), Sp3 (sc-644 from Santa Cruz Biotechnology), or β-actin (C-15 form Sigma-Aldrich) followed by horseradish peroxidase-labeled secondary antibodies (GE Healthcare Life Science). The binding was detected by Chemi-Lumi One Super (Nacalai Tesque) and visualized using Ez-Capture MG (ATTO).

Table 1
PCR primer sequences.

Subjects	Primers	Sequence
Reporter assay[a]	Clone #1 F	5′-GAGGTACCAGGTTTGATGTTATCAG-3′
	Clone #2 F	5′-GAGGTACCCTACCCATTGAAAATGAG-3′
	Clone #3 F	5′-GAGGTACCTGGATTCCCAGTTTTCAGAG-3′
	Clone #4 F	5′-GAGGTACCAATCCTGAAGGAGTGG-3′
	Clone #5 F	5′-GAGGTACCAGGTCCTTGCACAACACCTAAG-3′
	Clone #6 F	5′-GAGGTACCTGTGTACAAACTGCGCGGC-3′
	Clone #7 F	5′-GAGGTACCTGCCAATCAGGCGATC-3′
	Clone #8 F	5′-GAGGTACCTTCTCTCGCGGAGGTCG-3′
	Clone #9 F	5′-GAGGTACCAGGACGTTGGCGTTTACGTG-3′
	Clones #1–9 R	5′-GATCTCGAGCGGGTGGACTCCTCA-3′
Mutagenesis[b]	mGC1 F	5′-GGCGATCGGGCCCCG**AA**CCCCCGGAGTTGGGT-3′
	mGC2 F	5′-GTTGGGTGAAATAGA**TT**CG**TT**CGTCAAGTGTC-3′
	mGC3 F	5′-AGTGTCAGTAGTCGCGG**AT**C**TA**GTACGTGCGCTC-3′
	mGC4 F	5′-TCTCTCGCGGAGGTC**TT**CGGT**TT**CGGGAGCGG-3′
	mGC5 F	5′-GCGGAGGTCGGCGGT**TT**CGGGAGCGGGCTCCG-3′
	mGC6 F	5′-AGAGCACGGTGG**TT**CGG**TT**CGGGAGAAAGTGG-3′
	mGC1 R	5′-ACCCAACTCCGGGGG**TT**CGGGGCCCGATCGCC-3′
	mGC2 R	5′-GACACTTGACG**AA**CG**AA**TCTATTTCACCCAAC-3′
	mGC3 R	5′-GAGCGCACGTAC**TA**G**AT**CCGCGACTACTGACACT-3′
	mGC4 R	5′-CCGCTCCCG**AA**ACCG**AA**GACCTCCGCGAGAGA-3′
	mGC5 R	5′-CGGAGCCCGCTCCCG**AA**ACCGCCGACCTCCGC-3′
	mGC6 R	5′-CCACTTTCTCCCG**AA**CCG**AA**CCACCGTGCTCT-3′
RT-PCR	Sp1 F	5′-CCCCAGGTGATCATGGAGC-3′
	Sp3 F	5′-CGGAGGGTAGCTTGCACCTGTC-3′
	GAPDH F	5′-GTCAGTGGTGGACCTGACCT-3′
	Sp1 R	5′-CTTCTCACCTGTGTGTGTACG-3′
	Sp3 R	5′-GGATATCTTGATTGCTGGTGGC-3′
	GAPDH R	5′-AGGGGAGATTCAGTGTGGTG-3′
ChIP[c]	Endogenous F	5′-TCTCCCTGCTCATAGGCTGG-3′
	Endogenous R	5′-ACGTAAACGCCAACGTCCTCCG-3′
	Clone #7 F	5′-TGGCTGCCTCTCTCCCTGCTCATAG-3′
	Clone #7 R	5′-ACTCTTCCGCTCTTCCACAC-3′

Forward primer (F), reverse primer (R).
[a] *Kpn* I-susceptible and *Xho* I-susceptible sequences in forward and reverse primers were underlined, respectively.
[b] Substitution of nucleotides in GC box mutants (mGCs) were shown in bold.
[c] Primers for amplification of the minimal essential region of *KLF5* in endogenous gene and clone #7 were used for conventional and quantitative ChIP analyses, respectively.

2.4. Chromatin immunoprecipitation (ChIP) analysis

Approximately 5×10^6 HSC2 cells were fixed with 1% formaldehyde for 10 min at 37 °C and collected in ice-cold lysis buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, pH 8.0, 1% SDS, 0.8 μM aprotinin, 50 μM bestatin, 20 μM leupeptin, 10 μM pepstain A, 1 mM PMSF). DNA was isolated and sonicated to an approximate length of 500–1000 bps. Sonicated samples precleared with Dynabeads M-280 (Life Technology) were immunoprecipitated overnight by 2 μg of anti-Sp1 or -Sp3 antibodies, or normal rabbit IgG (Santa Cruz Biotechnology). Immune complexes were captured with Dynabeads M-280 slurry for 2 h at 4 °C, and washed by low salt buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton-X100, 0.1% SDS, 0.1% sodium deoxycholate), high salt buffer (50 mM Tris-HCl, pH 8.0, 500 mM NaCl, 1 mM EDTA, 1% Triton-X100, 0.1% SDS, 0.1% sodium deoxycholate), Li-Cl buffer (10 mM Tris-HCl, pH 8.0, 0.25 M LiCl, 1 mM EDTA, pH 8.0, 0.5% NP40, 0.5% sodium deoxycholate), and TE buffer. Antibody-DNA complexes were reverse cross-linked for 10 h in elution buffer (10 mM Tris-HCl, pH 8.0, 300 mM NaCl, 5 mM EDTA, pH 8.0, 0.5% SDS). After treatments with DNase-free RNase and proteinase K, DNA was purified by phenol/chloroform extraction. Precipitated DNA was amplified with PCR using primer sets (Table 1).

To quantify the Sp binding, precipitated DNA was applied for quantitative PCR using KOD-SYBR premix (TOYOBO) and StepOne Real-