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Transcriptional activation of the senescence regulator Lsh by E2F1

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ABSTRACT

Lsh, a protein related to the SNF2 family of chromatin-remodeling ATPases, is a major epigenetic regulator that is essential for DNA methylation and histone acetylation at repetitive elements. Lsh represses endogenous $p16^{INK4a}$ expression by recruiting HDAC to the $p16^{INK4a}$ promoter, which in turn delays cell senescence. However, the molecular mechanisms that govern loss of Lsh expression during cellular senescence have yet to be elucidated. Here we investigate the transcriptional regulation of the human Lsh promoter. We find that the minimal Lsh promoter is located between positions −216 and −119 relative to the transcription start site, and contains two putative E2F binding sites. Ectopic E2F1 increases expression of Lsh at both transcriptional and translational levels. E2F1 physically interacts with the Lsh promoter by binding to each of the two putative binding sites and transactivates the Lsh promoter. E2F1 also induces Lsh protein expression and transactivates the Lsh promoter in 2BS cells. At the same time, E2F1-induced Lsh promoter activity is reduced in senescent cells compared to young cells. These results indicate that E2F1 plays a crucial role in transcriptional control of the human Lsh gene and the decrease of Lsh expression in senescent cells is related to the repression of E2F1.

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1. Introduction

Lsh (lymphoid specific helicase; also known as PASG or Hells) is a member of the SNF2/helicase family of chromatin remodeling proteins (Geiman et al., 1998; Jarvis et al., 1996). Lsh is ubiquitously expressed during embryogenesis and Lsh-deficient mice display multiple developmental defects and early lethality (Dennis et al., 2001; Fan et al., 2003; Geiman and Muegge, 2000; Geiman et al., 2001; Sun et al., 2004). Lsh plays an important role in CpG methylation in mice and may be directly involved in control of *de novo* methylation of genomic DNA (De La Fuente et al., 2006; Dennis et al., 2001; Muegge, 2005; Xi et al., 2009; Zhu et al., 2006). Lsh is also linked to cell proliferation and disruption of Lsh in mice leads to premature aging which is associated with decreased proliferation and increased replicative senescence (Raabe et al., 2001; Sun et al., 2004). Imperfect maintenance of genome integrity has been postulated to be an important cause of senescence and premature aging (Hasty et al., 2003). Recently, Zhou et al. (2009) reported that the level of Lsh protein in senescent 2BS cells is much lower than that in young cells. The decrease of the level of Lsh protein reduces repression of $p16^{INK4a}$ in senescent 2BS cells, which leads to cellular senescence. However, little is known at present as to why Lsh expression declines during cellular senescence.

The E2F1 transcription factor plays a central role in cell cycle control, apoptosis and differentiation (Bell and Ryan, 2004; DeGregori et al., 1997; Muller et al., 2001). Recent studies concerning the functions of E2F1 gene seem to be contradictory as this gene was described both as an oncogene as well as a tumor suppressor gene (Phillips et al., 1997; Xu et al., 1995). As an oncogene, overexpression of E2F1 can promote progression of cells from the G_0 phase to the S phase (Johnson et al., 1993; Kowalik et al., 1995). Cells transfected with E2F1 can form colonies in soft agar, induce tumor formation in nude mice and, in cooperation with activated *ras*, transform rat embryo fibroblasts (REFs) (Johnson et al., 1994). E2F1 overexpression has been observed in HEL erythroleukemia cells and its overexpression can cause neoplastic transformation in astrocytes *in vitro* (Miyajima et al., 1996; Saito et al., 1995). However, in other circumstances, E2F1 possesses the ability to induce p53-dependent and p53-independent apoptosis, a function which implies a putative tumor suppressor activity (Hsieh et al., 2002; Kowalik et al., 1995; Nahle et al., 2002; Wu and Levine, 1994). The nature of this duality is likely to depend on the cellular context.

Cellular senescence was first described in normal human fibroblasts by Hayflick and Moorhead for cells able to enter a state of irreversible growth arrest after serial cultivation (Hayflick, 1965; Pazolli and Stewart, 2008). Compared to young cells, the

Abbreviations: Lsh, lymphoid specific helicase; RT-PCR, reverse transcription-polymerase chain reaction; ChIP, chromatin immunoprecipitaion; EMSA, electrophoretic mobility-shift assay.

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expression levels of several genes are altered during cellular senescence. Cell cycle regulatory proteins such as Cyclin A, CAK, Cdc2, E2F1, E2F2 and PCNA showed decreased expression. However, Cyclin-dependent kinase (CDK) inhibitors, including $p16^{INK4a}$, $p21^{Cip1}$and $p27^{Kip1}$ have increased expression levels (Wang et al., 2001, 2005; Zheng et al., 2006). E2F DNA binding activity and E2F1 mRNA expression are markedly increased in epithelial carcinoma cells (Dicker et al., 2000; Jones et al., 1997). When E2F1 function or E2F DNA binding activity is blocked, cell growth is inhibited (Chiang et al., 1997, 1998; Ishizaki et al., 1996; Maeshima et al., 1998; Morishita et al., 1995).

We show here that E2F1 is a transcription factor regulating Lsh expression. In senescent 2BS cells, reduced expression of Lsh is associated with decreased expression of E2F1.

2. Materials and methods

2.1. DNA constructs

A 1894 bp fragment ranging from position −1849 to +45 relative to the transcription start site of the human Lsh gene was amplified and cloned into the KpnI and HindIII restriction sites of the PGL3-basic vector (Promega, Madison, WI, USA). The resulting construct was designated pGL3-1849. As template for PCR, genomic DNA was isolated from the 2BS (Human embryonic lung diploid fibroblast) cell line. Using a standard PCR technique, sequential deletions from the 5′ end of the human Lsh promoter were performed to generate ten truncated forms of the promoter, designated pGL3-1478, pGL3-1226, pGL3-885, pGL3-519, pGL3-247, pGL3-216, pGL3-183, pGL3-119, pGL3-103, pGL3-49. Primers for the construction of various reporter plasmids are shown in Table 1. To obtain a pGL3-1894 clustered mutation, point mutations were introduced into the putative E2F binding sites using the QuikChange Directed Mutagenesis Kit (Stratagene). Synthetic oligonucleotides that contained the desired bases were used for mutagenesis. The sequence of mutations for nucleotides at positions −136 and −135 (GC-AA mutation) and at positions −209 and −208 (CG-TC mutation) are listed in Table 1. DNA containing the desired mutations was transformed into XL1-Blue supercompetent cells. The constructs were designated Lsh mt1 and Lsh mt2 respectively. All plasmids were verified by restriction digestion and/or DNA sequencing. All primers were synthesized by Sunbio Biotechnology (Beijing, China).

2.2. Cell culture and gene transfection

Human embryonic kidney HEK293 cells, and human embryonic lung diploid fibroblast 2BS cells were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO BRL, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 units/ml streptomycin at 37 °C in 5% CO_2. 2BS cells were previously isolated from female fetal lung fibroblast tissue and have been fully characterized (Tang et al., 1994). Gene transfection was conducted using Lipofectamine 2000 (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's protocol. Pools of stable transformants were obtained by sustained selection with the G418 reagent (Life Technologies, Grand Island, NY, USA).

2.3. Reverse transcription-PCR (RT-PCR)

Total RNA was isolated from HEK293 cells using the RNeasy kit (QIAGEN). After denaturing the total RNA at 70 °C for 10 min, cDNA was synthesized with oligo-dT primer and reverse transcriptase. PCR amplification was performed using specific primers: for Lsh (F: 5′-CGTTTATGCTCCACTTTC-3′; R: 5′-GTCCACCTCTGGCTGTAT-3′); for E2F1 (F: 5′-ACTGAATCTGACCACCAAGCG-3′; R: 5′-CAGGGTCTGCAATGC-TACGA-3′); for GAPDH (F: 5′-CGAGTCAACGGATTTGGTGGTAT-3′; R: 5′-AGCCTTCTC-CATGGTGAAGAC-3′). PCR products were electrophoretically separated on an agarose gel and stained with ethidium bromide for visualization.

Table 1
Primer sequences used for DNA constructs.

Plasmids name	Primer sequence
pGL3-1849	F: 5′-CGGGGTACCAGACAAAGTGTAGCTTTATCA-3′
pGL3-1849	R: 5′-CCCAAGCTTTTTTCCTCACTCACCGCCGCT-3′
pGL3-1478	F: 5′-CGGGGTACCGTGGCACGCACCTGTAGT-3′
pGL3-1226	F: 5′-CGGGGTACCCCCAGGTTCAGGCGATTC-3′
pGL3-885	F: 5′-CGGGGTACCAGAGGGAGACAAAGGAAATA-3′
pGL3-519	F: 5′-CGGGGTACCCTGTTTCTGACATCCTCGTG-3′
pGL3-247	F: 5′-CGGGGTACCGGTTGCCACAGGTCAGTC-3′
pGL3-216	F: 5′-CGGGGTACCCAAGCTGCGCCAAATCTC-3′
pGL3-183	F: 5′-CGGGGTACCGAGGGCGGGTGGGGAATGT-3′
pGL3-119	F: 5′-CGGGGTACCTTTTTTCCCTGGCGGGGG-3′
pGL3-103	F: 5′-CGGGGTACCGATTTGGCTAGAAGGCTG-3′
pGL3-49	F: 5′-CGGGGTACCTTGCAGGCTCTGAGAGGA-3′
Lsh mt1	F: 5′-TGACAGGATTTTCCCAAGAAGGAGAAG-3′
Lsh mt1	R: 5′-TTGGGAAAATCCTGTCATCTCGCGATA-3′
Lsh mt2	F: 5′-GAAAACCGCAAGCTGTCCCAAATCTCG-3′
Lsh mt2	R: 5′-GACAGCTTGCGGTTTTCGTCAGGACTG-3′

2.4. Western blotting

Cells were lysed in modified Radioimmune Precipitation Assay (RIPA) Buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.25% Na-deoxycholate, 1% NP-40, 0.2 mM sodium orthovanadate, 1 mM NaF) containing a protease inhibitor cocktail (Roche, Indianapolis, IN). Forty μg of total protein were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membrane (Millipore). Immunodetection was performed by incubating overnight at 4 °C with primary antibodies anti-p16 (sc-468), anti-p21 (sc-2646), anti-p53 (sc-126), anti-Lsh (sc-28202), or anti-E2F1 (sc-251), followed by rinses and addition of HRP-conjugated secondary antibodies (Zhongshan, China). Incubation with anti-β-actin (sc-1616) (Santa Cruz) served as a loading control. Proteins were visualized using SuperSignal WestPico Chemiluminescent Substrate (Pierce) according to manufacturer's instructions.

2.5. Luciferase assay

Cells were plated in 24-well culture plates in triplicate for each condition at an initial concentration of 5×10^4 cells/well. Cells were co-transfected with 0.8 μg of Lsh reporter constructs (or 0.2 μg Lsh reporter constructs and 0.6 μg pcDNA-E2F1/pcDNA-E2F1-132E expression plasmid) and 8 ng (or 2 ng) of Renilla luciferase reporter plasmid pRL-CMV vector which served as an internal control. Luciferase activity was assessed with a dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. The enzyme activity was normalized for efficiency of transfection on the basis of Renilla luciferase activity levels and reported as relative light units (RLU). All reporter assays were performed in triplicate on at least two individual experiments and standard errors are denoted by bars in the figures.

2.6. Chromatin immunoprecipitation (ChIP)

ChIPs were performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions with minor modifications. 1×10^6 cells were used for each experimental condition. Cells were sonicated and lysates immunoprecipitated using the indicated antibodies anti-E2F1 (KH-95) (Santa Cruz). DNA isolated from the immunoprecipitates was amplified by traditional PCR using as forward primer the oligo (F: 5′-TACGGTTGCCACAGGT-CAGT-3′) from position −250 of the Lsh promoter, and as reverse primer the oligo (R: 5′-CAGCCTTCTAGCCAAATCCC-3′) from position −85 of the promoter. Input DNA as well as DNA immunoprecipitated by anti-IgG served as positive or negative control, respectively. The primer for β-actin promoter were: F: 5′-ACGCCAA AACTCTCCCTCCTCCTC-3′, S: 5′-CATAAAAGG CAACTTTCGGAACGGC-3′.

2.7. Electrophoretic mobility shift assay (EMSA)

Human GST-E2F1 fusion protein was produced from growing *Escherichia coli* BL21 transfected with pGEX-4T-E2F1. EMSA probes were generated by end-labelling 30 bp duplexes using T4 polynucleotide kinase and [γ-^{32}P]ATP. For E2F1-binding activity analysis, the sequences of the sense strand of double-stranded oligonucleotides used as probes or competitors were as follows: −124 to −153 wild type, 5′-GATGACAGGATTTTCCCGCGAAGGAGAAGC-3′, and −124 to −153 mutated sequence, 5′-GATGACAGGATTTTCCC**AA**GAAGGAGAAGC-3′. For −192 to −221 probe, the sequence of wild type and mutant response element (sense strand) were 5′-AACCGCAAGCTGCGCCAAATCTCGCGCAGC-3′ and 5′-AACCGCAAGCTG**TC**C-CAAATCTCGCGCAGC-35′, respectively. ^{32}P-labeled probes were incubated with purified recombinant GST-E2F1 fusion protein in DNA binding reaction buffer. The reaction mixtures were incubated at room temperature for 30 min, then resolved in a 4% polyacrylamide gel and exposed for autoradiography.

3. Results

3.1. 5′ deletion mutants to confirm the minimal promoter of human Lsh gene

In order to study the transcriptional regulation of the Lsh gene, we generated an Lsh promoter luciferase construct containing an Lsh promoter spanning the region from position −1849 (5′ end) to position +45 (3′ end) (designated pGL3-1849). To further localize the minimal promoter sequence, ten progressive 5′ deletion constructs were generated, and were designated as pGL3-1478,

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