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Oct-1 is involved in the transcriptional repression of the p15^{INK4b} gene

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Abstract p15^{INK4b} functions as a tumor suppressor and implicated in cellular senescence. Here, we show that the Oct-1 binding site in the human p15^{INK4b} gene promoter functions as a silencer. Oct-1 specifically interacts with this binding site *in vitro* and *in vivo* and SMRT and HDAC 1 are present in the p15^{INK4b} proximal promoter region. Moreover, mouse embryo fibroblasts (MEFs) lacking Oct-1 have shown significantly increased levels of p15^{INK4b} protein compared to their normal counterparts. Treatment with a histone deacetylase (HDAC) inhibitor has activated the expression of p15^{INK4b} in wild-type MEFs but has no effect in MEFs lacking Oct-1, suggesting that Oct-1 represses p15^{INK4b} gene expression in an HDAC-dependent manner. Finally, we show that the expression of Oct-1 protein significantly decreases, whereas p15^{INK4b} protein significantly increases with the cellular aging process. Taken together, these results suggest that Oct-1 is an important transcriptional repressor for p15^{INK4b} gene and the transcriptional repression of the p15^{INK4b} gene by Oct-1 may be one of the regulatory mechanisms of cellular senescence.
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Keywords: p15^{INK4b}; Oct-1; Transcriptional repressor; HDAC inhibitor; Cellular senescence

1. Introduction

The p15^{INK4b} protein is one of the cyclin-dependent kinase (CDK) [1] inhibitors called INK4 family proteins, which bind directly to CDK4/6 and are specific inhibitors of the cyclin D-dependent kinases [2,3], with the result that p15^{INK4b} arrests cells in the Gl phase of the cell cycle. Furthermore, p15^{INK4b} functions as a tumor suppressor gene [4–8]. p15^{INK4b} deficiency enhances the susceptibility to retro virus-induced acute myeloid leukemia (AML) in mice [7,8] and p15^{INK4b} is inactivated with a high frequency in hematopoietic neoplasms in humans [5]. A high proportion of T-cell childhood acute lymphoblastic leukaemias have deletions of p15^{INK4b} [6]. On the other hand, the expression of p15^{INK4b} increases in several models of cellular senescence [6,9,10]. Overexpression of p15^{INK4b} induces replicative senescence, and inhibits telomerase activity [11], suggesting that p15^{INK4b} is involved in cellular senescence.

We previously cloned the 5′-flanking region of the human p15^{INK4b} gene [12]. In this study, we performed functional analysis of the promoter region, and found that the Oct-1 binding site in the human p15^{INK4b} gene promoter functions as a silencer in a histone deacetylase (HDAC)-dependent manner. Several endogenous genes thought to be regulated by Oct-1 were reported not to be altered in Oct-1-deficient mouse embryo fibroblasts (MEFs)[13,14]. However, interestingly, MEFs lacking Oct-1 have shown significantly increased levels of p15^{INK4b} protein compared to their normal counterparts. Finally, we show that expression of Oct-1 protein significantly decreases, whereas protein significantly increases with the cellular aging process. Taken together, these results suggest that Oct-1 is an important transcriptional repressor of the p15^{INK4b} gene, and raise a possibility that the transcriptional repression of the p15^{INK4b} gene by Oct-1 may be a new regulatory mechanism of cellular senescence.

2. Materials and methods

2.1. Plasmid constructions

The construction of the human p15^{INK4b} gene promoter-luciferase fusion plasmid, p15 (−7787/−1), has been described previously [12]. A DNA fragment of p15 (−559/−1) was generated by PCR using p15 (−7787/−1) as a template, and it was inserted between the *Kpn*I site and *Nhe*I site in front of the luciferase reporter gene in pGVB2. The 3′-deletion mutants of p15 (−559/−1), such as p15 (−559/−80), p15 (−559/−143), p15 (−559/−196) and p15 (−559/−210), were also generated using PCR. These sense primer and antisense primers used are listed as below. p15 (−559) sense:

5′-GGTACCGCAGCCAGCATTCCTGGCGG-3′; p15 (−1) antisense: 5′-GCTAGCTCCGCAGCCCCCAGACGCGC-3′; p15 (−80) antisense: 5′-GCTAGCACGCTGCTCCGGCGCACTCT-3′; p15 (−143) antisense: 5′-GCTAGCCGTCCTTCTGCGGCTTGGGG-3′; p15 (−196) antisense: 5′-GCTAGCTCCGGGCTTTTCCTGGCGCT-3′; p15 (−210) antisense: 5′-GCTAGCGGCGCTCAAGAACCAGCGGG-3′. Reporter plasmids with mutations in the Oct-1 binding site at −137/−125 were generated by site-directed mutagenesis using a Quick Change XL Site-Directed Mutagenesis Kit (Stratagene, CA, USA). The top strand of the oligonucleotides was as follows, p15 (−559/−80) Oct-1 mutant andp15 (−559/−1) Oct-1 mutant: 5′-GGACGACGGGAGGGGGGTGAAGCTGAGCCCAG-3′. The generated constructs were confirmed by sequencing.

2.2. Cells

Human immortalized keratinocyte HaCaT cells (a kind gift from Dr. N.E. Fusenig, German Cancer Research Center, Heidelberg, Germany) and a human colorectal carcinoma cell line HCT116 (a kind gift from Dr. B. Vogelstein, Johns Hopkins University School of Medicine,

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MD, USA) have been described [12]. Wild-type and Oct-1-deficient mouse embryo fibroblasts (MEFs) were derived from 13.5-day embryos and were immortalized using a serial 3T3 protocol [13]. Human normal fibroblast TIG-1 cells (at 40 population doubling levels (PDLs) (JCRB0503) and 60 PDLs (JCRB0505)) were purchased from the Human Science Research Resources Bank (Osaka, Japan) [15]. TIG-1 cells were cultured in Eagle's minimum essential medium (Nissui, Tokyo, Japan) supplemented with 10% FBS.

2.3. DNA transfection and promoter assays

Reporter constructs were co-transfected with pRL-TK (Promega, WI, USA) to standardize by Renilla luciferase activity. HaCaT cells (5×10^4 cells) were seeded in 48-well plates, and plasmids (0.4 μg of reporter plasmid and 0.069 μg of pRL-TK) were co-transfected using LipofectAMINE and plus Reagent (Invitrogen, CA, USA). After 24 h of transfection, the cells were harvested. The luciferase activity of each cell lysate was measured using a Dual-Luciferase Reporter Plasmid System (Promega, WI, USA). The firefly luciferase activity of each sample was normalized by its Renilla luciferase activity and the fold activation was obtained by setting the control value to 1.0. Each experiment was repeated at least three times. Data were analyzed using the two-tailed Student's *t*-test and differences were considered significant from controls when $P < 0.05$.

2.4. Preparation of nuclear extracts and EMSA

Nuclear extracts of HaCaT cells were prepared as described previously [16]. Annealed oligonucleotides containing the sequences between positions −139 and −120 were labeled with [α-^{32}P] dCTP and were used as the probe (Fig. 2A). The reaction mixture for the EMSA contained 8 mM Tris–HCl (pH 7.9), 24 mM HEPES-KCl (pH 7.9), 120 mM KCl, 24% glycerol, 2 mM EDTA, 2 mM DTT, 1.5 μg of poly(dI–dC) and 9.25 μg of nuclear extract. Following preincubation, the indicated cold competitors or antibodies were added to the mixture, and the binding reaction was allowed to proceed at 4 °C for 20 min. The reaction mixture was further incubated for 20 min in the presence of [^{32}P]-labeled probe DNA. The antibodies used were anti-Oct-1 (sc-232, Santa Cruz Biotechnology, CA, USA) and anti-Sp3 (sc-644, Santa Cruz Biotechnology). The sequences of the top strands of oligonucleotides used in the competition analysis were as follows, with mutations indicated by underlining: Wild (−139/−120), 5′-AGCTGGGAGGGTAATGAAGCTGAG-3′; Mutant (−139/−120), 5′-AGCTGGGAGGGGGGAGCTGGGAGGGGGGTGAAGCTGAG-3′.

2.5. Chromatin immunoprecipitations

ChIP assays were performed as described previously [17]. After immunoprecipitation with an antibody against Oct-1 (sc-232, Santa Cruz Biotechnology), SMRT (sc-1612, Santa Cruz Biotechnology), HDAC1 (sc-8410, Santa Cruz Biotechnology), or an irrelevant control protein, GAPDH (5G4, Hy Test Ltd., Turku, Finland), the recovered DNA was analyzed using PCR amplification with the Gene *Taq* NT (Nippon Gene, Toyama, Japan). PCR was carried out as follows: 1 cycle at 95 °C for 5 min; 40 cycles at 95 °C for 10 s, 60 °C for 5 s, 72 °C for 30 s; and 1 cycle at 4 °C for 10 min, using primers (sense, 5′-ATTCTTTGCCGGCTGGCTCCCC-3′ and antisense, 5′-CCGGATAATCCACCGTTGGCCG-3′) that amplify the region between positions −373 and −26 from the first base of the translation initiation codon of the p15^{INK4b} gene. Three-fold diluted "Input" DNA and anti-GAPDH antibody served as positive and negative controls, respectively, and the products were analyzed by electrophoresis. The detected band was confirmed to be this region of the p15^{INK4b} gene by sequencing.

2.6. Protein isolation and western blot analysis

The protein isolation and western blot analysis have been described previously [18,19]. The protein extract was loaded onto a 7% or 12% polyacrylamide gel, electrophoresed, and transferred to a nitrocellulose membrane. A rabbit polyclonal antibody to p15^{INK4b} (sc-612, Santa Cruz Biotechnology) or Oct-1 (sc-232, Santa Cruz Biotechnology) was used as the primary antibody and α-Tubulin (Oncogene Research Product, CA, USA) was used as a loading control. The signal was then developed with the enhanced chemiluminescence system (Amersham Pharmacia Biotech, UK Limited). Bands were quantified using Scion Image software (Scion Co., MD, USA).

3. Results

3.1. The potential Oct-1 binding site in the p15^{INK4b} gene promoter functions as a silencer in HaCaT cells

To precisely know the regulatory mechanisms of the p15^{INK4b} gene, we have recently cloned and sequenced a 7.8 kb fragment of the human p15^{INK4b} gene promoter region [12]. The p15^{INK4b} gene promoter was previously cloned by Li et al. [20], and they reported the Sp1 binding sites from nt −432 to −423 are important for the basal promoter activity of p15^{INK4b}. However, their p15^{INK4b} regions lacked the upstream region containing bases −277 to −1 from the initiation codon. A computer search for potential regulatory elements in this region was performed using MatInspector V2.2 at the TRANSFAC WWW site [21]. It elucidated the existence of multiple potential transcription factor-binding sites such as the STATx, c-Rel and Oct-1 binding site (Fig. 1A), which may transcriptionally regulate p15^{INK4b} gene expression. To investigate any involvement of such a region in p15^{INK4b} gene transcription, we generated p15 (−559/−1) and its 3′-deletion constructs. The promoter activity of the p15 (−559/−80) construct was slightly increased compared with that of the p15 (−559/−1) construct, but the promoter activities of the 3′-deleted constructs of p15 (−559/−143), p15 (−559/−196) and p15 (−559/−210) markedly increased, by about 3.2- to 4.4-fold compared to that of the p15 (−559/−1) construct in human immortalized keratinocyte HaCaT cells (Fig. 1B). These results suggest that a silencer for the p15^{INK4b} promoter activity may exist in this region from nt −142 to −80. This region contains a potential Oct-1 binding site (5′-AGGGTAATGAAGC-3′, nt −137 to −125) (Fig. 1A). To clarify whether this potential Oct-1 binding site functions as the silencer, point mutations (sequence: TAA to GGG) were introduced into the element in the p15 (−559/−80) and p15 (−559/−1) construct. The promoter activity of the p15 (−559/−80) Oct-1 mutant increased 2.6-fold compared to that of the p15 (−559/−80) construct, and was almost equivalent to that of p15 (−559/−143) (Fig. 1B). Similarly, the promoter activity of p15 (−559/−1) Oct-1 mutant was enhanced 2.9-fold compared with that of p15 (−559/−1) in HaCaT cells (Fig. 1C). We confirmed that the same results were also obtained in the human colorectal carcinoma cell line HCT116 (data not shown). Taken together, these results suggest that the potential Oct-1 binding site functions as a silencer for the p15^{INK4b} gene promoter activity.

3.2. Oct-1 specifically interacts with potential Oct-1 binding site in vitro and in vivo and SMRT and HDAC1 are present in the p15^{INK4b} proximal promoter region

Several studies have recently reported that the transcriptional factor Oct-1 acts as a transcriptional repressor [22,23]. We then examined whether the Oct-1 protein can interact with this potential Oct-1 binding site using electrophoretic mobility shift assays (EMSAs). EMSAs using nuclear extracts isolated from HaCaT cells were performed with labeled wild-type (Wild) −139 to −120 or its mutant (Mutant) fragment as probes (Fig. 2A). As shown in Fig. 2B, the transcription factor Oct-1 binds to the potential Oct-1 binding site of the p15^{INK4b} gene promoter. Next, to directly examine whether Oct-1 was associated with the human p15^{INK4b} gene proximal promoter

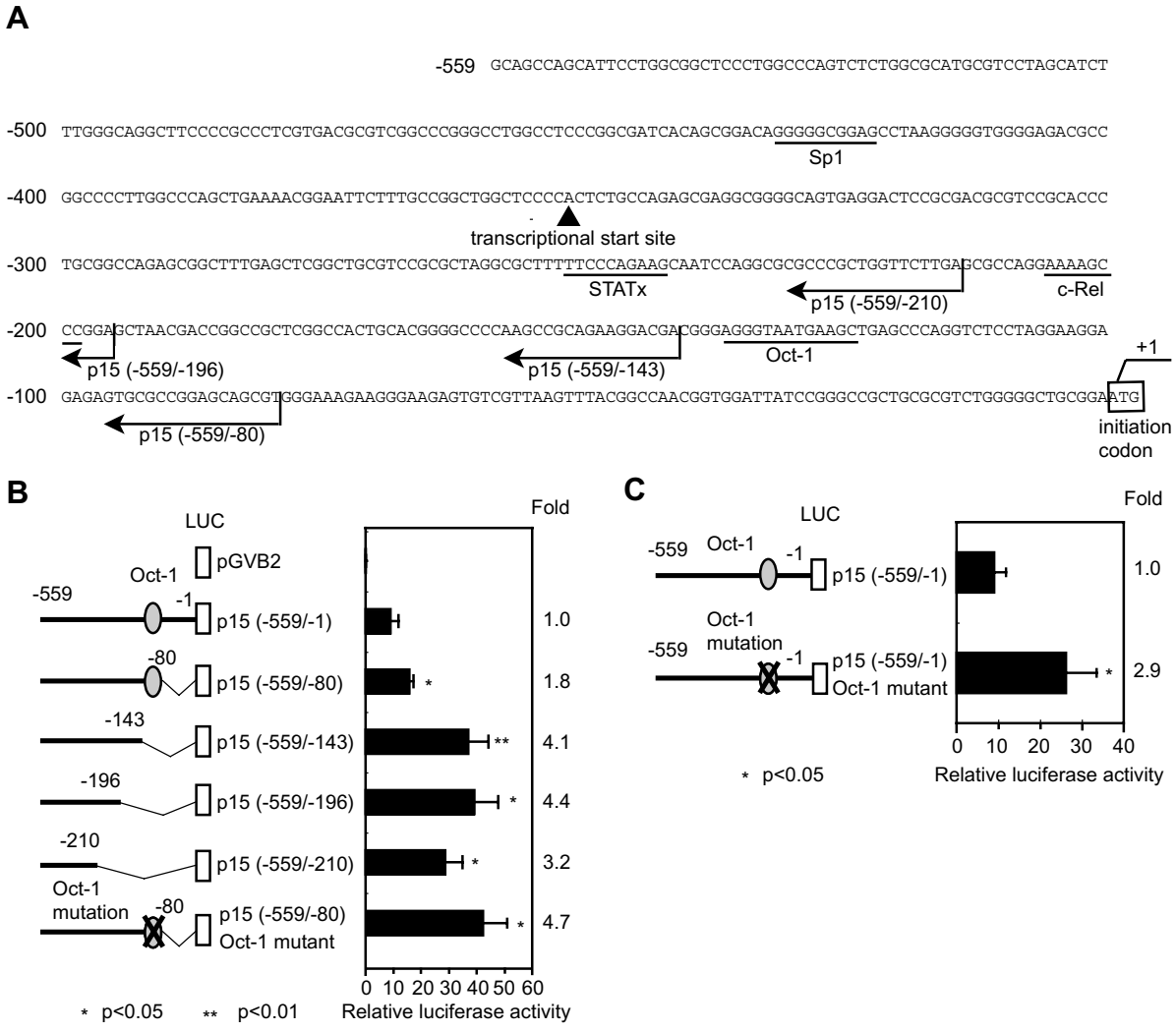

Fig. 1. p15^{INK4b} promoter activity in HaCaT cells. (A) Nucleotide sequence of the 5′-flanking region of the human p15^{INK4b} gene. The nucleotide number is counted from the first base of the initiation codon. The arrowhead indicates the transcriptional start site of the human p15^{INK4b} gene. Potential binding sites of transcriptional factors are underlined. The arrows indicate the 3′-end of the deletion constructs of the p15^{INK4b} gene promoters shown in (B) and (C). (B) Deletion analysis of the p15^{INK4b} promoter. (C) Mutation analysis of the p15^{INK4b} promoter. Data are shown as means ± S.D. (n = 3). *, $P < 0.05$; **, $P < 0.01$.

region *in vivo*, a chromatin immunoprecipitation (ChIP) assay was performed. The amplified 348-bp DNA fragment is indicated in Fig. 2C. Consistent with our EMSA results, the transcription factor Oct-1 was associated with the p15^{INK4b} gene proximal promoter region in HaCaT cells (Fig. 2D, lane 2). According to recent studies about the transcriptional regulation of Oct-1, a silencing mediator for retinoid and thyroid hormone receptors (SMRT) interacts with Oct-1 and acts as a transcriptional repressor [24,25]. Furthermore, SMRT is known to be a corepressor associated with histone deacetylases (HDACs) [26]. Therefore, we investigated whether SMRT and HDAC1 were also associated with the human p15^{INK4b} gene proximal promoter region containing the Oct-1 binding site *in vivo*. As shown in lanes 4 and 5 of Fig. 2D, not only Oct-1, but also SMRT and HDAC1, were also associated with the endogenous p15^{INK4b} gene promoter. These results suggest that these proteins may form complexes and suppress p15^{INK4b} gene expression.

3.3. Oct-1 represses the endogenous expression of the p15^{INK4b} protein in an HDAC-dependent manner

To further examine the relationship between Oct-1 and p15^{INK4b} gene expressions, we examined the expression levels of the p15^{INK4b} and Oct-1 proteins using wild-type mouse embryo fibroblasts (MEFs) and Oct-1 deficient MEFs [13]. The expression of the p15^{INK4b} protein in Oct-1 deficient MEFs significantly increased compared with that in its wild-type counterpart (Fig. 3A), suggesting that the Oct-1 protein represses endogenous p15^{INK4b} gene expression. In Fig. 2D, we showed that SMRT and HDAC1 were present in the p15^{INK4b} gene proximal promoter region. The repressive mechanism of Oct-1 is explained by the fact that its POU domain interacts with the SMRT, which is known to be a corepressor associated with HDACs [24–26]. Many studies have suggested that acetylation and deacetylation of histones have important roles in gene expressions [27]. HDACs form complexes with several transcriptional corepressors, including mSin3A, N-CoR and

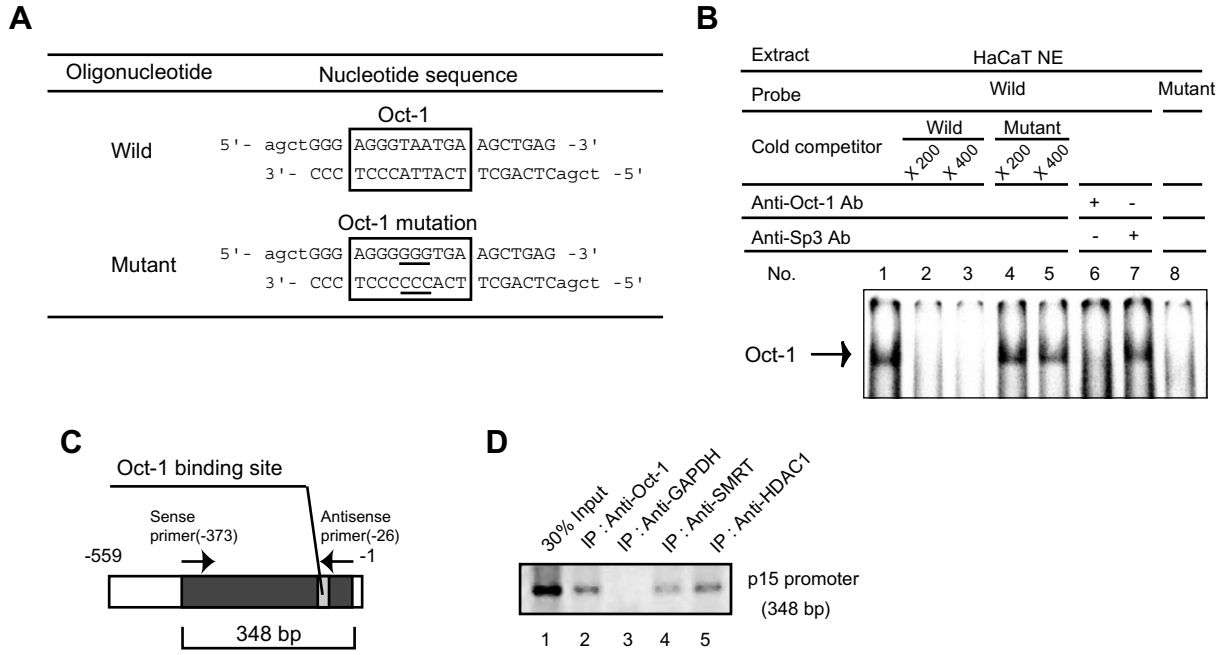

Fig. 2. Oct-1 protein binds to the potential Oct-1 binding site of the $p15^{INK4b}$ promoter *in vitro* and *in vivo*. (A) Sequences of each oligonucleotide (Wild or Mutant) used as a probe and competitors (the mutated nucleotides are underlined). (B) EMSAs showing the potential Oct-1 binding site-related band (arrow). (C) Schematic diagram of the human $p15^{INK4b}$ gene proximal promoter region (−559/−1). The $p15^{INK4b}$ promoter region including the Oct-1 binding site is amplified with sense and antisense primers indicated by arrows. (D) ChIP assays were performed to determine the presence of Oct-1, SMRT and HDAC1 at the human $p15^{INK4b}$ gene proximal promoter region.

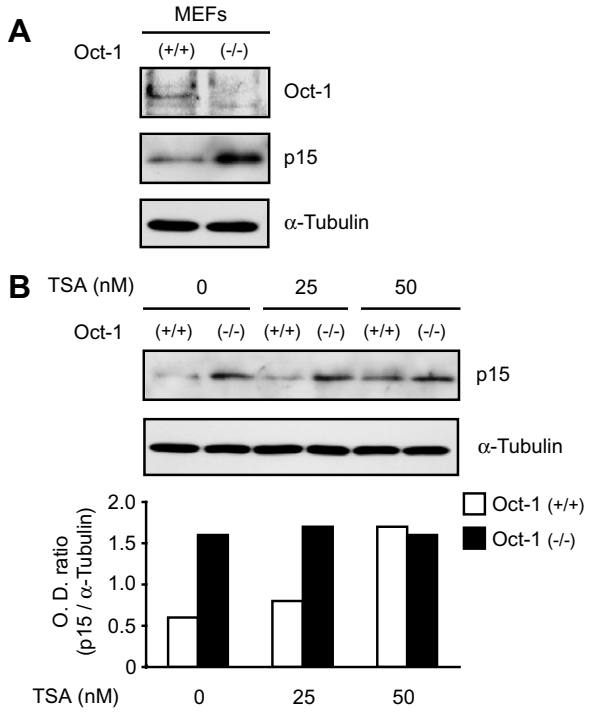

Fig. 3. Western blot analysis of $p15^{INK4b}$ protein in wild-type or Oct-1 deficient MEFs. (A) The endogenous expression of Oct-1 and $p15^{INK4b}$ proteins were examined in wild-type MEFs (Oct-1 (+/+)) or Oct-1 deficient MEFs (Oct-1 (−/−)). (B) Wild-type or Oct-1 deficient MEFs were treated in the presence of various concentrations of TSA for 24 h. The expression of $p15^{INK4b}$ protein was quantified using Scion Image software. α-Tubulin was chosen as a loading control in all blots.

SMRT, and HDAC-corepressor complexes act as transcriptional repressors and suppress gene expression [26]. Therefore, we investigated whether an HDAC inhibitor, trichostatin A (TSA), affects $p15^{INK4b}$ gene expression in MEFs. TSA activated the expression of $p15^{INK4b}$ in a dose-dependent manner in wild-type MEFs, but had no effect in MEFs lacking Oct-1 (Fig. 3B). These results suggest that Oct-1 negatively regulates the endogenous expression of the $p15^{INK4b}$ protein in an HDAC-dependent manner.

3.4. The expression of Oct-1 protein significantly decreases, whereas $p15^{INK4b}$ protein significantly increases with the cellular aging process in TIG-1 cells

Oct-1 represses the collagenase gene, one of the cellular aging-associated genes, and functions as a possible transcriptional repressor, whose function decreases with cellular senescence [28]. In addition, the expression of $p15^{INK4b}$ increases in several models of cellular senescence [6,9,10]. The overexpression of $p15^{INK4b}$ induces replicative senescence, and inhibits telomerase activity [11], suggesting that $p15^{INK4b}$ is involved in cellular senescence. However, no mechanisms as to the endogenous activation of $p15^{INK4b}$ expression were clarified. Therefore, to analyse the physiological relationship between Oct-1 and $p15^{INK4b}$ during the cellular aging process, we examined the expression levels of the Oct-1 and $p15^{INK4b}$ protein using young and senescent human normal fibroblast TIG-1 cells. In comparison with young TIG-1 cells, the expression of the Oct-1 protein significantly decreased and the expression of the $p15^{INK4b}$ protein significantly increased in senescent TIG-1 cells (Fig. 4A). To investigate whether the activation of the $p15^{INK4b}$ protein is mediated through the release of the Oct-1 protein on the Oct-1 binding site in the $p15^{INK4b}$

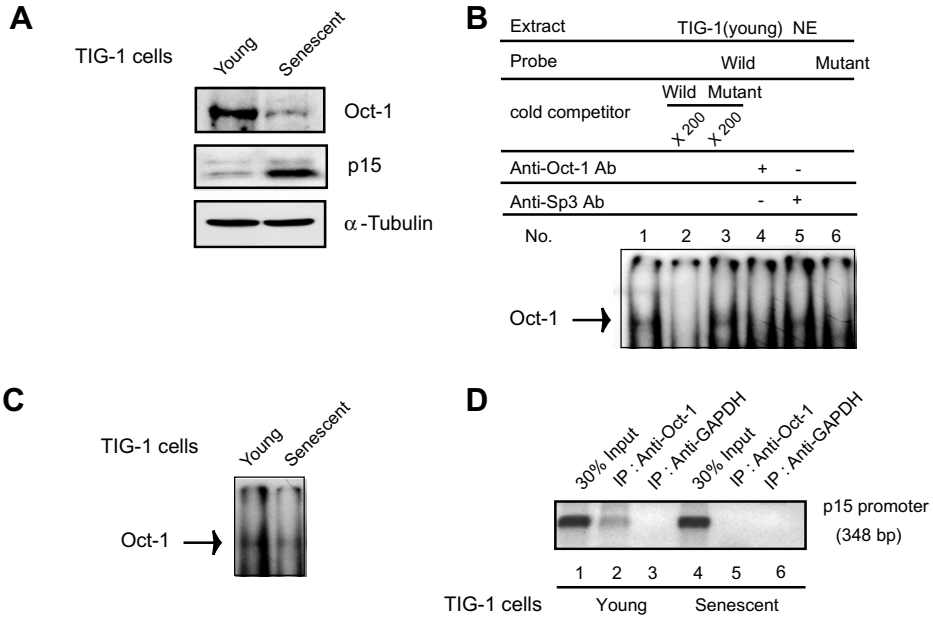

Fig. 4. The endogenous expression of Oct-1 and p15 ^{INK4b} in young (45–47 PDLs) and senescent (65 PDLs) TIG-1 cells. (A) Relative levels of Oct-1 and p15^{INK4b} protein expression in young and senescent TIG-1 cells using western blotting. α-Tubulin was chosen as a loading control in all blots. (B) EMSAs showing the potential Oct-1 binding site-related band (arrow) in young TIG-1 cells. (C) Comparison of the potential Oct-1 binding site-related band (arrow) between young and senescent TIG-1 cells. (D) ChIP assays were performed to compare the presence of Oct-1 at the human p15^{INK4b} gene proximal promoter region between young and senescent TIG-1 cells.

promoter, we performed EMSAs the same as in Fig. 2B using nuclear extracts isolated from young and senescent TIG-1 cells. The Oct-1 protein interacted with the Oct-1 binding site (Fig. 4B) and the interaction significantly decreased in senescent TIG-1 cells compared to young TIG-1 cells (Fig. 4C). Next, to examine whether Oct-1 was associated with the human p15^{INK4b} gene proximal promoter region *in vivo*, we performed ChIP assays using young and senescent TIG-1 cells. The transcription factor Oct-1 was associated with the p15^{INK4b} gene proximal promoter region in young TIG-1 cells compared to senescent TIG-1 cells (Fig. 4D, lanes 2 and 5). These results suggest that the increased expression of p15^{INK4b} in senescent cells is due to the decreased level of the Oct-1 and the release of Oct-1 protein from the Oct-1 binding site in the p15^{INK4b} promoter region.

4. Discussion

In this study, we showed that Oct-1 functions as a transcriptional repressor of the p15^{INK4b} gene. The repressive mechanism of Oct-1 is explained by the fact that its POU domain interacts with SMRT, which is known to be a corepressor associated with HDACs [24–26]. A lot of studies have suggested that the acetylation and deacetylation of histones have important roles in gene expression [27]. HDACs form complexes with several transcriptional corepressors, including mSin3A, N-CoR, and SMRT, and HDAC-corepressor complexes act as transcriptional repressors and suppress gene expression [29–31]. Therefore, our findings that not only Oct-1 but also SMRT and HDAC1 were associated with the p15^{INK4b} gene proximal promoter region *in vivo* suggest that these proteins may form complexes and suppress p15^{INK4b} gene expression. We have recently reported that HDAC inhibitors, TSA and sodium butyrate, activated the p15^{INK4b} gene expression through its promoter [12]. We speculate that recovery of the repressive function of Oct-1 by HDAC inhibitors may in part contribute to the up-regulation of p15^{INK4b} gene expression by HDAC inhibitors. However, Oct-1 binding site is the 5′UTR of p15^{INK4b} gene. Therefore, the mutation of the Oct-1 binding site could influence p15^{INK4b} gene mRNA stability and/or translation effects.

Recently, an Oct-1-deficient mouse was generated by gene targeting, and the role of Oct-1 *in vivo* was examined. However, Wang et al. have shown that several endogenous genes, such as Ig, histone H2B, U2 snRNA and U6 snRNA, thought to be regulated by Oct-1, underwent no change in their expressions in Oct-1 deficient MEFs compared with those in Oct-1 wild-type MEFs [13,14]. This apparent discrepancy suggested that the loss of Oct-1 protein might not be sufficient to affect the endogenous expressions of these genes, possibly due to the existence of binding sites for multiple transcriptional factors in endogenous promoters. However, in this study, we found that the p15^{INK4b} protein expression significantly increases in Oct-1 deficient MEFs compared to its wild-type counterpart. Our results suggest that Oct-1 is an important transcriptional regulator for the endogenous expression of the p15^{INK4b} gene.

Finally, we clearly indicated that there is an inverse correlation between Oct-1 and p15^{INK4b} expressions during the cellular aging process. As described above, the p15^{INK4b} gene is implicated in cellular senescence [6,9–11]. For example, T-lymphocytes display high levels of p15^{INK4b} protein as they enter into replicative senescence [6]. Furthermore, p15^{INK4b} functions as a tumor-suppressor gene [4–8]. These findings suggest that the inactivation of the p15^{INK4b} gene enables hematopoietic cells to avoid senescence and leads to malignancies. Taken together with the previous findings that p15^{INK4b} plays important roles

in cellular senescence and tumor suppression, transcriptional repression of the $p15^{INK4b}$ gene by Oct-1 may be one of the important regulatory mechanisms of senescence and malignant transformation.

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