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Creg1 And Its Enhancement Of P16ink4a-Induced Senescence

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CREG1 AND ITS ENHANCEMENT OF P16INK4A-INDUCED SENESCENCE

by

BENCHAMART MOOLMUANG

DISSERTATION

Submitted to the Graduate School

of Wayne State University,

Detroit, Michigan

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for the degree of

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2010

MAJOR: CANCER BIOLOGY

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Advisor   Date

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_____

DEDICATION

To my family, my parents and my sister,

who always provided mental support at all time during my study

AGKNOWLEDGEMENTS

I would like to thank my thesis advisor, Dr. Michael A. Tainsky for his invaluable guidance and support for my dissertation research.

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CHAPTER 1

INTRODUCTION

1.1   Summary

Normal human somatic cells have a limited proliferative potential when cultured *in vitro* before entering irreversible cell cycle arrest or senescence (1). In this process referred to as replicative senescence, cells permanently stop dividing and undergo genetic, biochemical and morphological changes. Cancer cells have developed the ability to overcome senescence and have an indefinite lifespan, sometimes referred to as immortal. This is an important step in the development of cancer in humans (2). Cellular immortalization is, therefore, the process of bypassing senescence to obtain a long, indefinite lifespan and develop the capacity to gain additional genetic and epigenetic mutations leading to tumor formation. Understanding of the genes, mechanisms and pathways that regulate the immortalization and senescence processes has provided information underlying abnormal cell proliferation and cancer.

1.2 Cellular senescence

Cellular senescence was initially described as the Hayflick limit demonstrating that normal human cells had a limited ability to proliferate in culture (1). Cellular senescence appears to be one of the tumor suppressive mechanisms which is programmed to prevent cells from uncontrolled proliferation. Cellular senescence can be classified as replicative or induced (Figure 1). Replicative senescence refers to the internal clocking mechanism responding to the shortening of telomeres, the repetitive DNA sequence that caps the ends of chromosomes in cells (3). Shortening of telomere leads to loss of structural integrity of telomere nucleoproteins resulting in activation of the p53 and pRb tumor suppressor pathways and senescence (4). The second category of cellular senescence called induced or premature senescence results from exogenous factors including oxidative stress, DNA damage, and oncogene overexpression in normal cells (5-8).
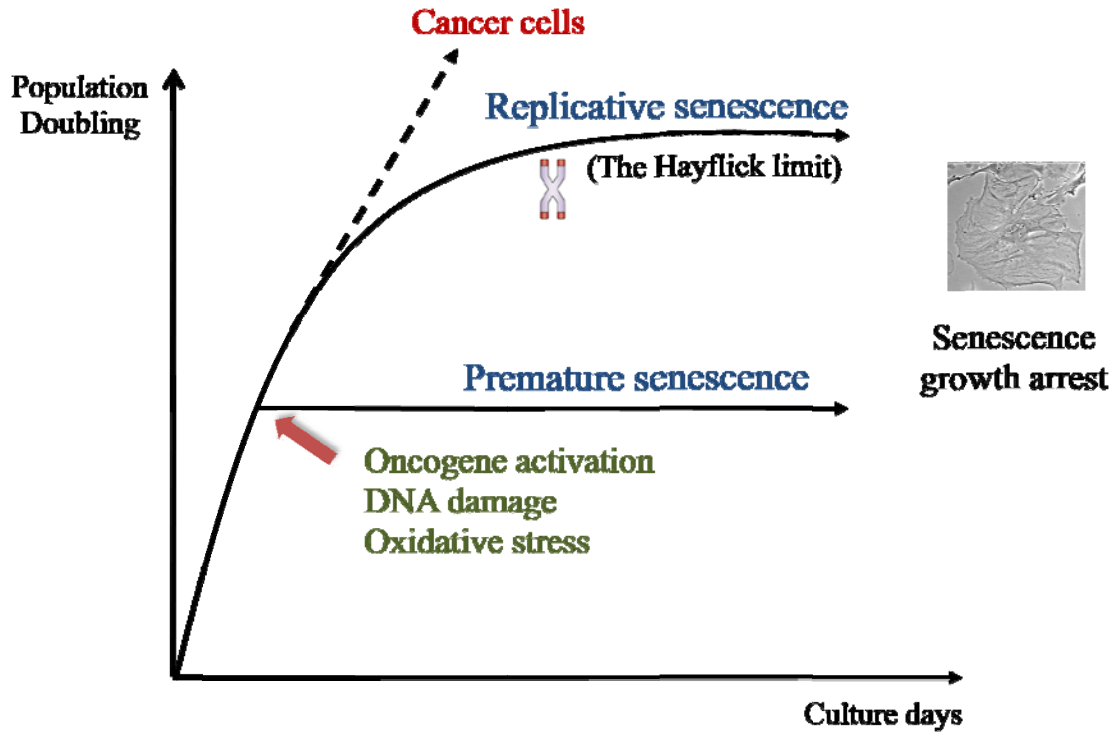
Figure 1. Cellular senescence. Replicative senescence is triggered by the dysfunction of telomere. Premature senescence can be induced by other cellular stresses. Bypass of senescence increases the susceptibility of cells to become tumorigenic. Figure is adapted from Ohtani et al. 2009 (9).

Senescent cells are characterized by an irreversible growth arrest in G1 phase of the cell cycle, an enlarged and flat cell morphology; although metabolically active, they are unresponsive to mitotic signals. Senescent cells have alterations in gene expression including increased expression of cell cycle inhibitors such as $p16^{INK4a}$ and $p21^{Cip1}$, the expression of senescence-associated-β-galactosidase at pH 6 (10, 11), and formation of senescent associated heterochromatic foci (SAHF) which coincides with the recruitment of heterochromatin proteins and the retinoblastoma (Rb) tumor suppressor for stable repression of E2F target genes (12).

There are two stages that have been defined for cellular senescence: mortality stage 1 (M1) or senescence and morality stage 2 (M2) or crisis. When cells encounter the shortening of their telomeres, a DNA damage checkpoint triggers the activation of proteins responsible for arresting the cell cycle, including the tumor suppressor protein p53. The $p16^{INK4a}$/pRb signaling pathway is another tumor suppressor pathway which is involved in regulating cell cycle arrest at the G1/S phase (12, 13). The inactivation of these tumor suppressor pathways permits progressive cell division until M2 (crisis) which is characterized by increasing cell death. The cells that continue through M2 generally become immortal (14-16). In contrast to most normal human somatic cells in which the telomerase activity is absent, most cancer cells (85-90%) have upregulated telomerase activity (17, 18); however, about 10-15% of tumors maintain telomere length by a mechanism called alternate lengthening of telomeres (ALT) (15). By permitting unlimited cell growth, immortalization increases the susceptibility of cells to tumorigenesis by providing more replicative capacity of cells so that cells can survive long enough to acquire multiple genetic alterations required for cancer.

Senescence was found to be the dominant trait over cellular immortalization. Somatic cell hybrids obtained from the fusion of normal human diploid fibroblasts with different immortal human cells exhibited limited cell proliferative potential, and that recessive changes of senescence genes, therefore, lead to immortalization (19-21). Moreover, a somatic cell hybridization study was performed to determine the complementation groups of cellular senescence and to narrow down the number of genes involved in this process (22). The series of cell fusion studies of different types of immortal cells regardless of cell types and tumor origins revealed four complementation groups: A, B, C, and D (22). The concepts behind this experiment were that if the immortal cell lines contain the same recessive changes, the hybrids of these two cell lines should be immortal and the cell lines would be assigned to the same complementation group. However, if the two cells had lost a different genetic function, the cell hybrid should have limited cell divisions and would be assigned to a different complementation group (23). Microcell-mediated chromosome transfers were later performed to identify chromosomes and genes altered in each complementation group. The results showed that chromosomes 1, 4, and 7 encode senescence genes inactivated in the complementation group C, B, and D, respectively (23, 24). Hensler et al (1994) showed that chromosome 1q carries genes altered in cell lines assigned in complementation group C, which is involved in cellular senescence. Moreover, the deletion of two regions located on chromosome 1q (1q25 and 1q41-42) appeared to be involved in escaping senescence (25). The presence of four complementation groups involved in cellular senescence indicates that there are a limited number of genes and pathways controlling this process.

The p53/p21^{Cip1} and p16^{INK4a}/pRb tumor suppressor pathways are the principal regulators of cellular senescence (26). In human fibroblasts, binding and inactivating of p53 and pRb by DNA tumor virus oncoproteins, for example simian virus 40 (SV40) large T antigen, can cause a bypass of senescence (26). The ability of the DNA tumor virus such as SV40, Adenovirus 16 and 18, and the human papillomavirus (HPV) to transform human fibroblasts was demonstrated over 30 years ago to target p53 and pRb by inactivating these important tumor suppressor proteins. The tumor suppressor p53 protein is bound and inactivated by the adenovirus E1B and simian virus 40 (SV40) large T antigen (27), and the human papillomavirus (HPV) E6 protein (28). Similarly, the E7 oncoprotein of HPV (29, 30), SV40 large T antigen(31), and the adenovirus E1A protein (32) have been shown to interact with and inactivate pRb protein. p53 is a transcriptional activator and repressor that regulates the expression of cell cycle arrested genes or apoptosis in response to DNA damage. The DNA binding and transcriptional activities of p53 are increased in high passage normal cells (33). pRb regulates the expression of cell cycle regulated genes indirectly by inactivating the E2F transcription factor and recruiting chromatin-remodeling proteins to genes that control cell cycle progression and differentiation (34). Recently, several genes and pathways were revealed to regulate the induction of senescence or being responsible for bypassing senescence. For instance, gene involved in interferon pathway, IFI 16, (35), PAI-1 (a critical downstream target of p53) (36), pRb (37), PML (a component of nuclear bodies) (38), prohibitin (39), HBP1 (40), p33ING (41), Bmi-1 (42), SUMO-2/3 (43), ING1a (44), IGFBP-rP1 (45), IRF5 and IRF7 (46), PPARγ (47) were shown to play a role in cellular senescence mechanism in mammalian cells.

1.3   Epigenetic regulation of genes and senescence

Epigenetic mechanisms regulate the expression of genes including many tumor suppressor genes without changing DNA sequences. The silencing of tumor suppressor genes can lead to abnormal cell growth, failure of cell cycle arrest checkpoints and prevention of programmed cell death, all of which drive the development of tumors. There are two well accepted epigenetic mechanisms involved in the regulation of gene expression. Changes in DNA methylation at cytosine bases of promoter-containing CpG islands, and histone acetylation both can affect the compactness of chromosomes (15). Methylation of DNA, covalent addition of methyl group, occurs at the cytosine base at CpG dinucleotides, which in clusters are called the CpG islands. CpG islands are usually located at the promoter region (200-2000 bp) around the transcription start site (48). This region is normally unmethylated in transcriptionally active genes; however, abnormal methylation frequently occurs in human cancers (49). Tumor suppressor genes involved in several pathways such as cell cycle, DNA repair, and apoptosis are hypermethylated and silenced, for example, pRb, p16^{INK4a}, p15^{INK4b}, and BRCA1 (50). In mammalian cells, DNA methylation is catalyzed by DNA methyltransferase (DNMT) (51). The inhibition of DNA methyltransferase can, therefore, restore gene expression silenced by DNA methylation. 5-azacytidine and 5-aza-deoxycytidine (5-aza-dC) are DNMT inhibitors widely used to demethylate DNA and restore gene expression (52). 5-azacytidine can be incorporated into DNA and RNA whereas 5-aza-dC can only be incorporated into DNA (53). Their structures are analogous to cytidine base; therefore, can be incorporated into DNA. DNMT is then irreversibly bound to 5-aza-dC residues in DNA resulting loss of activity (53). The treatment of immortal Li-Fraumeni Syndrome

(LFS) fibroblasts with 5-aza-dC can induce a senescence-like stage (54, 55). On the other hand, normal fibroblasts are not induced to senesce by treatment of 5-aza-dC because the promoters of tumor suppressor genes in normal cells are rarely silenced by promoter methylation. Certain genes were consistently found to be downregulated in immortal LFS fibroblasts and reactivated after 5-aza-dC treatment (55). This shows that genes involved in the immortalization and senescence processes can be regulated by DNA methylation (56).

1.4   Project background: Identification of genes associated with cellular immortalization and senescence in Li-Fraumeni Syndrome fibroblasts by microarray analysis

1.4.1 Li-Fraumeni Syndrome and established immortal LFS cell lines

The Li-Fraumeni Syndrome (LFS), described by Li and Fraumeni in 1969, is a rare familial inheritated cancer syndrome characterized by multiple primary tumors including breast carcinoma, soft tissue sarcoma, brain tumors, osteosarcoma, leukemia, lymphoma and adrenocortical carcinoma as well as early age of tumor onset (57). About 75% of LFS patients exhibit heterozygous germ line mutations of the tumor suppressor gene p53 (57).

Upon establishment in cell culture, dermal fibroblasts from seven LFS patients developed changes in morphology, anchorage-independent growth, and chromosomal abnormalities (58). These cells continued to proliferate and escaped senescence, and four of these (MDAH041, MDAH087, MDAH172, and MDAH174) spontaneously became immortal. Bischoff et al. established immortal LFS cell lines from these four individuals by skin biopsy and growth in culture (58). These immortal cells have several properties of transformed cells including an altered morphology and chromosomal anomalies. After becoming immortal, these cells can be transformed by an activated H-ras oncogene to form tumors in nude mice (59). In contrast, fibroblasts from normal donors almost never spontaneously immortalize.

The MDAH041 cell line was developed from a female patient, and the established immortal cells have lost their wild-type p53 allele and contain only a p53 allele with a

point deletion mutation at amino acid 184 which results in a truncated p53 protein (60). This cell line, after becoming immortal, contains active telomerase and the telomere length is stabilized at ~4 kb (population doubling [PD] 84) (60). The MDAH087 cell line was established from a 24-year-old male LFS patient containing one wild-type p53 and one p53 allele with a missense R→W point mutation at amino acid 248. Immortal MDAH087 cells have no detectable telomerase activity; however, the telomere length of this cell line, which is long (> 30 kb), is maintained by an alternative lengthening mechanism (ALT) (58, 60). The genomic analysis in these two cell lines showed that the remaining wild type p53 allele was lost in both cell lines. Subsequently, the three additional immortal LFS cell lines, MDAH087-1, MDAH087-10, and MDAH087-N, were independently developed from early passage precrisis MDAH087.

Four immortal LFS fibroblasts, MDAH041, MDAH087-N, MDAH087-1, MDAH087-10, were used to characterize genes, pathways, and also mechanisms involved in cellular senescence and immortalization. The advantage of studying these cells is that they are spontaneously immortal compared with other cell lines induced to immortalize by chemicals or oncogenes. Two additional LFS fibroblast cell lines from sisters, MDAH172 and MDAH174, were also independently established, and they became immortalized spontaneously (Bischoff et al., 1990). These immortal cells contain only a mutant p53 with an R175H mutation.

1.4.2 Identification of genes and pathways altered during immortalization and senescence in LFS fibroblasts

To identify gene and pathway changes during cellular immortalization and bypass of senescence, Fridman et al. used four immortal LFS cell lines and their precrisis counterparts to characterize gene expression by Affymetrix DNA microarray profiling analysis (55, 56). Gene expression of these spontaneously immortalized LFS cell lines was examined during immortalization by comparing gene expression changes of immortal LFS cell lines with their precrisis cells and gene expression changes of immortal LFS fibroblasts with senescence induced by treatment with 5-aza-deoxycytidine (5-aza-dC), an inhibitor of DNA methyltransferase. Since the senescence phenotype is dominant over immortalization, it was hypothesized that genes that are epigenetically silenced (downregulated) are the key regulators of cellular senescence. We identified genes downregulated during immortalization and upregulated after 5-aza-dC treatment as candidate senescence genes. In the microarray analysis, the immortal MDAH041 cell line was compared with the precrisis MDAH041 (MDAH041-PC) and the three immortal MDAH087 cells (MDAH087-1, MDAH087-10, and MDAH087-N) were compared with the precrisis MDAH087-PC cell line. Also, all four immortal cells treated with 5-aza-dC were compared with untreated immortal cells. In all four immortal LFS cell lines, the results showed that there were numerous genes downregulated during immortalization and upregulated after 5-aza-dC treatment. Among those genes, 187 genes downregulated during immortalization and 185 genes upregulated after 5-aza-dC treatment were commonly found in all four cell lines. Furthermore, 14 genes whose expression was decreased during immortalization and increased after 5-aza-dC treatment were common

among all four immortal cell lines. A schematic of microarray analysis is shown in Figure 2. Gene ontology analysis was then performed to analyze the biological, cellular, and molecular characteristics of the genes differentially expressed during immortalization and after 5-aza-dC treatment. Three pathways were identified: cell cycle, cytoskeleton, and interferon pathways. There are several genes regulating the cell cycle that are involved in cellular senescence. The well-known cell cycle-regulated genes, p53/p21^{Cip1} and p16^{INK4a}/pRb, have been shown to be increased in senescent cells and play an important role in immortalization and senescence (61-63). Among the fourteen genes epigenetically down-regulated during immortalization (56), four genes (CREG1, SERPINB2, IGFBPrP1, and ALDH1A3) were shown to regulate cell cycle. IGFBPrP1 or IGFBP3 overexpression suppresses cell growth and inhibits colony formation in the immortal LFS cell lines (64).
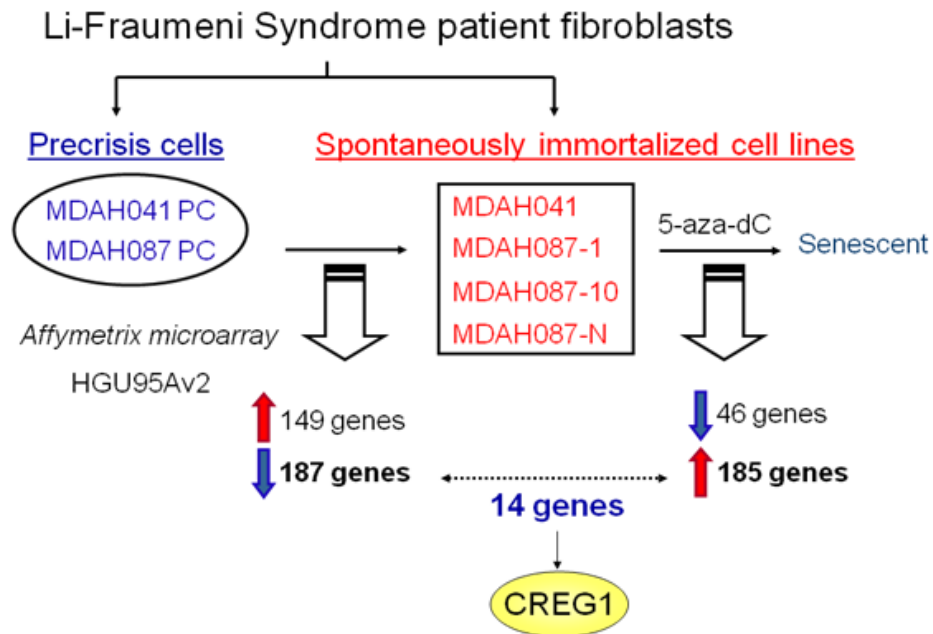
Figure 2. A schematic of microarray analysis from LFS fibroblasts (Adapted from Fridman et al., 2006). Gene expression profiles were identified by Affymetrix microarray analysis comparing immortal LFS with precrisis cells and 5-aza-dC-treated immortal with untreated cells. CREG1 was identified in the group of genes that were downregulated during immortalization and reactivated in 5-aza-dc-induced senescence.

1.5   CREG1

CREG1, the cellular repressor of E1A-stimulated genes 1, is a secreted glycoprotein containing 220 amino acids with three consensus N-glycosylation sites. The CREG1 gene is located at the 1q24 region of the chromosome. Human CREG1 DNA is 98%, 80%, and 47% identical to chimpanzee, mouse, and *Drosophila*, respectively. The alignment of CREG1 protein among species is shown in Figure 3. The first 31 amino acids of human CREG1 are predicted to be a cleavable signal sequence (65). The crystal structure of CREG1 shows that it forms a tight homodimeric complex (66). CREG1 was first identified by yeast two-hybrid screen for proteins that interacted with the *Drosophila* TATA-binding protein, TBP. In addition, CREG1 shares sequence similarity with a region of the adenovirus E1A protein (Figure 4) (67). Adenovirus E1A protein contains the Rb binding motif and shares the capacity to form a complex with pRb to disrupt the interaction between pRb and E2F transcription factors (68). This favors the transcriptional activity of E2F target genes and cell cycle progression. The human CREG1 was shown to bind to TBP and Rb-family pocket proteins, pRb/p105, RbL1/p107 and RbL2/p130 *in vitro* (67).

CREG1 functions as transcriptional repressor by repressing E2F transcription activation and antagonizing the ability of E1A and ras to transform primary cells (66, 67). It enhances differentiation of embryonal carcinoma cells and its expression increases in differentiated cells (65). Overexpression of CREG1 in human teratocarcinoma cells decreases cell cycle progression and cell proliferation (69). The secreted form of CREG1 interacts with its putative receptor, insulin-like growth factor 2 receptor (IGF2R), for endogenous localization and its growth suppression activity is IGF2R-dependent (69, 70).

Moreover, Han et al. demonstrated a role for CREG1 in vascular cells showing that CREG1 promotes differentiation and growth arrest in this cell type (71). Adenovirus-mediated CREG1 transfer to balloon-injured arteries reduces vascular hyperplasia (72), CREG1 overexpression was shown to attenuate cardiac hypertrophy in mice, involving downregulation of ERK signaling (73), inhibiting vascular smooth muscle cell migration as well as reducing activity and expression of MMP-9 protein with increased protein expressions of TIMP-1 and TIMP-2 (74).

```
Human        -----------------------------------------------------MAGLS 5
Chimpanzee   SNDLFLYKAIEKQPQHSLSPDTSRHHTSTFWVEGDFHTQLKTLGGCRQATRRIASQIGLS 120
Mouse        -----------------------------------------------------MAARA 5
Chicken      ------------------------------------------------------------
Drosophila   ------------------------------------------------------------

Human        RGSARALLAALLASTLLALLVSPARGRGGRDHGDWDEASRLPPLPPREDAARVARFVTHV 65
Chimpanzee   QGSERALLAALLASTLLALLVSPARGRGGRDPGDWDEASRLPPLPPREDAARVARFVTHV 180
Mouse        PELARSLLAALLAPALVALLVSPASGRGGRDHGDWDVDRRLPPLPPREDGPRVARFVTHV 65
Chicken      ----MVLLAFLCAAALAAL----ARG----------------TIPPPEEAARMARFVLHN 36
Drosophila   ---MKTFHSLLFALILALVSLDPSSGYSRRKD---ERIIREYKREQELNHAKIARDLVHR 54
                  : :  *  *   *  *  :        : *             : .::** : *

Human        SDWGALATISTLEAVRGRPFADVLSLSDGPPGAG--SGVPYFYLSPLQLSVSNLQENPYA 123
Chimpanzee   SDWGALATISTLEAVRGRPFADVLSLSDGPPGAG--SGVPYFYLSPLQLSVSNLRENPYA 238
Mouse        SDWGSLATISTIKEVRGWPFADIISISDGPPGEG--TGEPYMYLSPLQQAVSDLQENPEA 123
Chicken      CDWGALATLSAQEGLRGRPFANIFSISDGAPGPGGGSGVPYLYLTDMEISVQDLEVNSNA 96
Drosophila   ANWAAVGSLSTNERVKGYPMVNIISIDDSDANNRS-TGRIRFLLTDLDFTGPDWQKDNKV 113
             .:*.::.::**: : ::* *:.:::*:.*. ..    :*    : *: :: :  : . :  .

Human        TLTMTLAQTNFCKKHGFDPQSPLCVHIMLSGTVTKVNETEMDIAK--HSLFIRHPEMKTW 181
Chimpanzee   TLTMTLAQTNFCKKHGFDPQSPLCVHIMLSGTVTKVNETEMDIAK--HSLFIRHPEMKTW 296
Mouse        TLTMSLAQTVYCRNHGFDPQSPLCVHIMMSGTVTKVNKTEEDYAR--DSLFVRHPEMKHW 181
Chicken      SLTVSLAQTPYCKKHRYDPQNPLCAHIIFCGSIVKVNDSEAALAK--KALFTRHPEMESW 154
Drosophila   TLLFSDEQTLRCKEGGKDPMEPTCARSMISGQVKKMDPSDKSYQPSLDAYVRRHPAAINW 173
             :* .:  **  *::    ** .* *.: ::.* : *:: ::      .: . ***   *

Human        PSSHNWFFAKLNITNIWVLDYFGGPKIVTPEEYYNVTVQ 220
Chimpanzee   PSSHNWFFAKLNITNIWVLDYFGGPKIVTPEEYYNVTVQ 335
Mouse        PSSHNWFFAKLKISRIWVLDYFGGPKVVTPEEYFNVTLQ 220
Chicken      PKDHNWFYAKFNITNIWVLDYFGGLKIVTPEEYYNVKP- 192
Drosophila   VKAHNFYLCELEISNIFVLDFYGGPHKVSASDYYAVSN- 211
             .  **:: .:::*:.*:***::** : *:..:*: *.
```

Figure 3. The amino acid sequences alignment of CREG1 among species. The box shows position of homology to the amino acid sequence with E1A oncoprotein Rb binding site. The black line indicates the cleavage signal sequence in human homologue. The amino acid sequences of CREG1 were obtained from NCBI database (human NP_003842, chimpanzee XP_001157266, Mouse NP_035934.1, Chicken LOC768680, *Drosophila* NP_524385). Protein alignment was performed using ClusterW2 tool (http://www.ebi.ac.uk/Tools/clustalw2/index.html)

1.6   p16^{INK4a}/pRb pathway and its regulation of cellular senescence

The p16^{INK4a} tumor suppressor protein plays a very important role on cell cycle regulation. It binds to and inhibits the kinase activity of the CDK4-6/cyclin D complex preventing the phosphorylation of Rb-family members (75). Thus, p16^{INK4a} maintains Rb family proteins in a hypophosphorylated state to promote the inactivation of E2F transcription factor, preventing entry into the cell cycle. The p16^{INK4a} gene (*CDKN2A*) is located at the INK4a/ARF/INK4b locus on chromosome 9p21 which encodes three related genes and distinct tumor suppressor proteins (ARF, p15^{INK4b}, and p16^{INK4a}) (76). Genetic alterations (gene deletion, intragenic mutation and methylation-mediated silencing) of p16^{INK4a} and its related INK4 family members, are among the most frequent found in human cancer (75). The expression of p16^{INK4a} can also be repressed by several factors including the polycomb complexes (77), the helix-loop-helix Id1 protein (78).

The retinoblastoma protein or pRb and its homologues RbL1/p107 and RbL2/p130, bind and inhibit several transcription factors including the E2F family of transcription factors. Different Rb family members regulate different sets of E2Fs. pRb preferentially binds to activator E2Fs, E2F1-3, whereas p107 and p130 bind the repressor E2Fs, E2F4 and E2F5 (79). Progression through cell cycle requires phosphorylation of Rb family members by the CDK4-6/cyclin D complex to favor the transcriptional activity of E2F transcription factors. Accordingly, the function of p16^{INK4a} is essential for a functional Rb checkpoint and overexpression of p16^{INK4a} in cells with a wild type pRb can result in cell cycle arrest or senescence (80, 81).

1.7   Purpose of the study

The loss of senescence response appears to be an important step in the development of cancer. To gain more knowledge on cellular senescence, and further understand mechanisms that regulate this process, the Tainsky lab had previously identified genes and pathways altered to bypass senescence (56). To investigate the role of a candidate senescence-associated gene, I present here the functional study of CREG1 gene and its implication in cellular senescence using immortal LFS fibroblasts and cancer cells as a model. Its function related to cell cycle regulation, negative growth effects, and the transcriptional repression of E2F, indicated that this gene may be a pleiotropic regulator of cellular senescence.

CHAPTER 2

METHODS

2.1   Cell lines and cell culture conditions

Six independent immortal LFS cell lines were derived from primary fibroblasts by skin biopsy from four independent LFS patients, MDAH041, MDAH087, MDAH172 and MDAH174, and were spontaneously immortalized *in vitro* (58). Immortalized LFS fibroblasts, normal fibroblast CRL-1502, HT1080 fibrosarcoma, and SaOS2 osteosarcoma cell lines were grown in Modified Eagles Medium (MEM, Invitrogen), and the U2OS osteosarcome cell line was cultured in RPMI medium 1640 (Invitrogen). All cell lines were supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen) and were maintained at $37^{o}C$ in 5% humidified CO_2.

2.2   Plasmids and DNA transfection

A CREG/pRC plasmid was kindly provided by Dr. Grace Gill from Tufts University. A 675-bp cDNA fragment of CREG1 was amplified from the CREG/pRC plasmid with primers flanking EcoRI and BamHI restriction sites (forward primer 5'-GATATCGAATTCCATATGGCCG and reverse primer 5'-GGGATCCTCTAGACCACAGTCT) and was cloned into a pIRESpuro vector. CREG/pcDNA3.1-Hygromycin was generated by ligation of a CREG fragment digested from CREG/pRC into HindIII and XbaI sites of pcDNA3.1-Hygro. p16^{INK4a} was cloned into a pcDNA3.1HisB vector.

In the functional study of CREG1, immortal LFS cell lines MDAH041 and MDAH087-1, as well as U2OS, SaOS2, and HT1080 cancer cell lines (6-9 x 10^5 cells in 10-cm culture plate) were transfected with 2 μg of CREG1-pIRESpuro and pIRESpuro empty vectors using lipofectamine transfection reagent and plus reagent (Invitrogen). For stable transfection, transfected cells were selected in standard culture media containing 0.5 μg/ml puromycin (Sigma-Aldrich) at 48 h after transfection. Stably transfected cells were used for cell growth assays including cell count, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric and colony formation assays after 1 week of selection. Expression of genes was confirmed by Western blot analysis

In the coexpression of CREG1 and p16^{INK4a} experiment, cells were transfected with four expression conditions, 1) empty vector pIRES and pcDNA3.1, 2) CREG1-pIRES, 3) p16-pcDNA3.1, 4) CREG1-pIRES and p16-pcDNA3.1. Two μg of each vector were cotransfected using lipofectamine transfection reagent and plus reagent (Invitrogen). At 48 h posttransfection, the cells were selected in combination of 0.5 μg/ml puromycin (Sigma-Aldrich) and 200 μg/ml G418 (Gibco) (double drug selection). Control untransfected cells died at day 3 after selection. At 5 days after selection, stable transfected cells were harvested for western blotting, flow cytometry for cell cycle analysis, seeded for colony formation assay, or fixed and senescence-associated β-galactosidase stained to detect senescence.

2.3   5-aza-dC treatment and gene expression analysis by Q-RT-PCR

5-aza-deoxycytidine (5-aza-dC) (Sigma-Aldrich) treatment of immortal LFS fibroblasts was performed as described by Fridman et al.(56). Briefly, immortal LFS

fibroblasts ($3x10^5$ cells) were treated with 1 µM 5-aza-dC in H_2O every other day for 1 week (media containing 5-aza-dC was changed on day 2, 4, 6) and cells were harvested for protein and RNA at day 8 of the experiment. Total RNA was isolated from immortal and 5-aza-dC-treated immortal LFS cell lines using the QIAGEN RNeasy Kit (QIAGEN). cDNA was prepared from 2 µg of RNA using Superscript II (Invitrogen). Q-RT-PCR was performed using SYBR Green PCR Detection Kit (PE Biosystems) and analyzed on the ABI 5700 Sequence Detection System (Applied Biosystems). Primers for CREG1 Q-RT-PCR were Forward-5' CAGCTTCAGCCAGGGACAAA and Reverse-5'GGGCAGTTGAGGAAGCCTTAG, and GAPDH was used as an internal control. Primers for cyclin A2 Q-RT-PCR were forward-5' AGTGATGTTGGGCAACTCTG and reverse-5'TCCGGGTTGATATTCTCCTG. The relative fold change was calculated using C_T method as follows: $2^{-\Delta\Delta CT}$, where, $\Delta\Delta C_T = (C_{T\ Gene\ of\ interest} - C_{T\ GAPDH})_{experiment} - (C_{T\ Gene\ of\ interest} - C_{T\ GAPDH})_{control}$ (82). The calculated relative fold change between 0 and 1 was divided by -1.

2.4 Methylation analysis of CREG1 promoter

CpG islands in the promoter of CREG1 were identified using MethPrimer software (http://mail.ucsf.edu/urolab/methprimer/index1.html). The region of gene, -1000 to +500 regarding the transcription start site, was identified using UCSC Golden Path (http://genome.ucsc.edu). Genomic DNA prepared by Puregen DNA isolation kit (Gentra System) was modified with bisulfite as described by Li Q et al (2008). Briefly, 1 µg of genomic DNA was denatured and treated with 3.6 mol/L sodium bisulfite. The modified DNA was resuspended in water and amplified by PCR with two nested PCR reactions. The two sets of primers were, F1 5'-TTAAAAGGAGGGGGTGTGTG (-274), and R1

5'-CGAACCCCAACTCACCTACA (+1297); F2 5'-TGCGGAGTTGTAGAGGGATT (-65) and R2 5'-CCGCCTCCAACGTAAAAATA (+316). The numbers in parentheses indicate the position relative to the transcriptional start site of CREG1 (NM_003851). The PCR products were cloned into pCR2.1-TOPO vector (Invitrogen) using the TOPO-TA cloning kit (Invitrogen). Sequencing of ten colonies was performed using M13-reverse and T7 primers. The sequencing results were analyzed by MethTools software (http://genome.imb-jena.de/methtools/).

2.5   Western blotting

To isolate protein, cells were washed with cold PBS and cold lysis buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1% NP40, 0.25% Na-deoxycholate, 1 mM EDTA), 1 ml lysis buffer containing 10 µl of the following; protease cocktail inhibitor, PMSF (10 mg/ml in isopropanol), Sodium pyrophosphate (0.1M), Sodium fluoride (1M) and Sodium orthovanadate (1M), was added and the cell lysates were collected by scraping cells from plates and centrifugating at 14000 rpm for 15 minutes. Equal amounts of protein (40-60 µg) were loaded onto 10 or 12% polyacrylamide gels, electrophoresed, and transferred onto nitrocellulose membrane. The membrane was blocked in 5% dry milk in Tris-buffered saline tween-20 (TBST). Proteins were detected by incubating overnight with primary antibodies diluted in 5% dry milk in TBST following by 1 h incubation with secondary antibodies. The primary antibodies used (mostly 1:1000 dilution) are shown in Table 1. The Alexa Fluor 680-labeled secondary antibodies were from Molecular Probes or the Li-Cor IRDye 800-labeled secondary antibodies were from Rockland Immunochemical. The working dilution for secondary antibodies was 1:10,000.

The images were detected using the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Table 1. The primary antibodies used

Antibody	Dilution	Catalog number	Supplier	Host
CREG1	1:1000	AF2380	R&D system	Goat
p16^{INK4a}	1:1000	554079	BD Biosciences	Mouse
p21^{WAF1}	1:1000	05-345	Upstate	Mouse
Cyclin A2	1:1000	611268	BD Biosciences	Mouse
Cyclin B1	1:1000	Ab72	Abcam	Mouse
Cyclin D1	1:1000	2926	Cell Signaling	Mouse
IGF2R	1:1000	Sc-25462	Santa Cruz	Rabbit
pRb	1:1000	9309	Cell Signaling	Mouse
p107/Rbl1	1:1000	Sc-318	Santa Cruz	Rabbit
p130/Rbl2	1:1000	Sc-317	Santa Cruz	Rabbit

2.6 **Senescence-associated β-galactosidase assay**

Senescent cells express high β-galctosidase activity which can be detected through 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) hydrolysis at pH 6 (10). A senescence detection kit (BioVision) was used for staining according to the manufacturer's instructions. Briefly, stably tranfected cells were stained for senescence-associated β-galactosidase activity after 5-7 days of drug selection. In order to count the senescence-associated β-galactosidase positive cells, stably transfected cells were seeded at equal amount ($1x10^5$ cells in 60-mm culture plate) after stable transfection and stained 24 h later. Cells were washed twice with PBS and fixed with fixative solution for 10-15 minutes. The fixed cells were stained with the staining solution containing X-gal and incubated at 37^oC overnight.

2.7 **Colony formation assay**

To determine the ability of stably transfected cells to form colonies, cells stably transfected with CREG1 and p16, alone and in combination, were seeded at low density (1000-5000 cells depending on cell lines) on 10-cm culture plates in triplicate and cultured under drug selection, as described in section 2.2, for 2 weeks. The colonies were washed twice with PBS, fixed with 4 ml cold methanol for 10 minutes, air dried, stained with 5 ml diluted Giemsa (1:10 v/v in H_2O) (Sigma-Aldrich), and incubated at room temperature for 30 minutes. The plates were then rinsed with water and air dried. The colonies (>1mm diameter) were counted and mean percentages were calculated from at least two independent experiments.

2.8   MTT cell proliferation assay

The MTT tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) colorimetric assay was used to quantify metabolically active cells. After stable transfection, cells were seeded at a density of 2000 cells per well in a 12-well plate and the MTT assay was measured every 3 days for two weeks. Stock 2 mg/ml filtered MTT (Sigma-Aldrich) solution was diluted 1:10 in culture media. One ml diluted MTT was added to each well and the plates were incubated 1.5 h at 37 $^{\circ}$C, 5% CO_2. MTT was aspirated and 1 ml DMSO was added to each well. The plates were shaken at room temperature for 10 minutes. 100 µl of cell extract was pipetted into a 96 well plate and the absorbance was measured at 540 nm on a Dynatech-MR-500 plate reader (Dynatech Laboratories). Absorbance is directly proportional to the number of live cells.

2.9   Flow cytrometry analysis

Stably transfected cells were trypsinized, counted, and 10^6 cells were washed in 5 ml phosphate buffered saline (PBS) and centrifuged at 200 x g. The resuspended cells (0.3 ml PBS) were fixed by adding 1 ml of cold 95% ethanol with mixing and incubated at -20 $^{\circ}$C overnight. The fixed cells were centrifuged at 200 x g and the ethanol was removed by pipetting. The cell pellets were stained with 1 ml of propidium iodide (PI) staining solution containing 50 µg/ml PI (Invitrogen), 200 µg/ml RNaseA and 0.1% Triton X-100 in PBS and incubated at room temperature for 30 minutes. The DNA content was measured at the Karmanos Cancer Institute Flow Cytometry Core using the Becton-Dickinson FACSCalibur ^{TM} Cytometer.

2.10   Reporter gene assay

The cyclin A2 wild-type promoter reporter was generated by cloning of a 347-bp fragment (-75 to +272) of the cyclin A2 promoter into the KpnI and HindIII restriction sites of the pGL3-basic vector containing luciferase reporter gene (83). The following primers were used to PCR amplify the promoter fragment from genomic DNA of normal fibroblast CRL-1502, forward 5'-GCAGGGTACCTGTCGCCTTGAATGACGTCA-3'and reverse 5'-GCAGAAGCTTCACTGCTCCCGGGAGTGGAC-3'. To generate the cyclin A2Δ with deletion of the CRE element of the cyclin A2 promoter, the following primers were used, 5'-GCAGGGTACCGGCCGCGAGCGCTTTCATTG-3' and reverse 5'-GCAGGGTACCGGCCGCGAGCGCTTTCATTG-3'. The E2F reporter was constructed by cloning the fragment of oligonucleotide containing four consensus E2F binding sites with TATA promoter sequence into KpnI and HindIII sites of pGL3-basic vector. The E2F1 promoter reporter containing E2F1 promoter-driven luciferase gene was kindly provided by Dr. Gustavo Leone from Ohio State University. The E2F1/CMV expression plasmid was kindly provided by Dr. Joseph R. Nevins from Duke University. U2OS cells (40,000) were transfected with 2 μg of each expression vector in a six-well cultured plate in triplicate. Selective media was added at 48 hours after transfection. Two μg of the cyclin A2 reporter construct and 0.01 μg of TK-renilla expression vector for normalizing the transfection efficiencies were transfected at day 3 after selection. In the E2F-TA reporter experiment, 1 μg of E2F-TA reporter, 0.5 μg of E2F1/CMV expression vector and 0.01 μg TK-renilla were transfected after expression vector transfection as described above. The cells were then harvested for luciferase assay at 40 h after the transfection of reporter plasmids. The luciferase activity was tested with the dual-

luciferase reporter assay system (Promega) according to the manufacturer's instructions. The luciferase activity was calculated from three independent experiments.

2.11 Lifespan analysis

To analyze the lifespan of CREG1-transfected immortal LFS cells, MDAH087-1 was transfected with CREG1 or pIRES followed by drug selection for two weeks. Ten individual clones from CREG1 and pIRES transfected cells were selected and transferred to 24-well plate using cloning cylinders. The cells were then transferred to 10-cm plates and cultured to obtain a confluent plate for analysis. $1x10^5$ cells were seeded at day 1 and the cell number was counted at day 7. The population doublings were calculated from the total cell number by applying $\log_2$. The cells were re-plated at $1x10^5$ and continued counting until the PD exceeded 50.

2.12 Coimmunoprecipitation

Coimmunoprecipitation was performed to detect CREG1-interacting proteins. MDAH087-1 immortal cell clones stably transfected with CREG1 and pIRES vectors as well as U2OS cell line transiently transfected with CREG1 and p16 were used in this assay. The EZview Red Protein A and G Affinity gel beads (Sigma-Aldrich) were used according to the manufacturer's instruction. To prepare cell lysate, cells (70-80% confluency in 10-cm culture plate) were washed with ice cold PBS and lysed by scraping in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40, 0.25 % Na-deoxycholate, 1 mM EDTA) containing 10 μl protease inhibitor cocktail and 10 μl PMSF (10 mg/ml). Cell lysates were centrifuged for 15 minutes at 10,000 rpm at 4^oC and supernatant was retained. Cell lysates were pre-cleared by adding 50 ul of RIPA buffer-

washed beads followed by incubating at 4°C for 10 minutes with thorough mixing. The beads were removed by centrifugating at 14,000 rpm at 4°C for 10 minutes and the supernatant (cell lysate) was retained. For immunoprecipitation, one µl of antibody was added to cell lysate (500-1000 µg protein) and the total volumes of the reactions were adjusted to equal volumes by cold lysis buffer. The antibody-cell lysate mixtures were incubated with thorough mixing at 4°C overnight. In the washing step, 50 µl of the protein A or G agarose beads slurry (50%) was transferred into a microcentrifuge tube and washed twice with cold lysis buffer. The beads were resuspended in 50 µl of cold lysis buffer and transferred into the antibody-lysate mixture followed by incubation for 1 hour at 4°C. The mixture was then centrifuged for 30 seconds at 10,000 rpm and the supernatant was carefully aspirated. The beads were washed by pipetting with cold lysis buffer three times. To elute the antibody-antigen complexes for SDS-PAGE, 25 µl of lysis buffer and 25 µl of 2x loading buffer were added to the beads. The samples were then boiled for 5 minutes to dissociate the immunocomplex from the beads. Ten to twenty µl of the supernatant was subjected to denaturing gel electrophoresis.

2.13   Preparation of nuclear extracts

To detect whether CREG1 is mainly found in the nucleus or cytoplasm, nuclear extracts from MDAH087-1 cell clones stably expressing pIRES empty vector and CREG1 were prepared to characterize CREG1 localization. The protocol was adapted from previously published methods (84). Briefly, 10-cm culture plates (10^7 cells) were placed on ice for 10 minutes. Cells were then washed twice with cold PBS and washed once with 1.5 ml Buffer A (10 mM Hepes pH7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1% igepal, 1% protease cocktail inhibitor). One ml of Buffer A was added to

each plate followed by incubating on ice for 10 minutes with shaking. Cells were collected by scraping into a glass homogenizer and were homogenized with type B pestle for 10-15 strokes. The homogenized extracted from each plate were collected into a single 15 ml tube and were centrifuged at 7000 rpm, 4°C, for 10 minutes. The supernatant (cytosolic fraction) were saved. The nuclear pellet was resuspended in 40 µl Buffer C (20 mM Hepes pH7, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 1% protease cocktail inhibitor, 0.5 mM PMSF, 1mM DTT) by pipetting and incubated at 4°C for 2 h with shaking. The mixtures were centrifuged at 14,000 rpm at 4°C for 5 minutes and the supernatant (nuclear extract) were collected.

2.14 Knockdown of Rb family members and transfection

U2OS cells expressing three members of the Rb family were used in this experiment. Each of the Rb pocket proteins, pRb, p107 and p130 was knocked down using the trans-lentiviral GIPZ packaging system (OpenBiosystems). To produce the lentiviral particles targeting each Rb pocket protein and non-silencing control, 9 ug of the pGIPz-shRNAmir constructs for each Rb member (pRb# RHS4430-98853387, p107#RHS4430-98851876, p130#RHS4430-99160980) as well as a non-silencing control vector obtained from Karmanos Cancer Institute shRNA shared resource together with the trans-lentiviral packaging plasmid mix (OpenBiosystems) were transfected into TLA-HEK293T cells using Arrest-In transfection reagent (OpenBiosystems) according to the manufacturer's instruction. The supernatant containing the virus was harvested and combined at 48 h and 72 h post-transfection. The virus stock was concentrated by ultracentrifugation using SW28 ultracentrifuge tube and the virus particles were resuspended into 100-200 µl media. U2OS cells were infected by the virus followed by

drug selection (0.5 µg/ml puromycin) beginning at 48h after infection. The cells expressing GFP proteins were examined by microscope to confirm the infection efficiency. One week after infection, the colonies were pooled (>20 colonies) and used for further experiments.

To study the role of each Rb family member in the enhancement of CREG1 on p16-induced cellular senescence, U2OS stably infected with lentivirus targeting each of the Rb pocket proteins, non-silencing control (passage 4-8) and uninfected cells were used in coexpression of CREG1 (CREG/pcDNA3.1-hygro) and p16 (p16/pcDNA3.1-Neo) experiments as mentioned above. Three selection drugs, puromycin (Sigma-Aldrich) (0.5 µg/ml), hygromycin (Invitrogen) (200 µg/ml), and G418 (Gibco) (200 µg/ml) were used to select transfected cells at 48h after transfection.

2.15 Statistical analysis

The student's two-tailed t-test for independent samples (unpaired t-test) was used to test the statistic significant of the effect of CREG1 and control pIRES overexpression on cell growth by colony formation assay. The same method was applied to test for differences in cell growth (colony formation assay), senescence-associated β-galactosidase activity and reporter gene assay of p16 overexpression alone and in combination of CREG1 and p16. One way analysis of variance (ANOVA, Tukey's test) was used to test for differences on coexpression of CREG1 and p16 experiment (vector, CREG1, p16, CREG1+p16 transfection conditions). A P-value ≤ 0.05 was considered to be statistically significant.

CHAPTER 3

FUNCTIONAL ANALYSIS OF CREG1

3.1   Summary

CREG1 was identified from gene expression profiling by microarray analysis as a gene whose expression fits the criteria of a senescence-associated gene: downregulated during immortalization and reactivated by 5-aza-dC induced senescence. Moreover, CREG1 was attractive for this study because it is involved in control of E2F transcription activity. CREG1 downregulation was validated by quantitative real-time PCR and western blot analysis from six independent immortalized LFS cell lines. CREG1 expression was reactivated after treatment of immortal LFS fibroblasts with 5-aza-dC, which can be used to induce senescence in immortal LFS cells. Promoter analysis of CREG1 revealed the presence of CpG islands and bisulfite sequencing of a selected region on the CpG island showed that hypermethylation of CREG1 promoter in immortal LFS cells compared with precrisis counterparts was related to the level of gene expression. Ectopic expression of CREG1 in immortal LFS cell lines and related cancer cell lines, osteosarcoma and fibrosarcoma cells, decreased cell proliferation but did not induce senescence.

3.2   CREG1 expression in immortal LFS fibroblasts

3.2.1   Identification of CREG1 as a candidate senescence-associated gene by microarray analysis

Fridman et al. previously identified the gene expression profiling changes during cellular immortalization in four independent, spontaneously immortalized LFS cell lines by Affymetrix microarray analysis to compare gene expression profiles of precrisis with immortal LFS fibroblasts as well as 5-aza-dC-treated immortal LFS with untreated cells (56). This study identified groups of genes and pathways including interferon, cell cycle and cytoskeleton altered in the process of immortalization. Genes that were downregulated during immortalization and upregulated after 5-aza-dC treatment to induce senescence are of the most interest for further study because cellular senescence is a tumor suppressive mechanism which can be bypassed by changes in tumor suppressor genes. In addition, the cell hybridization study revealed cellular senescence is dominant over immortalization, and that treatment of 5-aza-dC induces a senescent phenotype; therefore, genes epigenetically silenced are more critical in bypassing cellular senescence to become immortal.

CREG1 is one of fourteen genes whose expression decreased during immortalization and increased in 5-aza-dC-induced senescence. The Affymetrix microarray data obtained by Fridman et al. (56) of CREG1 expression (fold change) from four independent immortal LFS cell lines is shown in Figure 4. CREG1 expression, downregulation during immortalization and upregulation after 5-aza-dC treatment which was demonstrated to induce senescence in immortal LFS cells (55), fit the criteria of

senescence associated genes and led to further investigation of the CREG1 role in cellular
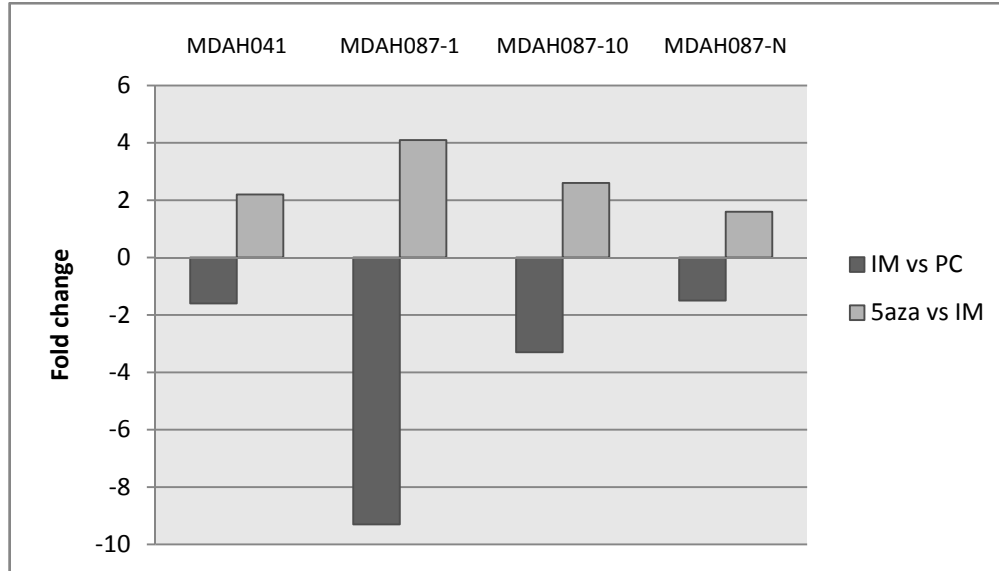
senescence.

Figure 4. Expression of CREG1 by Affymetrix microarray analysis. The dark bars show the fold changes (log$_2$) of CREG1 expression in immortal LFS cell lines (IM) compared with precrisis cells (PC) and the light bars represent the fold changes in 5-aza-dC treated immortal cells (5aza) compared with untreated LFS cells (IM). The data was obtained from Fridman et al (56). CREG1 expression is downregulated in immortal cells and is upregulated after 5-aza-dC treatment.

3.2.2 **Validation of CREG1 expression by quantitative real-time PCR**

To verify the microarray data of CREG1 expression, quantitative real-time PCR (Q-RT-PCR) was performed to measure CREG1 expression at mRNA levels in four immortal LFS cell lines used in microarray analysis, MDAH041, MDAH087-1, MDAH087-10 and MDAH087-N. Two additional immortal LFS cell lines, MDAH172 and MDAH174, which were not included in gene expression profiling study provided an independent validation. Total RNA was isolated from precrisis (PD<30) and immortal (PD>150) LFS fibroblasts as well as 5-aza-dC-treated and untreated immortal cells for cDNA preparation and Q-RT-PCR. The Q-RT-PCR analysis of CREG1 was performed in triplicate from two independent RNA preparations. The average fold changes of gene expression by Q-RT-PCR are shown in Figure 5. The expression of CREG1 mRNA decreased in all immortal cell lines compared with their precrisis counterparts and increased after treatment with a DNA demethylating agent compared with untreated cells, which was consistent with the microarray data.
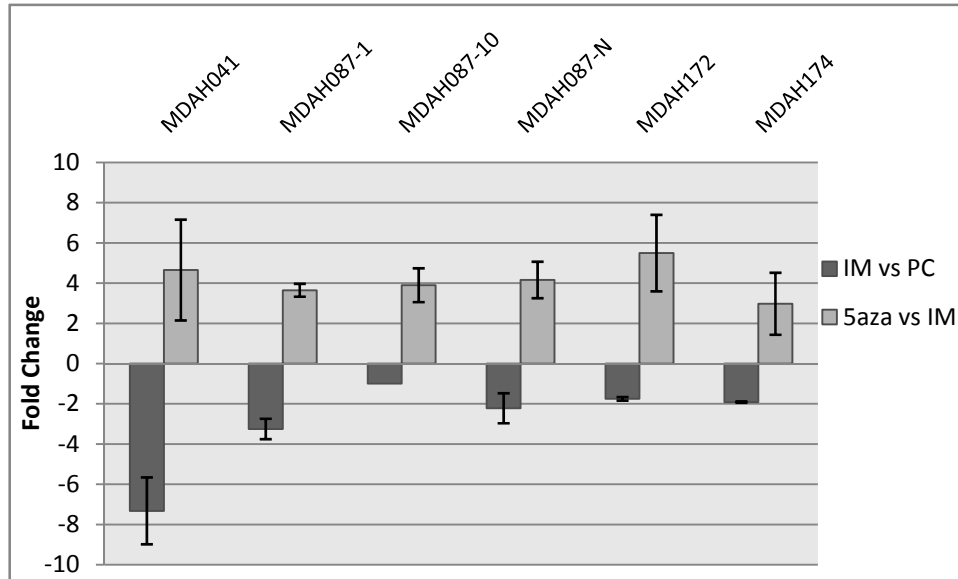
Figure 5. The expression of CREG1 by Q-RT-PCR. CREG1 expression was examined by Q-RT-PCR analysis in six independent immortalized LFS fibroblasts. The dark bars show fold changes of CREG1 in immortal LFS (IM) cells compared with precrisis (PC) fibroblasts derived from the same patient. The light bars represent the fold changes of immortal cells treated with 5-aza-dC compared to untreated immortal cells. GAPDH mRNA was detected from the same sample for normalization. The bar graphs represent the mean fold changes from two different sets of RNA preparation with three replicate for each reaction. CREG1 mRNA levels decrease in immortal LFS fibroblasts and reactivate after 5-aza-dC treatment.

3.2.3 Western blot analysis of CREG1

To expand on the Q-RT-PCR results, CREG1 protein levels were measured by western blotting from precrisis and immortal LFS cell lines (Figure 6) and 5-aza-dC treated immortal cells (Figure 7). Additionally, the protein levels of CREG1 were measured from normal and LFS cells at replicative senescence. The population doublings of replicative senescent LFS fibroblasts are slightly varied among four independent cells lines; MDAH041 (PD43), MDAH087 (PD61), MDAH172 (PD51), MDAH174 (PD52) and normal fibroblast CRL-1502 (PD46). Interestingly, the protein levels of CREG1 were upregulated in replicative senescent LFS and normal CRL-1502 fibroblasts, as shown in Figure 6.
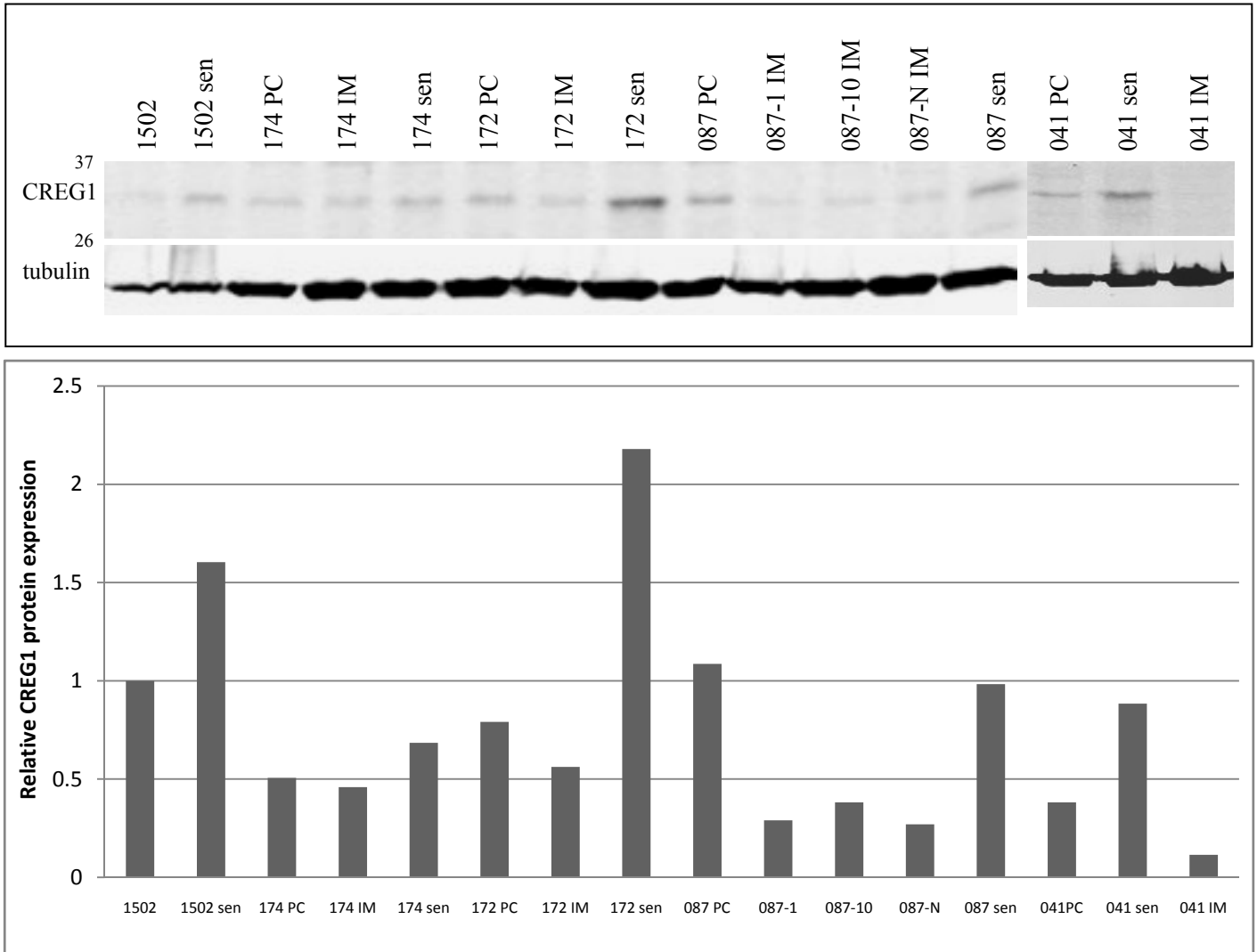
Figure 6. Western blot analysis of CREG1 in normal and LFS fibroblasts. CREG1 protein levels were examined in normally growing and in replicative senescent fibroblast CRL1502 and LFS precrisis (PC), immortal (IM) and senescent (sen) fibroblasts. The population doublings (PD) of examined fibroblasts are indicated as followed; 1502 (PD21), 1502 sen (PD46), 174 PC (PD13), 174 IM (PD132), 174 sen (PD52), 172 PC (PD14), 172 IM (PD172), 172 sen (PD51), 087 PC (PD26), 087-1 IM (PD298), 087-10 IM (PD258), 087-N IM (PD250), 087 sen (PD61), 041 PC (PD 19), 041 IM (PD351), 041 sen (PD 43). CREG1 protein levels are increased in replicative senescent fibroblasts compared with precrisis and immortal cells. The expression of CREG1 protein in MDAH087 senescent cells is somehow similar to precrisis cells.
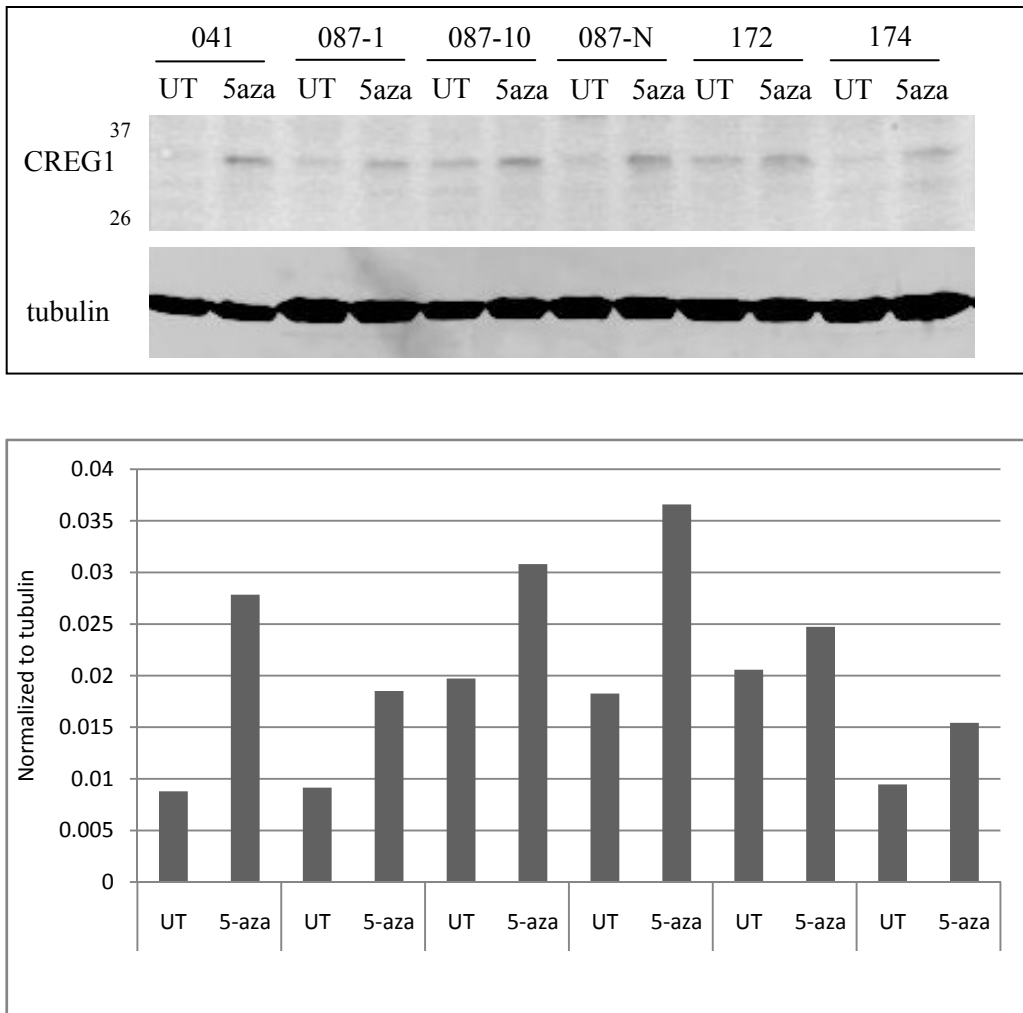
Figure 7. Western blot analysis of CREG1 in 5-aza-dC treatment of immortal LFS cell lines. CREG1 proteins were detected in six independent immortal LFS cell lines untreated (UT) and treated (5aza) with 1μM 5-aza-dC. The bar graphs show CREG1 level normalized to tubulin. CREG1 protein levels are upregulated after 5-aza-dC treatment in immortal LFS fibroblasts.

3.3   Analysis of CREG1 promoter and bisulfite sequencing

Treatment with DNA demethylating agent 5-aza-dC can reactivate CREG1 expression in immortal LFS cells. DNA methylation at promoters of genes has been shown to silence gene expression, including many tumor suppressor genes in cancer cells (49). To investigate whether CREG1 expression is regulated by promoter methylation, we analyzed the region of CREG1 promoter, -1000 to +800 bp relative to the transcription start site using MethPrimer software. We found that the promoter of CREG1 contains CpG rich islands (Figure 8). Sodium bisulfite-modified genomic DNA samples from immortal and precrisis LFS fibroblasts were PCR amplified followed by sequencing at the CpG rich region. The region of CREG1 promoter analyzed (383 bp) is highly methylated in immortal MDAH041 cells (60%) and partially methylated in MDAH087-1 (30%) and MDAH087-10 (10%) compared to their precrisis counterparts (Figure 9). The degree of promoter methylation seemed to correlate with the expression level of CREG1; lower CREG1 expression and higher methylation status was observed in MDAH041 than those in MDAH087, and this was also true for degree of promoter methylation and expression levels in MDAH087-1 compared with MDAH087-10. However, CREG1 expression may not only be regulated by promoter methylation as we observed the partial methylation in MDAH087-1 and low methylation in MDAH087-10. In this case, other transcriptional mechanisms may also regulate CREG1 expression.
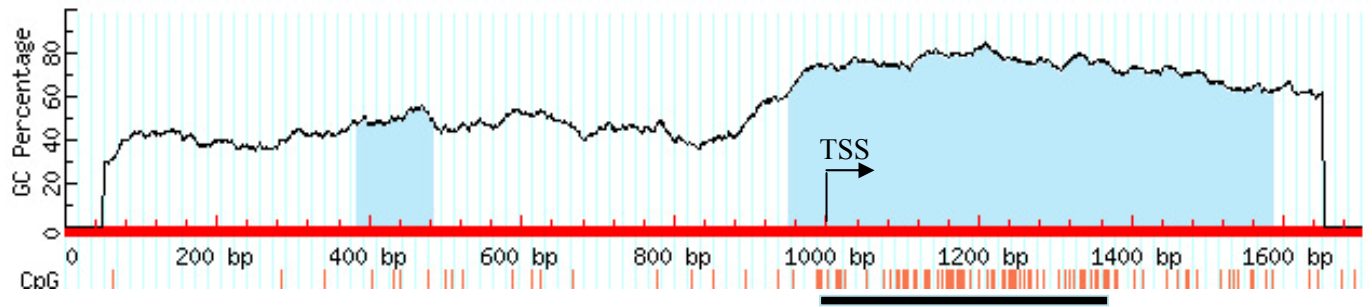
Figure 8. Schematic of CREG1 promoter from -1000 to + 800 bp relevant to the transcription start site (TSS). The blue area shows the CpG rich region and the red vertical bars represent the CpG sites. The black line represents the region analyzed by bisulfite sequencing.
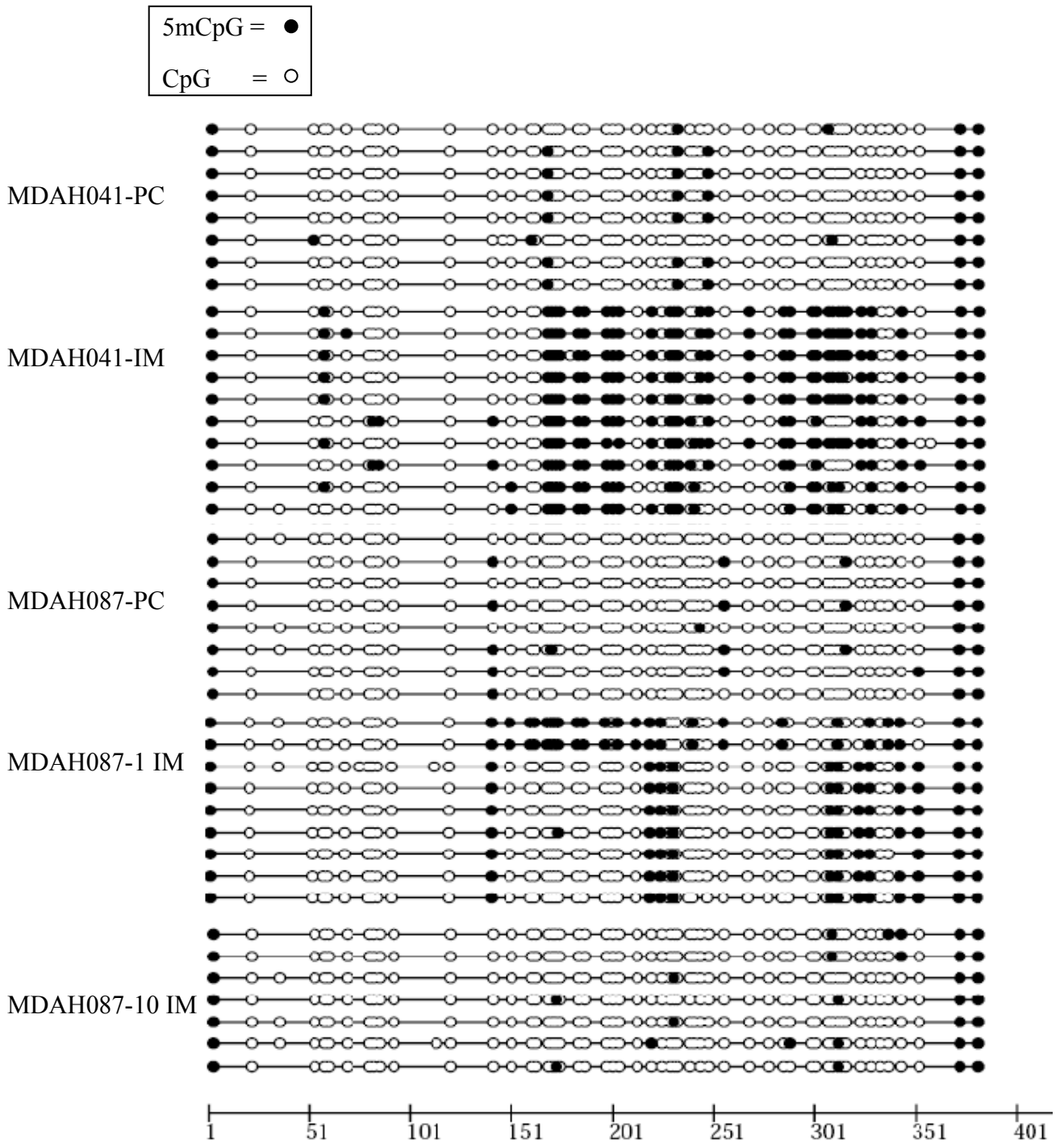
Figure 9. Bisulfite sequencing of the CREG1 promoter from precrisis (PC) and immortal (IM) MDAH041, MDAH087-1 and MDAH087-10. The black circles represent the methylated CpG sites, whereas the white ones represent the unmethylated CpG sites.

3.4   CREG1 overexpression in immortal LFS and cancer cell lines

3.4.1   Overexpression of CREG1 decrease cell proliferation in immortal LFS cells

The upregulation of CREG1 in replicative and 5-aza-dC-induced senescent fibroblasts and its silencing by promoter methylation led us to further investigate whether CREG1 was functionally involved in cellular senescence. We transfected CREG1 and a control empty vector into two immortal LFS cell lines, MDAH041 and MDAH087-1, and investigated consequent growth properties and senescence. CREG1 was cloned into the pIRESpuro expression vector. This vector is a bicistronic expression plasmid containing an internal ribosome entry site (IRES) which enables two genes of interest (CREG1 and puromycin resistant gene) to be expressed simultaneously. For stable transfection, two µg of CREG/pIRES or pIRES empty vectors were transfected into MDAH041 and MDAH087-1 immortal LFS cell lines. Forty eight hours posttransfection, transfected cells were selected with puromycin (0.5 µg/ml) and selective media was changed every 3 days for 2 weeks. Drug selection maintained expression in the transfected cells containing gene of interest and control empty vector for further investigation of the effects of the transfected gene. Stably transfected cells were split for growth assay, including cell counting, MTT cell proliferation assay, colony formation assay and lifespan analysis. Transfected cells (2000 cells) were seeded into 24-well plate and cultured over two weeks for cell growth analysis by cell counting and MTT assay. Cell proliferation assay using both cell counting (Figure 10) and MTT assay (Figure 11) of stably transfected MDAH041 and MDAH087-1 immortal cells showed decreased cell growth of CREG1 transfected cells compared with pIRES transfected control cells. To further confirm the effect of CREG1 on cell proliferation, the stably transfected cells

were seeded at low density (1000-5000 cells in 10-cm culture plates) to measure the ability to form colonies under the condition of drug selection for two weeks followed by Giemsa staining. As shown in Figure 12, CREG1 overexpression in immortal LFS cells exhibited decreased colony number compared with pIRES transfected cells.
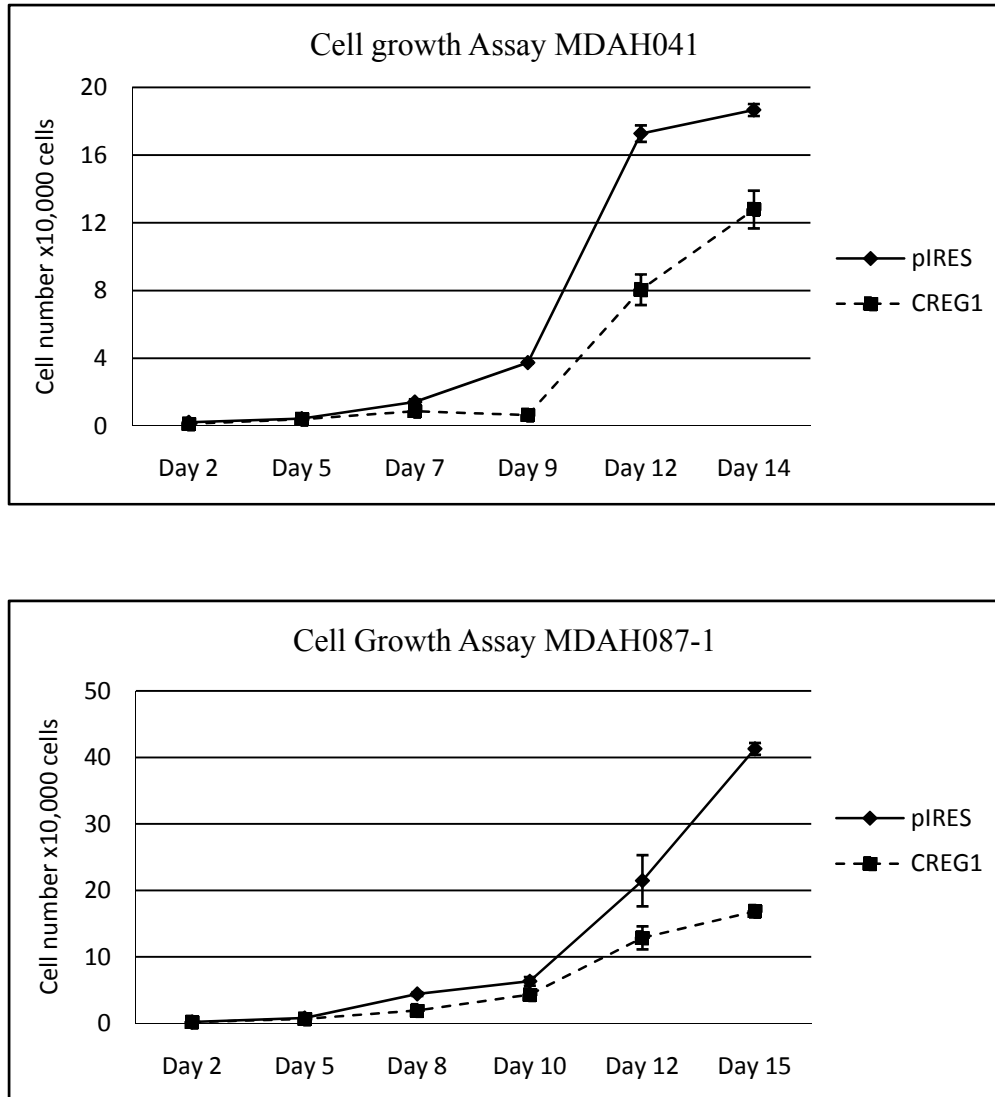
Figure 10. Cell growth assay of MDAH041 and MDAH087-1 transfected with CREG1 and control pIRES vectors. After stable transfection, 1000 cells were seeded into 24-well plates in triplicate and cell number was counted every 2 days. The data point with the standard deviation shows the result from the representative experiment of at least two independent experiments. CREG1-overexpressing cells grow slower than control cells in two independent LFS immortal cell lines.
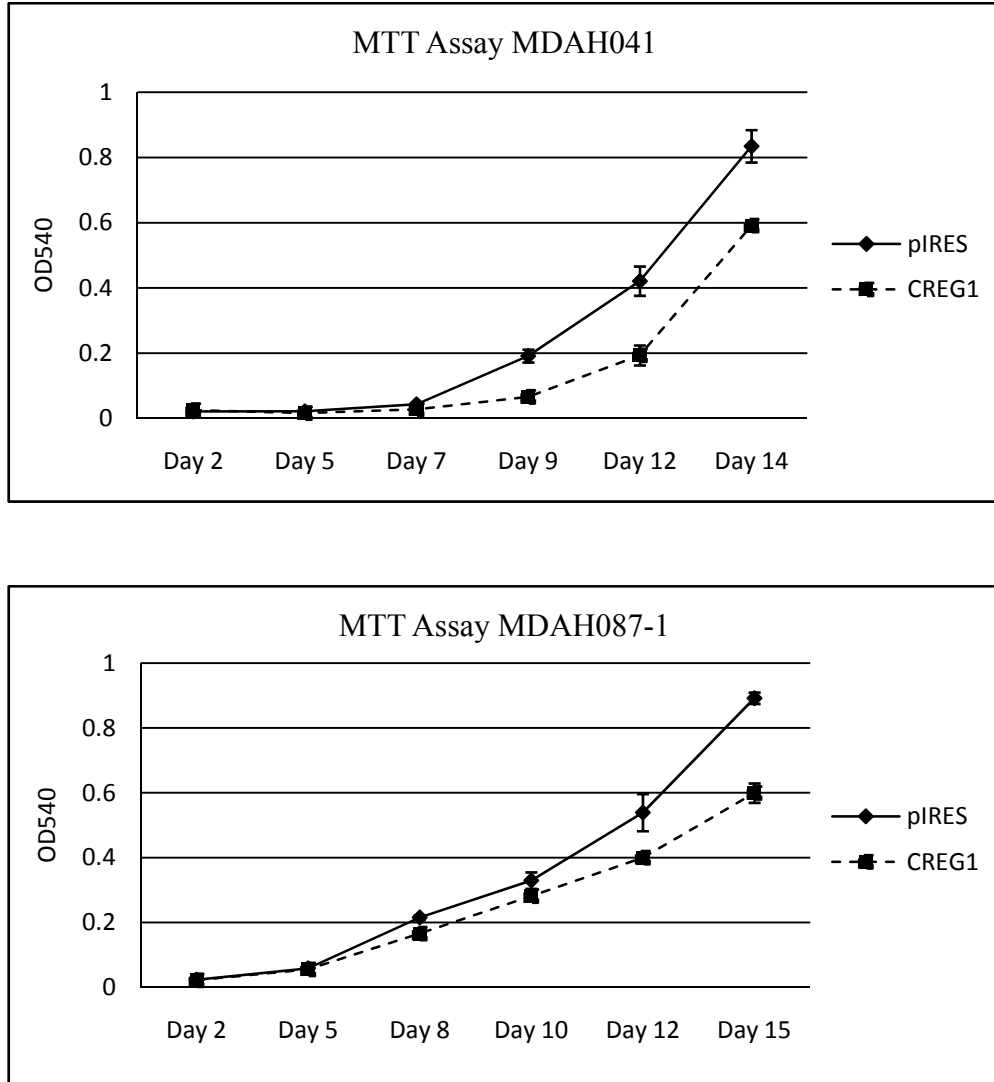
Figure 11. MTT cell proliferation assay of MDAH041 and MDAH087-1 immortal cells. After stable transfection, 2000 cells were seeded into 24-well plates in triplicate and the MTT assay was conducted every 2 days for 2 weeks. The data point with the standard deviation shows the result from the representative experiment of at least two independent experiments. Overexpression of CREG1 decreases cell proliferation in two independent immortal LFS cell lines.
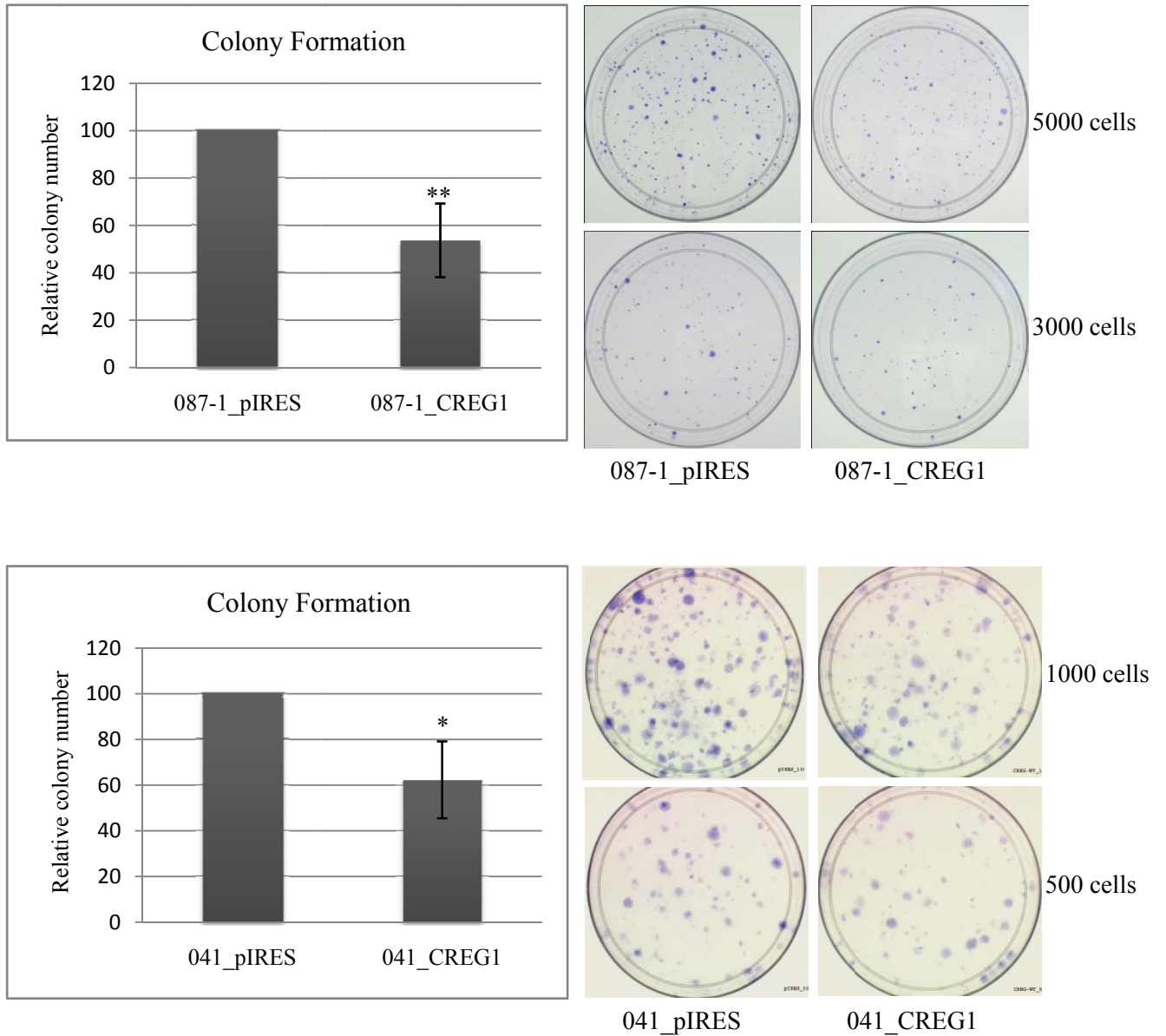
Figure 12. Colony formation assay of MDAH041 and MDAH087-1 cells. After stable transfection, cells (as indicated, depending on cell type) were seeded into 10-cm culture plates in triplicate and the colonies were stained by Giemsa after 2 weeks. The bar graphs represent the relative colony number normalized to control pIRES vector that were set to 100% from at least two independent experiments. Overexpression of CREG1 in immortal LFS cells decreases colony number compared with pIRES vector control. (** *P*<0.01; * *P*<0.05)

3.4.2   Lifespan analysis of CREG1 overexpressing immortal LFS cells

To further investigate the negative growth effects of CREG1 and whether stable expression of CREG1 affect the lifespan of immortal cells, the lifespan (population doubling) of cells expressing vector control and CREG1 was examined. The population of stably transfected cells was homogenous and derived from a single clone. MDAH087-1 immortal cells were transfected with CREG1 and pIRES vectors followed by drug selection for two weeks. Ten individual clones were selected and isolated from CREG1 and pIRES transfected cells by cloning cylinders and transferred to a 24-well plate for further growth. Lifespan assay from four of each CREG1 and empty vector cell clones was performed as described in section 2.11. The calculated population doublings (PD) of CREG1 and vector control clones were obtained from over three months in culture on drug selection. At the end of the lifespan assay, the PD from three of the vector control clones exceeded 60 and the growth rate seemed to increase constantly, whereas three of CREG1 clones had PD $\leq$ 50 (Figure 13). The effect of CREG1 on stably transfected clones over a period of time seemed to slow growth compared with pIRES empty vector but did not induce growth arrest of the cells because they maintained their initial growth rate. To detect if CREG1 overexpressing cells induce a senescence-like phenotype, senescence-associated β-galactosidase staining was examined in pIRES- and CREG1-expressing MDAH087-1 clones from passage 6 (Figure 14). CREG- expressing clones did not have higher activity of senescence-associated β-galactosidase than cells from vector control clones except clone no. 8 (8C8), which had high senescence-associated β-galactosidase activity. This might be due to clonal variation and a higher spontaneously induced senescence of MDAH087-1 clone 8 fibroblasts. The protein level of CREG1 in pIRES and CREG1 expressed clones is shown in Figure 15.
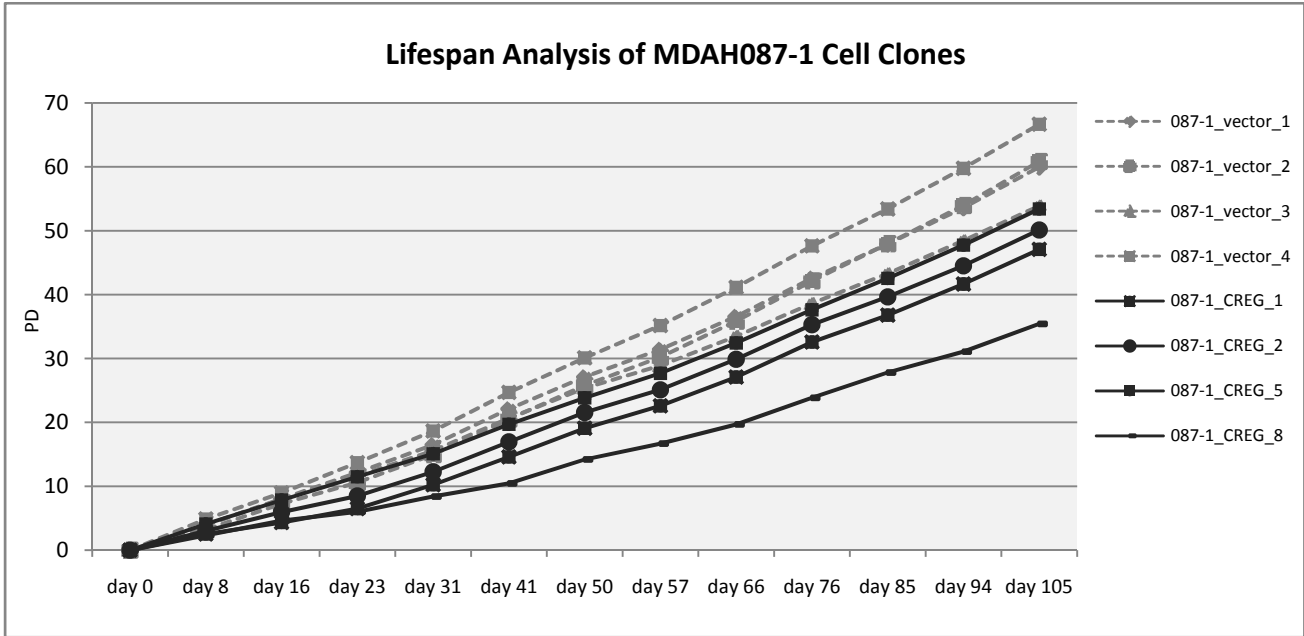
Figure 13. Lifespan analysis of CREG1 overexpressing immortal cells. The population doublings of MDAH087-1 from pIRES and CREG1 expressing clones were calculated from cell number counted every week over a period of 105 days in culture.
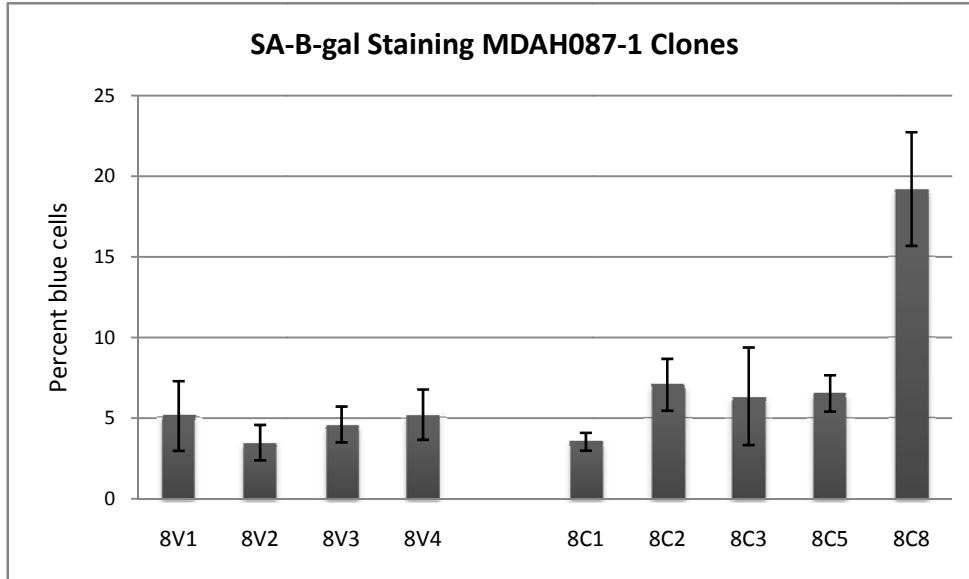
Figure 14. Senescence-associated β-galactosidate staining of MDAH087-1 cell clones stably transfected with pIRES empty vector and CREG1. MDAH087-1 cell clones stably expressed CREG1 (8C1, 8C2, 8C3, 8C5, and 8C8) and pIRES (8V1, 8V2, 8V3, and 8V4) and were stained at passage 6. Student's t-test of percent mean of β-gal stained cells in the group of vector control (8V1, 8V2, 8V3, and 8V4) and CREG1 (8C1, 8C2, 8C3, and 8C5) shows $P=0.043$.

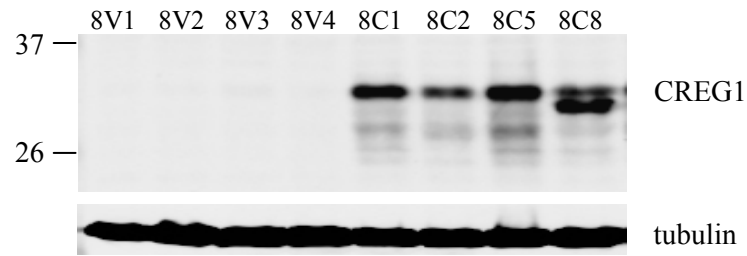**Figure 15.** CREG1 protein levels in MDAH087-1 cell clones stably expressed CREG1. The protein levels were examined at passage 12 from pIRES (8V1, 8V2, 8V3, and 8V4) and CREG1 (8C1, 8C2, 8C5, and 8C8) expressed clones. The different variant of CREG1 protein observed in clone 8 (8C8) may due to the different form of glycosylation or protein cleavage which was not observed in later passage (passage 19 shown in Figure 19)

3.4.3 CREG1 overexpression slows cell growth in cancer cell lines

To confirm the negative growth effects of CREG1, cancer cell lines relevant to Li-Fraumeni Syndrome including osteosarcoma (U2OS;pRb$^+$ and SaOS2;pRb$^-$) (85) and fibrosarcoma (p53-deficient) (86) cell lines were investigated. The status of pRb, p16 and p53 in these cell lines as well as immortal LFS fibroblasts are shown in Table 2. Two μg of CREG1 and pIRES empty vectors were transfected into osteosarcoma U2OS (pRb$^+$) and SaOS2 (pRb$^-$) cells and fibrosarcoma HT1080 cells followed by drug selection (0.5 μg/ml puromycin in standard culture media) for one week. Cell proliferation assays by cell counting were performed in the U2OS cells and the results showed that CREG1 transfected U2OS cells grew slower cell than pIRES control cells (Figure 16). In addition, colony formation was examined in U2OS, SaOS2, and HT1080 cell lines transfected with CREG1 and pIRES control vectors followed by drug selection. Transfected cells were seeded at low density (500-5000 cells depending on cell lines) on 10-cm culture plates in triplicate and cultured under drug selection for 2 weeks. The colonies were stained by Giemsa after two weeks in culture. The colonies (size≥ 1 mm) were counted and the relative colony number was calculated from normalization to the vector control. Overexpression of CREG1 decreased colony formation in U2OS and HT1080 cells and slight decreased colony formation in SaOS2 cells (Figure 17).

Table 2. The status of pRb, p16 and p53 in cell lines studied. LFS; Li-Fraumeni Syndrome immortal fibroblasts, U2OS and SaOS2; osteosarcoma, HT1080; fibrosarcoma cell lines.

Cell line	pRb	p16	p53
LFS			Gene mutation
MDAH041	Expressed	No expression	Codon 184/ FS stop
MDAH087	Expressed	No expression	Codon 248 MS Arg-Trp
MDAH172	N/A	N/A	Codon 175 MS Arg-His
MDAH174	N/A	N/A	Codon 175 MS Arg-His
U2OS	Expressed	No expression	Expressed
SaOS2	No expression	Expressed	No expression
HT1080	Expressed	No expression	Gene mutation
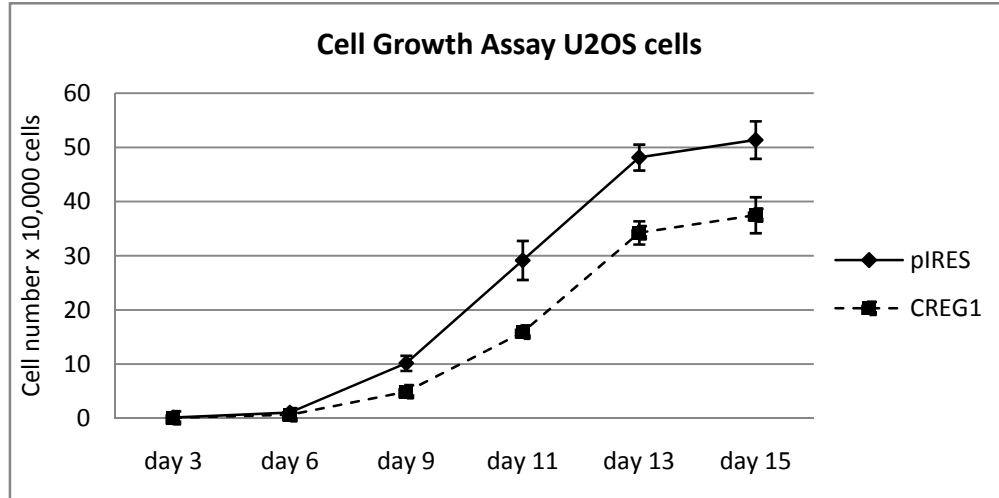
Figure 16. Cell growth assay of U2OS cells stably transfected with CREG1 and pIRES empty vectors. CREG1 overexpression slows cell growth in U2OS cells. After stable transfection, 1000 cells were seeded into a 24-well plate in triplicate and cell number was counted every 2 days. The data point with the standard deviation shows the result from a representative experiment. The experiments were repeated twice.

Colony number

U2OS_pIRES

U2OS_CREG

5000 cells

3000 cells

Colony number

HT1080_pIRES

HT1080_CREG1

1000 cells

500 cells

Colony number

SaOS2_pIRES

SaOS2_CREG1

5000 cells
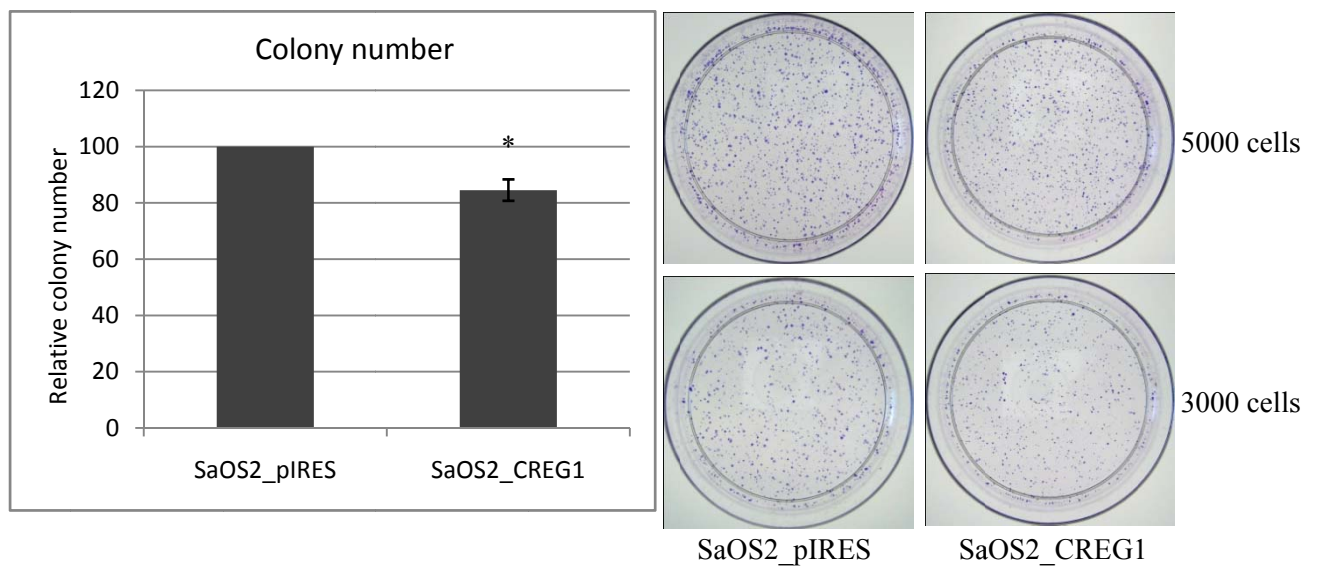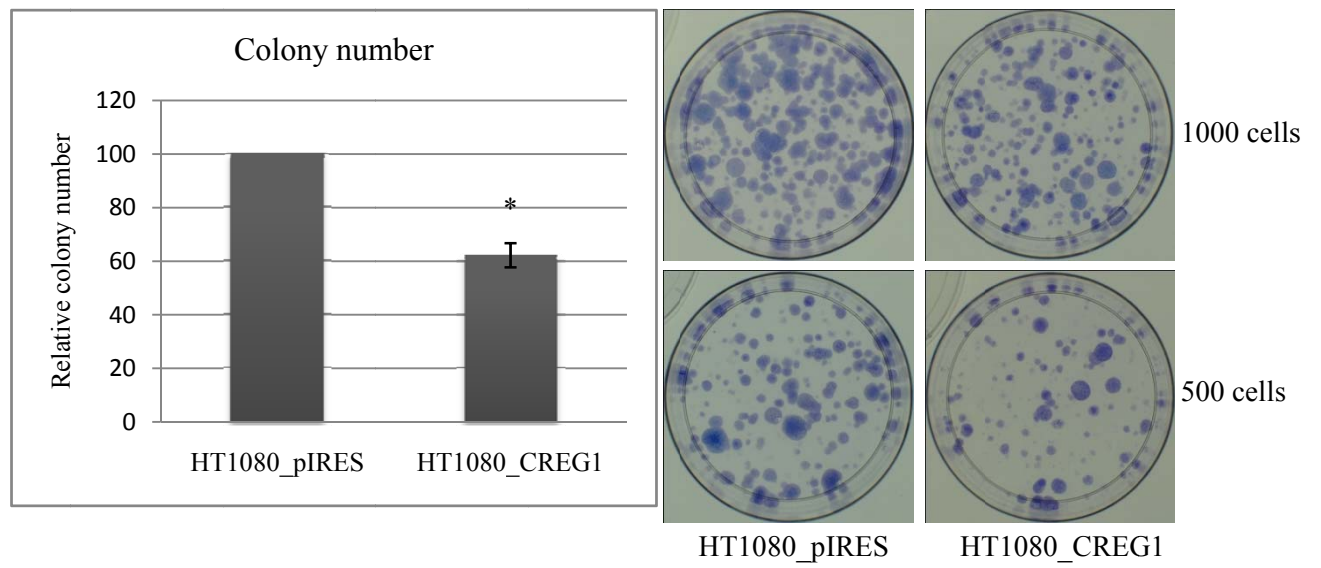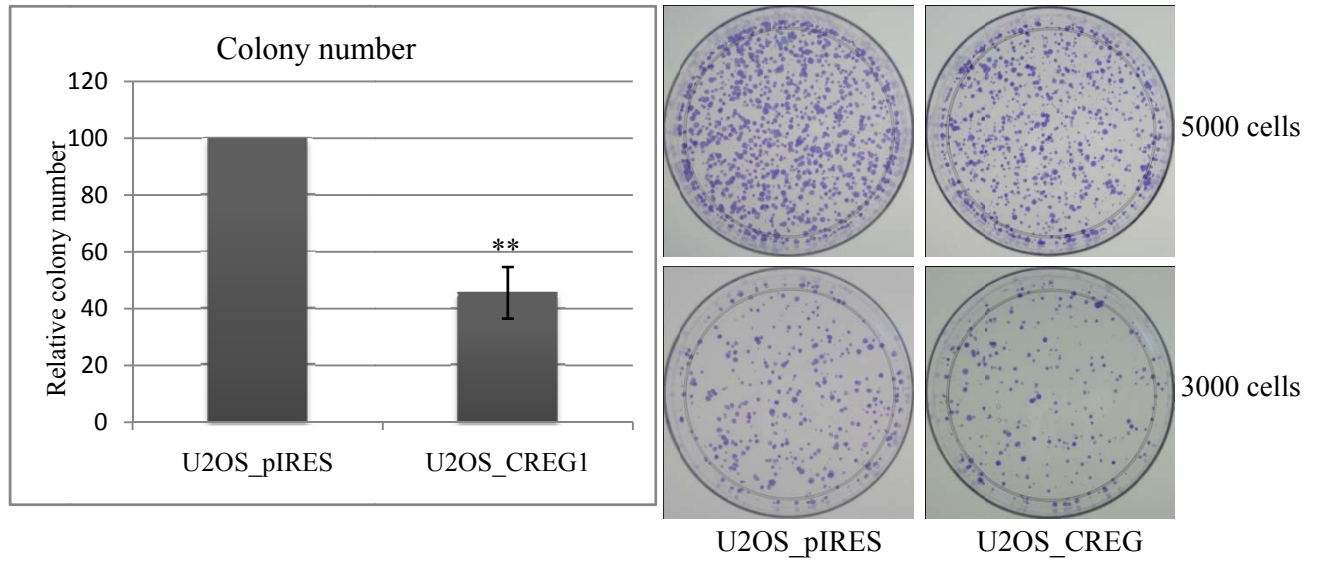
3000 cells

Figure 17. Colony formation assay of U2OS, HT1080, and SaOS2 cell lines. Cells were seeded as indicated (500-5000 cells) in triplicate and cultured for two weeks. The bar graphs demonstrate the relative colony number normalized with control empty vector. The error bars are standard deviation from at least two independent experiments. CREG1-overexpressing cells reduce colony number relative to vector control in U2OS and HT1080 cell lines and slightly decrease colony number in SaOS-2 cells. (** $P<0.01$; * $P<0.05$)

3.5 **Analysis of CREG1 protein; glycosylation modification, cellular localization, and interacting proteins**

CREG1 was initially identified by the yeast two-hybrid method for proteins that interact with TATA-binding protein (TBP) in the *Drosophila* cDNA library (67). It was also shown to bind TBP and Rb family pocket proteins, pRb, p107 and p130 *in vitro* by GST pull down assay by the same group. CREG1 is a glycoprotein which contains three N-glycosylation sites (65). To determine whether there were glycosylated forms of CREG1 in our cells, we treated cell lysates from MDAH087-1 cells that overexpressed CREG1 with peptide-N-glycosidase F (PNGaseF) (BioLabs) following the manufacturer's instructions. Western blot analysis of PNGaseF treated cell lysates demonstrated a deglycosylated form of CREG1 as shown in Figure 18. The size of CREG1 protein changed from glycosylated forms (~25-30 kD) to single form of protein (~20 kD), the predicted size of the protein calculated from amino acid sequence.

To further investigate the localization of CREG1 within the cells, cytosolic and nuclear fractions from CREG1 stably transfected-MDAH087-1 cell clones were prepared and analyzed by western blotting. As shown in Figure 19, CREG1 was not observed in the nuclear fraction but mainly found in the cytosolic fraction. Nevertheless, detection of the majority of CREG1 in the cytosol does not exclude the possibility that CREG1 could function in the nucleus to antagonize the transcriptional activity of E2F and interact with TATA binding protein and Rb family pocket proteins, as revealed by Veal et al. (67). It is possible that the amounts of CREG1 present in the nucleus may be below the sensitivity of detection or that it accumulates in the nucleus under specific cellular conditions.

To investigate CREG1-interacting proteins, coimmunoprecipitation was performed in MDAH087-1 cell clones stably expressing CREG1 and control pIRES vectors. The concept of this method is to use the antibody targeting the protein of interest, which binds to the antigen or the target protein that may form the complex with its binding partners in the intact cell lysate. The immunocomplex is then analyzed for its known or unknown binding proteins. The antibodies of known CREG1 interacting proteins including CREG1, IGF2R and pRb were used in coimmunoprecipication (co-IP) experiments followed by western blot analysis. CREG1 was observed to directly interact with IGF2R by this method. However, we did not observe direct interaction between CREG1 and pRb by coimmunoprecipitation assay (Figure 20). Nevertheless, there are several aspects of concern regarding this assay, including the specificity of the antibody to the antigen (protein of interest), exposure of binding sites, and the tightness of the interaction with the proteins to be detected.
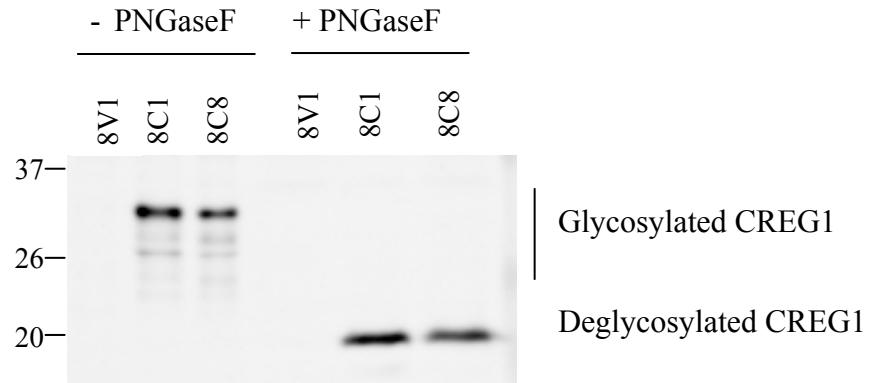
8V1 = MDAH087-1 pIRES stably transfected clone 1

8C1 = MDAH087-1 CREG1 stably transfected clone 1

8C8 = MDAH087-1 CREG1 stably transfected clone 8

Figure 18. Deglycosylation of CREG1. Total cell lysate from MDAH087-1 cell clones stably expressing vector control (8V1) and CREG1 (8C1 and 8C8) at passage 19 were treated and untreated with PNGaseF followed by western blotting. Treatment with PNGaseF deglycosylates CREG1.
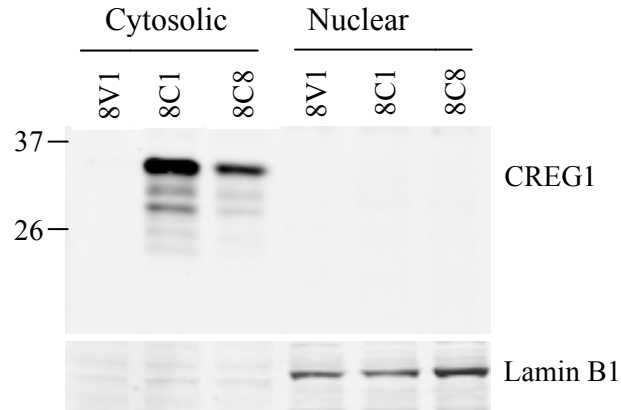
Figure 19. CREG1 is mainly found in the cytosolic fraction. Cytosolic and nuclear fractions of pIRES (8V1) and CREG1 (8C1 and 8C8) overexpressing MDAH087-1 clones individually isolated from CREG1 and pIRES transfected MDAH087-1 cells. Lamin B1 was used as the control for the nuclear fraction.
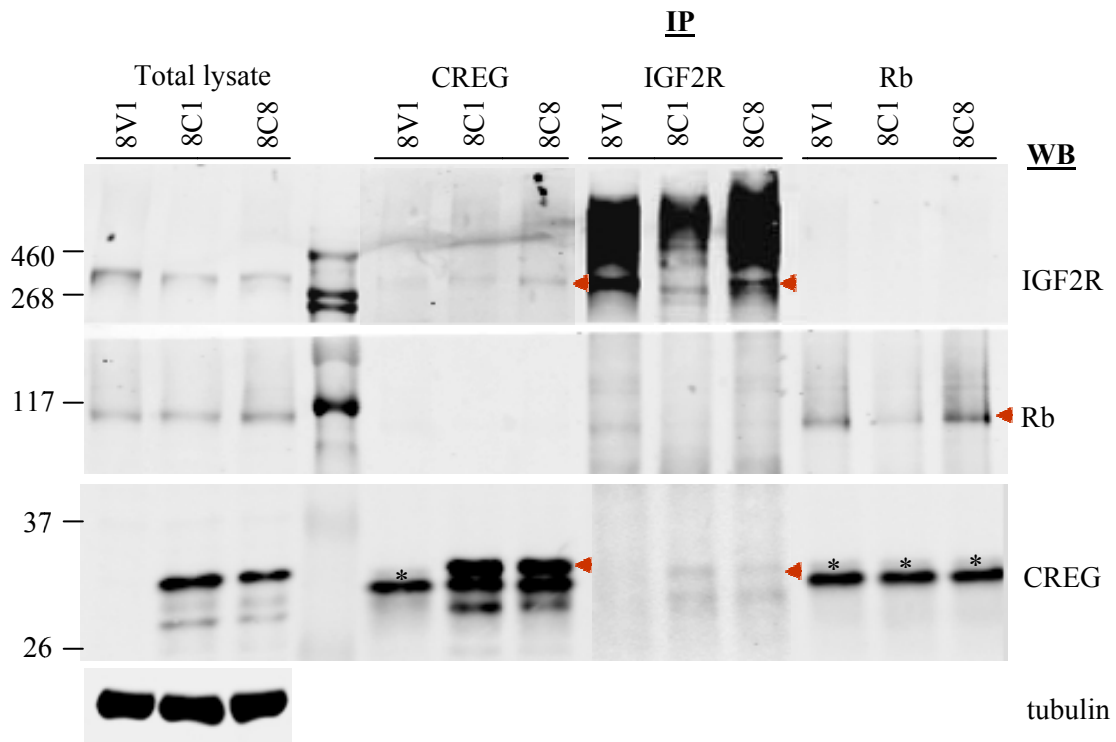
Figure 20. Western blot analysis of total cell lysate coimmunoprecipitated with indicated antibodies in CREG1 and pIRES stably expressed MDAH087-1 cell clones. CREG1 directly interacts with IGF2R as it can be detected with IGF2R immunoprecipitation and *vice versa*. The red arrows indicate the corresponding protein that can be detected following immunoprecipitation and the asterisks indicate the non specific bands due to agarose beads.

3.6   Discussion

The goal of this study was to investigate the role of CREG1 in cellular immortalization and senescence. This gene was identified from the gene expression profiling analysis (56) of genes and pathway changes during cellular immortalization and senescence in Li-Frameni Syndrome fibroblasts harboring p53 mutations. CREG1 is one of the genes that was shown to be downregulated during immortalization and upregulated after 5-aza-dC-induced senescence. CREG1 was demonstrated to be a negative regulator of E2F transcription factors that antagonizes the ability of E1A to transform the cells to be tumorigenic (67). This background information indicated that CREG1 was an interesting candidate gene for study.

We then investigated the role of CREG1 in cellular senescence in immortal LFS and related cancer cell lines. Verification of CREG1 expression in four spontaneously immortalized LFS cell lines used in microarray study and additional two LFS cell lines revealed that CREG1 levels decreased in immortal fibroblasts compared with each precrisis counterpart cell strain and its expression was upregulated in replicative and 5-aza-dC-induced senescence of fibroblasts. Analysis of the CREG1 promoter identified a CpG island that was highly methylated in MDAH041, partially methylated in MDAH087-1 and poorly methylated in MDAH087-10 immortal fibroblasts. It is possible that the two alleles of CREG1 in MDAH087-1 and MDAH087-10 are differentially methylated reflecting the intermediate expression level of CREG1 in these cells when compared with heavily methylated promoters from MDAH041. In addition, CREG1 is located at the 1q24.1 region and it has been shown that chromosome 1q carries genes altered in cell lines assigned in complementation group C, which is involved in cellular

senescence (87). We demonstrated for the first time that CREG1 expression is epigenetically silenced by promoter methylation in immortal fibroblasts and this may contribute to cellular immortalization.

To examine whether CREG1 can induce cellular senescence, CREG1 and pIRES empty vectors were stably transfected into two immortal LFS cell lines, MDAH041 and MDAH087-1, which express the lowest levels of CREG1. We found that CREG1 overexpression in immortal LFS cells resulted in slow cell growth compared with control pIRES transfected cells. These results support the growth suppression function of CREG1 from previous studies (69, 70). The negative growth effects of CREG1 were also observed in cancer cell lines including U2OS osteosarcoma (wild-type p53) and HT1080 fibrosarcoma (p53-deficient). However, the growth suppression effect of CREG1 was lower in SaOS2 cells, another osteosarcoma cell line which has no pRb expression, compared to two other cell lines studied that express pRb protein. In addition, the expression level of CREG1 in this cell line, SaOS2, seems to be higher than others. This may indicate that the growth suppressive function of CREG1 depends on the Rb signaling pathway. Moreover, analysis of the lifespan (population doubling) of MDAH087-1 immortal cells expressing CREG1 revealed that CREG1 expressing cells continued to grow at a slower rate than empty vector expressing cells. Nevertheless, we did not observe the induction of cellular senescence in cells transfected with CREG1 alone as judged by cellular morphology or the senescence-associated β-gactosidase assay. Although, we observed the slight increase of senescent cells in MDAH087-1 cell clones expressing CREG1 when compared with vector control clones (Figure 14), this may not conclude that those senescent cells caused by CREG1 expression but spontaneously

senescing, because we also observed the basal level of senescent cells in cells transfected with vector control. This can be due to stress associated with cell culture conditions and changes of pH in the β-galactosidase staining assay which influence an increase of β-galactosidase activity (10, 88). Despite the epigenetically downregulation of CREG1 may be one of the steps during immortalization, the ectopic expression of CREG1 in immortal LFS and cancer cells was not sufficient to induce senescence. This can be partly due to the absence some other partner proteins associated with CREG1 function or proteins that regulate CREG1 in some way.

Cell fractionation analysis of immortal cells overexpressing CREG1 showed that the majority of CREG1 is found in the cytosolic fraction. However, no previous study has clearly elucidated the localization of CREG1 in the nucleus as expected for a transcriptional repressor. It is possible that the signaling pathway involved in the negative growth effects of CREG1 occurs outside the nucleus. The putative receptor of CREG1 is IGF2R, the scavenger transmembrane protein that plays a role in regulation of insulin-like growth factor II (IGF-II) endocytosis. Supporting this observation, a study from Han et al. showed that CREG1 plays a role in the inhibition of smooth muscle cells migration by mediating IGF-II endocytosis via the IGF2R receptor (74). It is therefore possible that the growth suppressive function of CREG1 may depend on the IGF2R-IGF2 signaling pathway. We examined CREG1 interacting proteins by coimmunoprecipitation using CREG1 antibody and found that CREG1 physically interacts with IGF2R. However, we did not observe the interaction of CREG1 and Rb pocket proteins as demonstrated by Veal et al. 1998, who used an *in vitro* GST pull-down assay. To further investigate

CREG1 interacting proteins, an alternative and more sensitive technology such as mass-spectrometry may be needed to identify proteins that interact with CREG1.

CHAPTER 4

CREG1 ENHANCEMENT OF P16^{INK4a} IN GROWTH SUPPRESSION AND SENESCENCE

4.1   Summary

The functional analysis of CREG1 in immortal LFS and related cancer cell lines revealed the growth suppression function of CREG1. However, CREG1 overexpression did not directly induce senescence. We hypothesized that CREG1 function may depend on the phosphorylation status of pRb which is phosphorylated by cyclin/CDK complex. p16^{INK4a}, a CDK 4/6 inhibitor, is an upstream regulator of Rb phosphorylation and plays an important role in cellular senescence. We found that p16^{INK4a} expression was downregulated in immortal LFS fibroblasts and its expression was epigenetically regulated by promoter hypermethylation (56). In addition, p16^{INK4a} is not expressed in U2OS and HT1080 cell lines. SaOS2 cell line expresses p16^{INK4a} but not pRb. Interestingly, coexpression of CREG1 and p16^{INK4a} in immortal LFS, U2OS and HT1080 cell lines induced a senescence-like phenotype including changes of cell morphology, decreased cell growth, increased staining of senescence-associated β-galactosidase, decreased transcriptional activity and expression levels of cyclin A and B, regulators of cell cycle progression. We further investigated the role of Rb-family members, pRb, p107 and p130 in cellular senescence induced by cooperation between CREG1 and p16 by using lentivirus-mediated knockdown of each Rb family protein in U2OS cells. p16 alone or coexpression of CREG1 and p16 induced senescence in U2OS cells stably infected with lentivirus-mediated knockdown of p107, p130 and non-silencing control cells.

However, this effect was diminished in Rb knockdown cells. This indicates that pRb is critical in cellular senescence induced by coexpression of CREG1 and p16.

4.2 Coexpression of CREG1 and p16^{INK4a} has a great effect to suppress cell growth

To investigate whether coexpression of CREG1 could enhance the effect of p16-induced senescence in immortal LFS fibroblasts, U2OS and HT1080 cell lines were used. Plasmids containing CREG1 and p16^{INK4a} were cotransfected into these cell lines, alone or in combination using the empty vectors to balance the amount of transfected DNA. Cells were selected using double drug selection in all cases: puromycin (0.5 µg/ml) and G418 (200 µg/ml), to ensure similar transfection efficiency. Control untransfected cells died after 3 days of drug selection. After 5 days of selection, U2OS cells were expanded for growth and senescence. Cell cycle analysis by flow cytometry showed an increase of cells in G0-G1 arrest and a decrease of S phase cells in cells cotransfected with CREG1 and p16 compared to cells transfected with either CREG1 or p16 alone (Figure 21 and Figure 22). The data were obtained from three independent experiments. The histograms are shown from flow cytometry of representative experiments. There was no obvious sign of apoptosis (sub-G1 peak of PI histogram) observed from the cell cycle analysis results (Figure 22). The additional independent results of cell cycle analysis by flow cytometry (histograms of cell populations) are shown in Appendix 1 and 2.
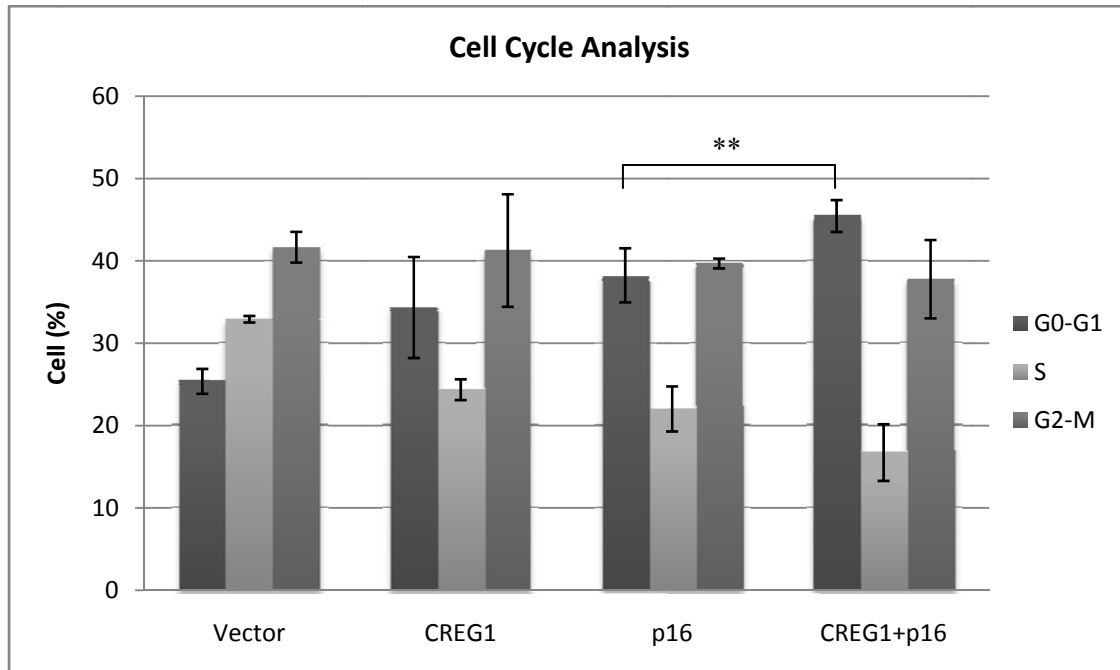
Figure 21. Cell cycle analysis of U2OS cells cotransfected with CREG1 and p16. Cells were analyzed at day 5 after selection. The bar graphs are average percent cell population in G0-G1, S and G2-M phases from three independent experiments. ANOVA analysis was performed to test the differences of each phases in all transfection conditions and showed significance differences of G0-G1 ($P<0.01$) and S phases ($P < 0.01$) but not G2-M ($P=0.68$). Student's t-test was performed to compare the effect of p16 and combination of CREG1 and p16 in G0-G1 phase. ** $P<0.01$
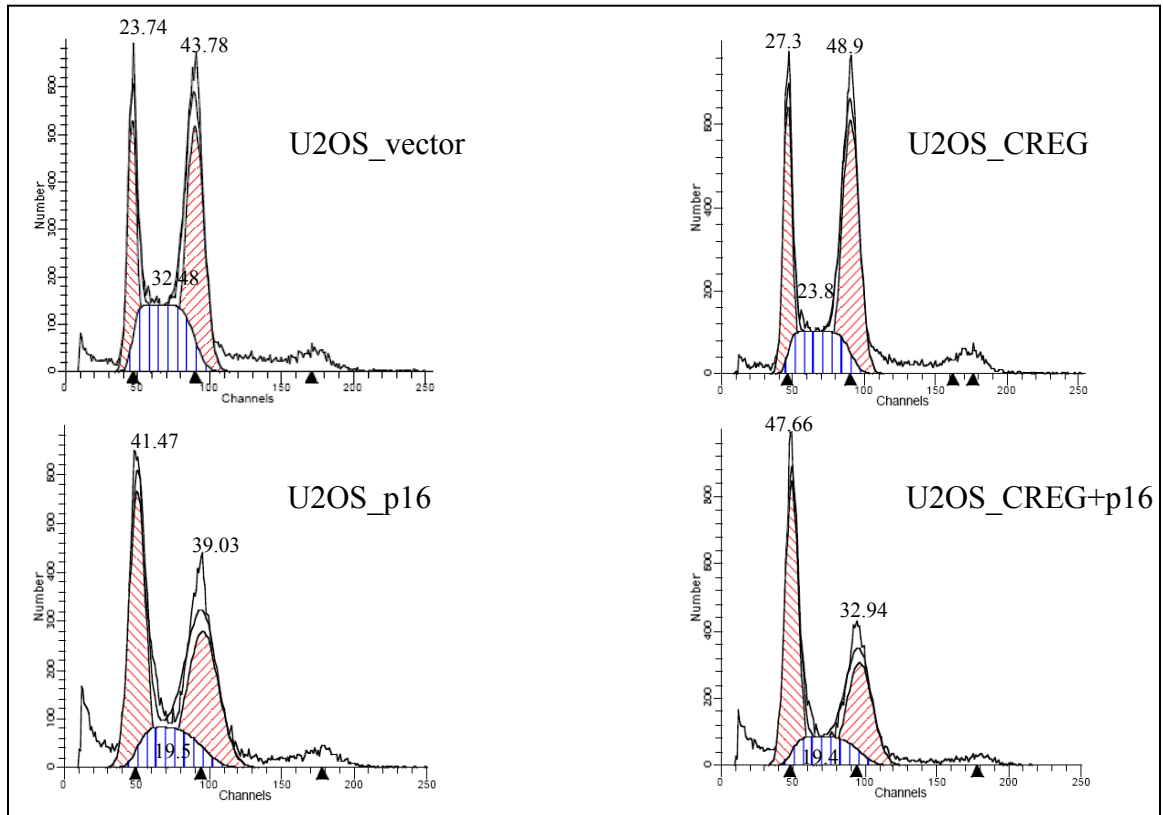
Figure 22. Histograms of cell cycle analysis by flow cytometry of U2OS cells transfected with CREG1 and p16 from the representative experiment. Cells were analyzed at day 5 after drug selection. The numbers indicate the percent of cell population in G0-G1, S and G2-M phases.

To confirm the growth inhibition upon CREG1 and p16 coexpression, a colony formation assay was performed after stable transfection. U2OS cells were transfected with 2 µg each of CREG1 and p16, alone or in combination followed by double drug selection at 48h after transfection for 5 days. Transfected cells were trypsinized and seeded at low density (2000 cells in 10-cm cultured plates) in triplicate and cultured under drug selection for 2 weeks. The colonies were stained with Giemsa and the colony number was counted and compared to the vector control. As shown in Figure 23, colony formation was inhibited in cells cotransfected with CREG1 and p16 more than cells transfected with p16 or CREG1 alone. The results were obtained from three experiments.
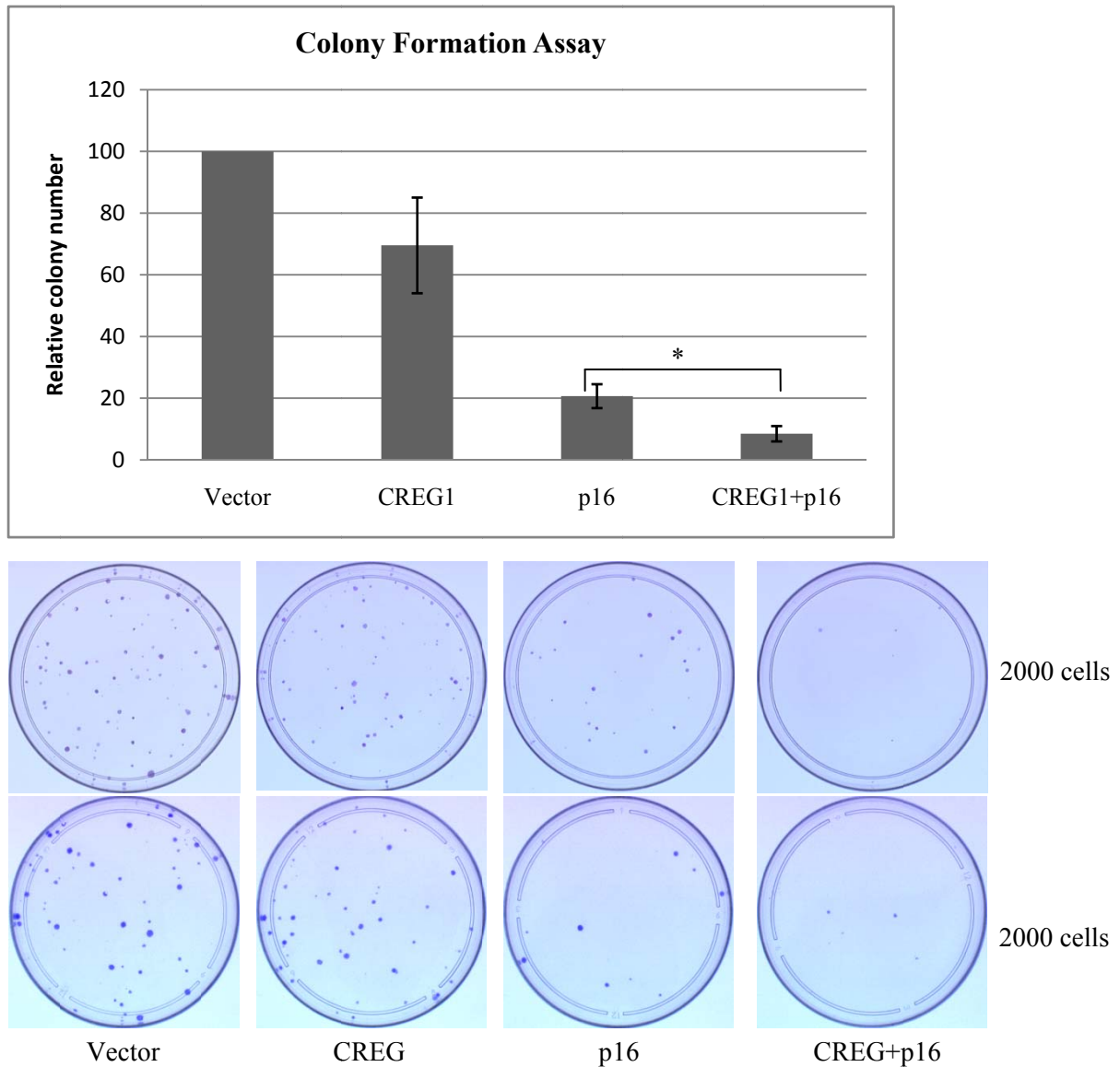
Figure 23. Colony number of U2OS cells transfected with CREG1 and p16. The bar graphs show the average relative colony number normalized to the vector control from three independent experiments. 2000 cells were seeded into 10-cm cultured plate in triplicate at day 5 after double drug selection and the colonies were stained with Giemsa after 2 weeks. The pictures are from two independent experiments. ANOVA analysis was performed to test the differences of relative colony number to vector control ($P<0.01$). Student's t-test was performed to compare the effect of p16 and combination of CREG1 and p16 on colony formation. Coexpression of CREG1 and p16 has the strongest effect on inhibiting colony formation. * $P<0.05$

4.3 CREG1 enhances p16^{INK4a}-induced senescence: cell morphology changes and senescence-associated β-galactosidase staining

The senescent-like phenotype can be observed by changes of cell morphology (large and flat cells) and an increase of senescence-associated β-galactosidase activity at pH 6. These characteristics, as well as the growth arrest phenotype, were used as indicators of senescent cells in this study. U2OS cells were transfected with CREG1 and p16, alone and in combination, followed by double drug selection for 5 days as mentioned above. In p16-transfected cells, and more so in cells cotransfected with CREG1 and p16, we observed cell characteristics indicative of senescence as shown in Figure 24 (cell morphology) and Figure 25 (senescence-associated β-galactosidase staining) at day 5 after drug selection. Although it was shown previously that p16 overexpression alone induces cellular senescence in this cell line, U2OS, we showed here that coexpression of CREG1 with p16 induced an even stronger effect than p16 expression alone.
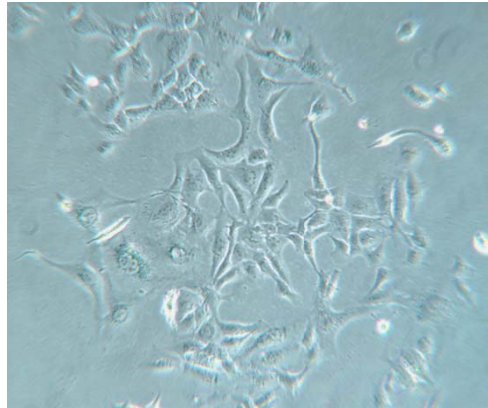
Vector

CREG1

p16
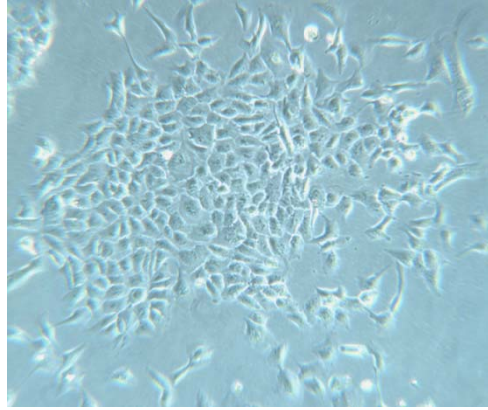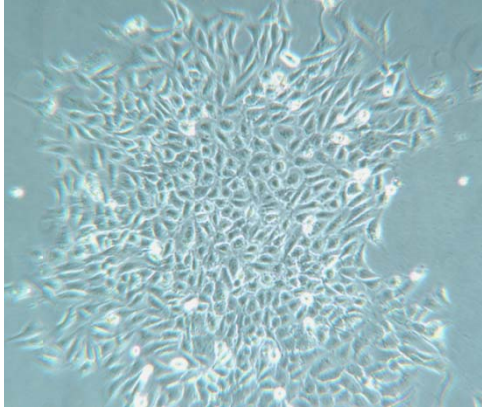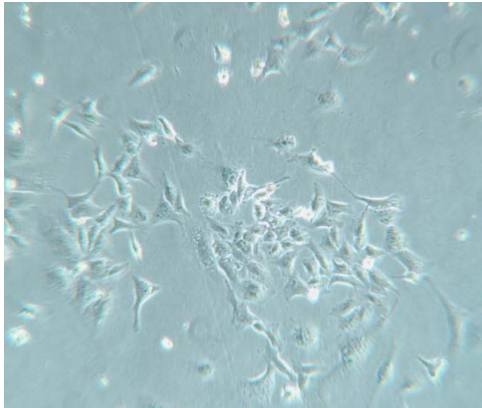
CREG1+p16
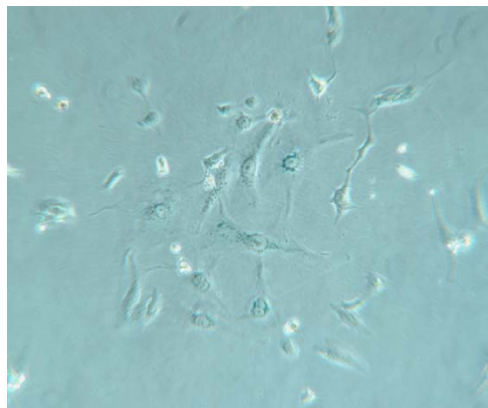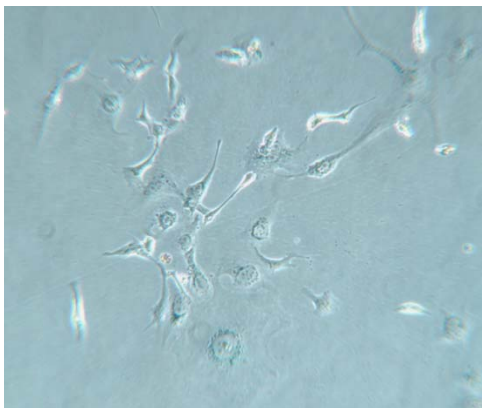
Figure 24. Cell morphology of U2OS cells cotransfected with CREG1 and p16, alone and in combination. Transfected cells were β-galactosidase stained and photographed at day 5 after selection. The pictures show two different fields on the culture plate from the representative experiment. Scale bar is 200 µm.

Figure 25. Senescence-associated β-galactosidase (SA-β-gal) staining of U2OS cells transfected with CREG1 and p16, alone and in combination. The bar graphs show the percent of SA-β-gal stained (blue) cells from three independent experiments. ANOVA analysis was performed to test the differences of percent stained cells relative to vector control ($P<0.01$). Student's t-test was performed to compare the effect of p16 and combination of CREG1 and p16 on colony formation. ** $P<0.01$

We also examined the effect of CREG1 and p16 coexpression in cellular senescence in the HT1080 fibrosarcoma cell line. As shown in Table 2, this cell line is p53-deficient, does not express p16 and contains intact pRb protein. Coexpression of CREG1 and p16 in this cell line was done similarly to U2OS cells as described above. Interestingly, the effect of CREG1 and p16 on cellular senescence was also observed in this HT1080 cell line. Coexpression of CREG1 and p16 showed an increase in senescence-associated β-galactosidase staining and cell morphology changes characteristic of senescent cells (Figure 26 and Figure 27). These results indicate that the effect of CREG1 and p16 coexpression on cellular senescence does not depend on the intact p53 protein.

| Vector | CREG1 | p16 | CREG1+p16 |
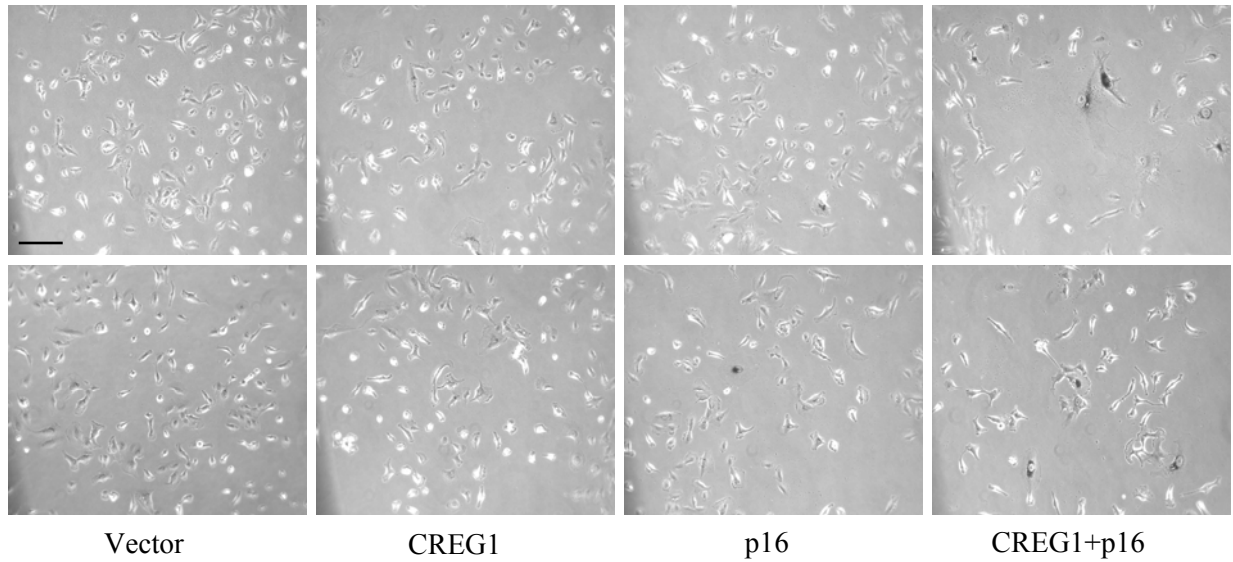
Figure 26. Cell morphology of HT1080 cells transfected with CREG1 and p16, alone and in combination. Cells were split for senescence-associated β-galactosidase staining at the same cell number after two weeks of drug selection. The pictures show two different fields on the plate from the representative experiment. Scale bar is 200 μm.
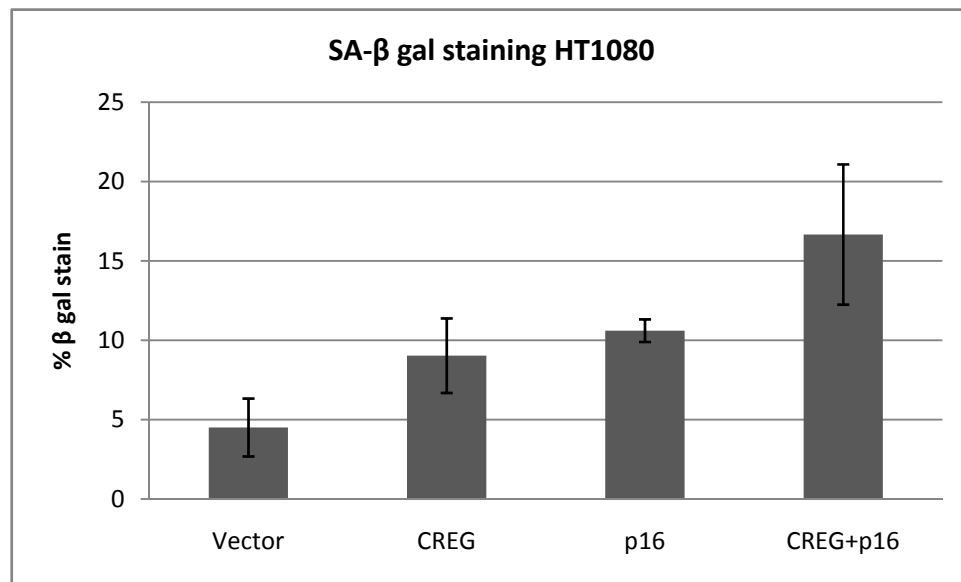
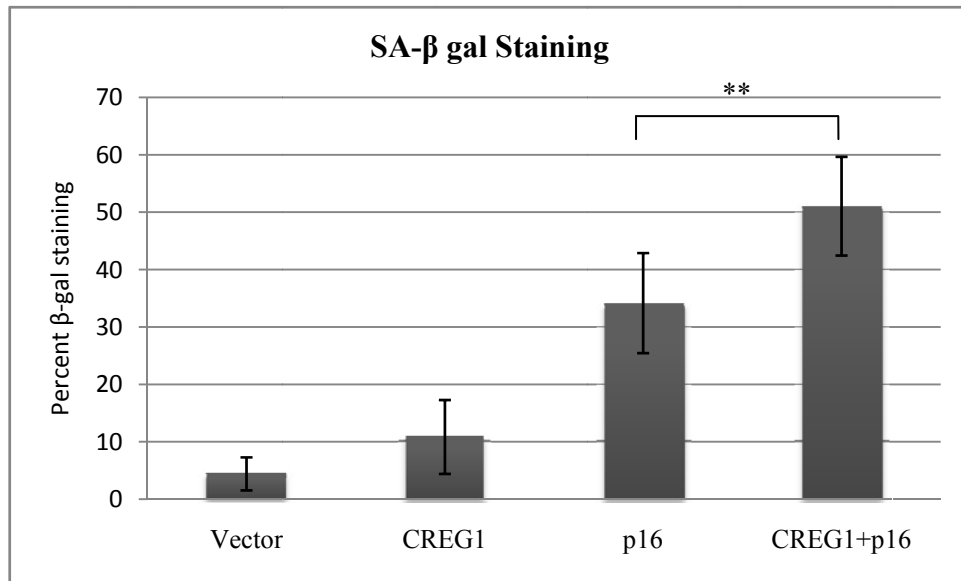**Figure 27.** Senescence-associated β-galactosidase staining of HT1080 cells coexpresssing CREG1 and p16. The bar graphs show the percent of β-gal stained (blue) cells from the representative experiment.

In addition, we also observed the additive effect of CREG1 and p16 to induce senescence in an immortal LFS cell line. The MDAH041 cell line was transfected with 2 μg each of CREG1 and p16, alone and in combination followed by double drug selection. After two weeks of selection, cells were trypsinized and lifespan analysis of stably transfected cells as well as MDAH041 untransfected cells was performed. In this assay, cells were seeded at $1x10^5$ cells in a 10-cm culture plate and the cell number was counted to calculate the population doubling by applying $\log_2$ to the total cell number after one week in culture. The cells were then re-seeded at the same amount and the population doublings were calculated. The cell morphology of MDAH041 transfected with CREG1 and p16 at day 5 after seeding for the lifespan assay is shown in Figure 28. The lifespan analysis showing the population doublings of MDAH041 cells over a month in culture is shown in Figure 29. Unexpectedly, cells stably expressing p16 grew at a similar growth rate as CREG1-expressing cells. It is possible that these data indicate the outgrowth of cells expressing lower amount of the proteins of interest, in this case CREG1 and p16, which would influence the results of this experiment. We speculate that culture of these cells over time may cause loss of the growth suppression effect of CREG1 and p16, alone and in combination.

Vector	CREG1	p16	CREG1+p16
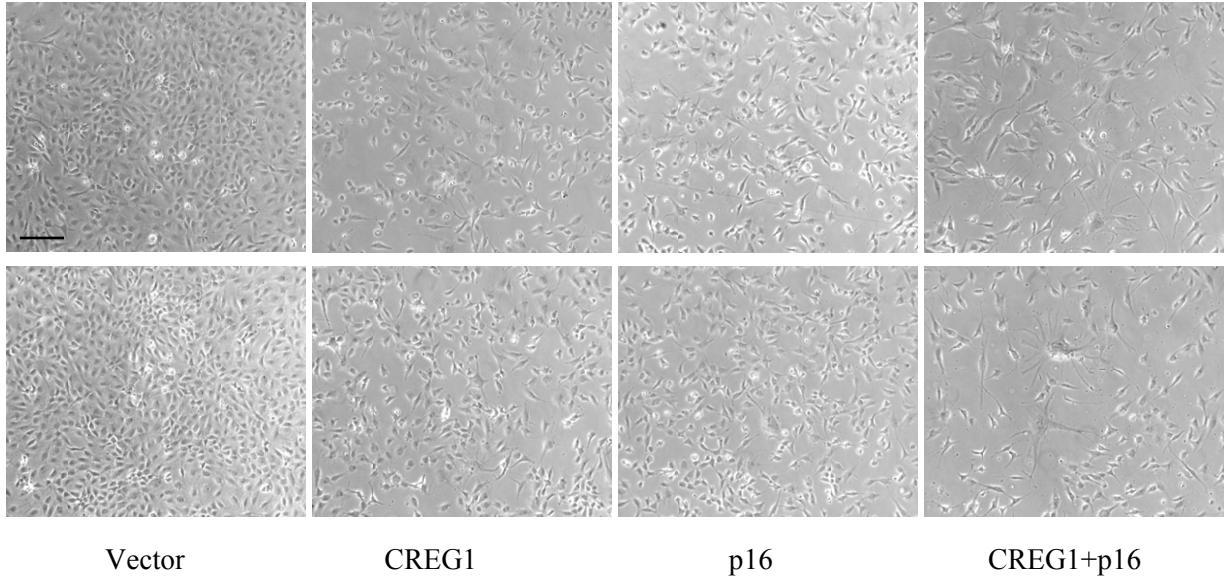
Figure 28. Cell morphology of MDAH041, an immortal LFS cell line expressing CREG1 and p16. MDAH041 cells were transfected with CREG1 and p16, alone and in combination, followed by double drug selection for two weeks. Transfected cells were trypsinized, seeded at $1x10^5$ cells and cultured for 5 days. The cell morphology was photographed at day 5 after seeding for lifespan assay. Scale bar is 200 μm.
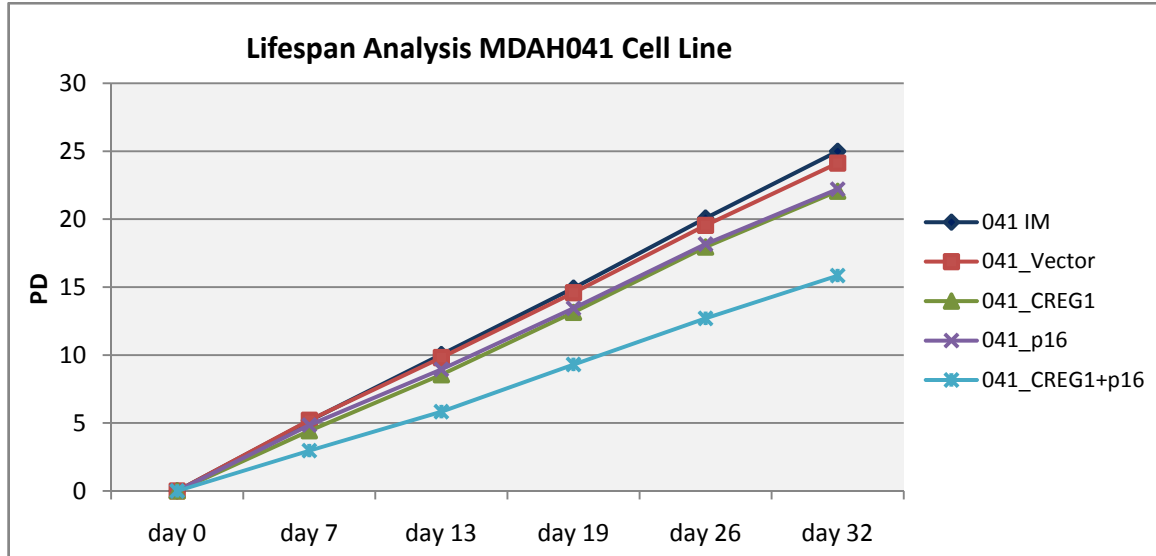
Figure 29. Lifespan analysis of MDAH041 cells stably transfected with CREG1 and p16, alone and in combination. The population doublings are calculated from total cell number counted every week. Coexpression of CREG1 and p16 decreased the population doubling compared to CREG1 and p16 expression alone.

4.4   The effect of CREG1 and p16 coexpression on cell cycle regulator expression

To investigate the effect of CREG1 and p16 on cell cycle control, the expression of genes involved in cell cycle progression were examined by western blotting. U2OS cells were transfected with CREG1 and p16 as mentioned above and the protein levels of Rb family pocket proteins and cyclins were determined after 5 days of drug selection. We found that cells coexpressing CREG1 and p16 showed a marked decline in protein levels of cyclin A and B (Figure 30). In addition, the protein levels of pRb/p105 and RbL1/p107 were decreased whereas RbL2/p130 expression was unchanged. The same results were obtained from HT1080 cells cotransfected with CREG1 and p16 (Figure 31). Additionally, as mentioned in section 4.2, no differences were detected in Parp protein cleavage, which is an indicator of apoptosis. The western blotting of Parp protein is shown in Appendix 3 (Exp#38). Fragments of cleaved Parp are observed in all transfected conditions, which may be caused by stressful cell culture conditions or the effect of drug selection.

Exp #35   **Exp #41**
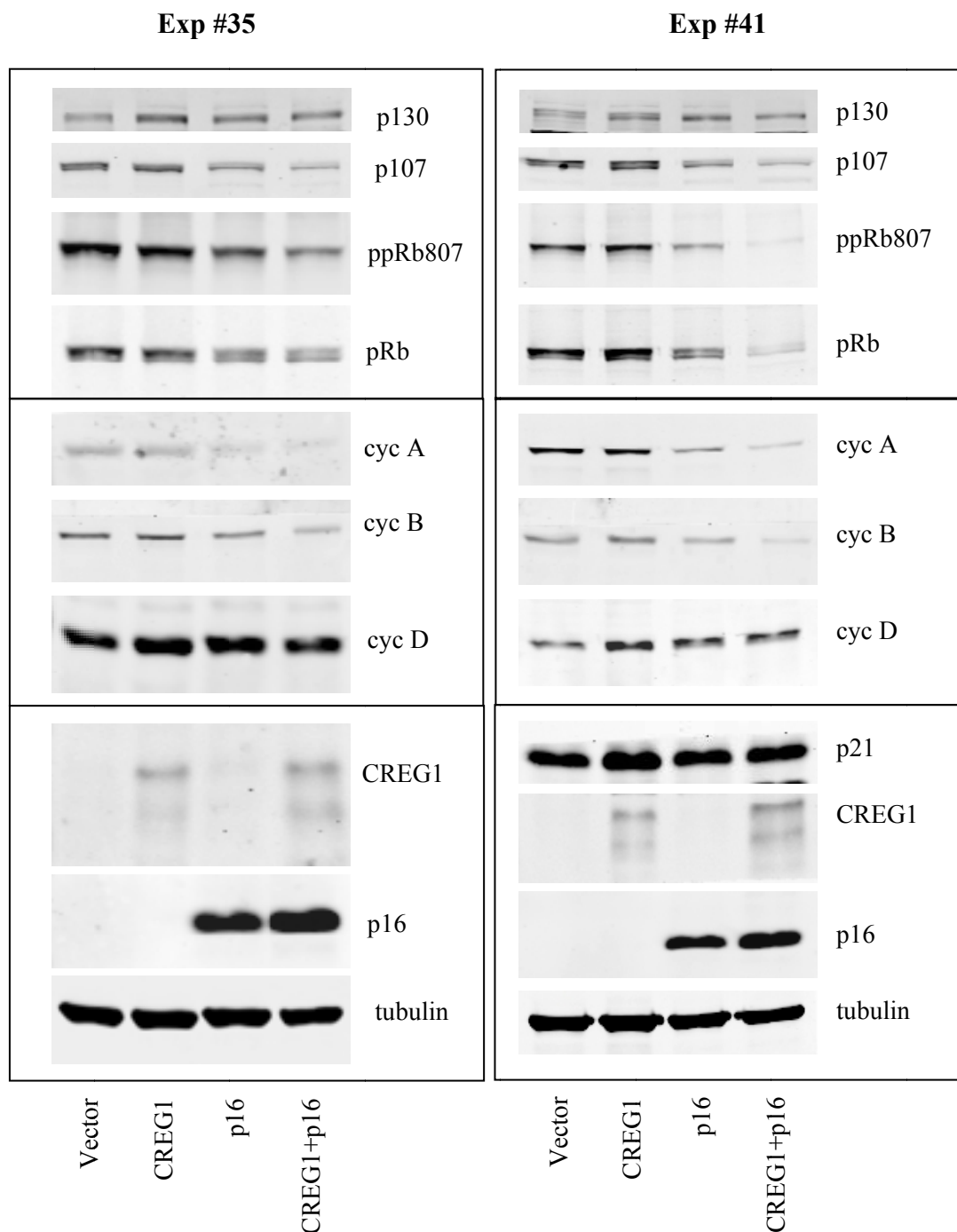
Figure 30. Western blot analysis of U2OS cells transfected with CREG1 and p16 at day 5 after selection. The results shown were obtained from two independent experiments. Coexpression of CREG1 and p16 reveals strong downregulation of cyclin A and B whereas cyclin D expression is still retained. Expression of pRb and p107 are downregulated and p130 is still expressed in CREG1 and p16 cotransfected cells.
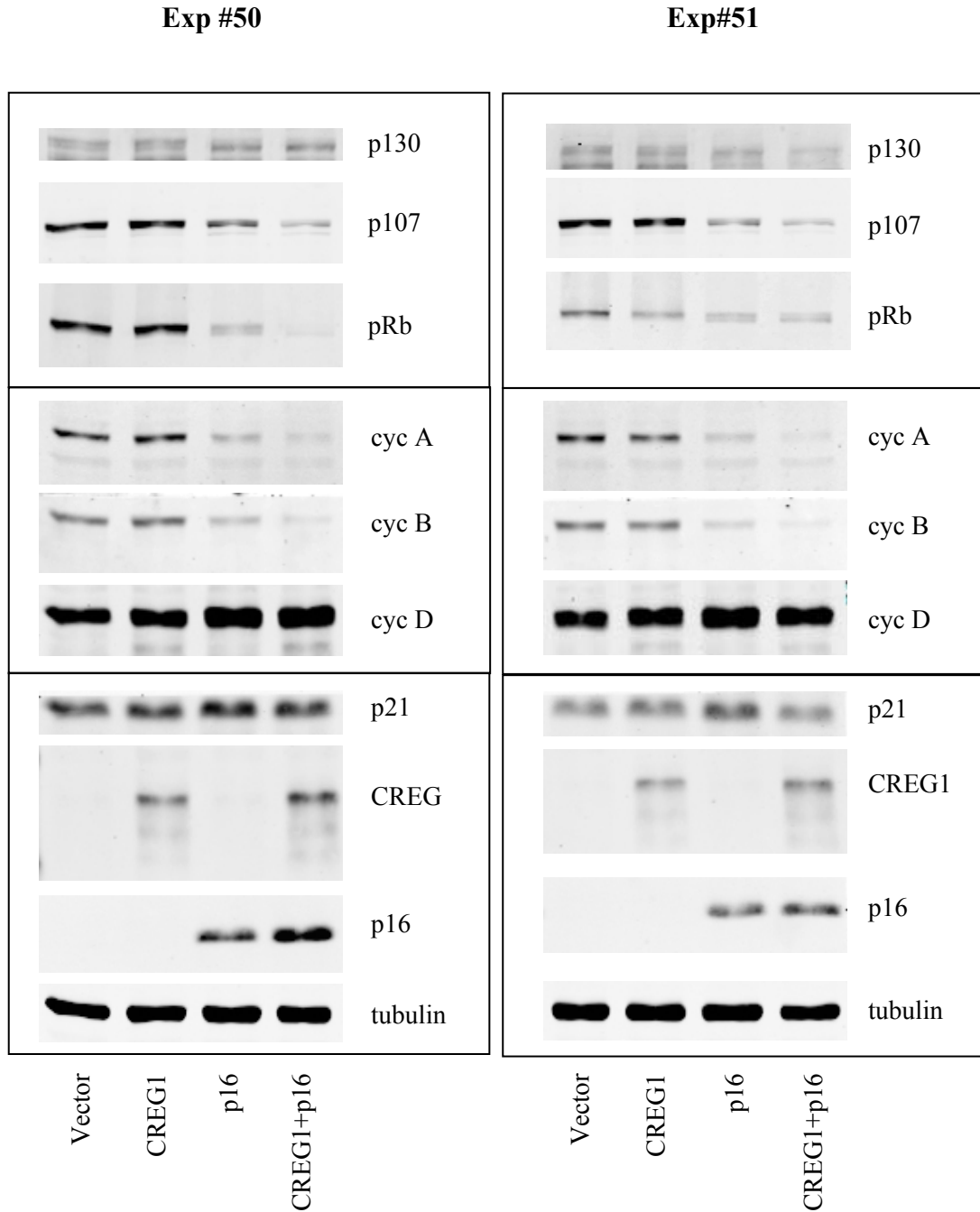
Figure 31. Western blot analysis of HT1080 cell line transfected with CREG1 and p16. The cell lysates were harvested at day 5 after drug selection. CREG1 and p16 coexpression reveals a marked decrease of cyclin A and B whereas cyclin D expression is still retained. Expression of pRb and p107 are downregulated and p130 is still expressed in CREG1 and p16 cotransfected cells.The results shown were obtained from two independent experiments.

4.5 **CREG1 and p16^{INK4a} coexpression inhibits the expression of cyclin A at the transcriptional level**

We found that coexpression of CREG1 and p16 in U2OS and HT1080 cell lines greatly repressed cyclin A and B protein expression levels. The expression level of cyclin A mRNA was also determined by Q-RT PCR. Total RNA was isolated and cDNA was prepared from U2OS cells transfected with CREG1 and p16 at day 5 after drug selection. The mRNA levels of cyclin A2 were examined (Figure 32) and were consistent with the protein levels.
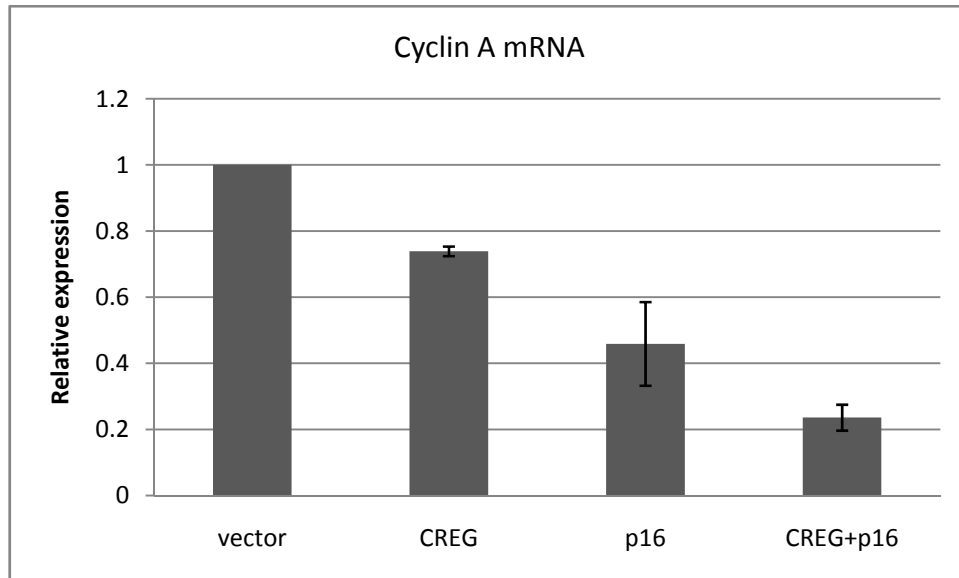
Figure 32. The expression of cyclin A2 by Q-RT-PCR in U2OS cells transfected with CREG1 and p16, alone and in combination. The data represents the average relative expression level of cyclin A to the vector control from two independent experiments.

To further examine the effect of CREG1 with and without cotransfection of p16 on cyclin A expression, we constructed a cyclin A reporter vector containing a fragment of cyclin A promoter driven luciferase reporter gene. The schematic of the cyclin A2 promoter fragment used for the luciferase reporter assay is shown in Figure 33 (83). The CCRE site on the cyclin A2 promoter is mediated by Rb pocket proteins and is activated by the E2F transcription factor. Mutation of this site abrogates the promoter activity (89). In addition, the CRE element on the promoter was revealed to regulate the promoter activity and its expression level of cyclin A2. Previous studies by Ahmed-Choudhury et al. 2005 (83) and Fajas et al. 2001 (90) demonstrated that p120^{E4F}, a transcription factor, binds to the CRE element of the cyclin A2 promoter and negatively regulates the promoter activity of cyclin A2. We then tested if the effect of CREG1 and p16 overexpression, alone and in combination, may depend on the transcriptional repression of the CRE site through p120^{E4F}. The cyclin A2Δ reporter vector was constructed as described in section 2.10. The schematic of this reporter is shown in Figure 33.

A.

B.

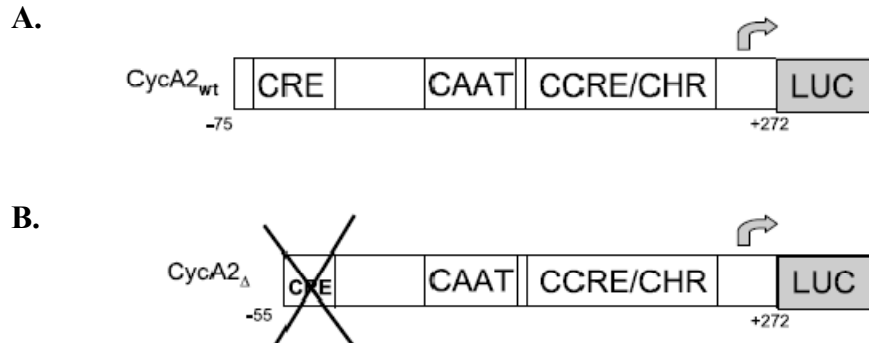**Figure 33.** The schematic of cyclin A2 promoter reporters. **A**. diagram of cyclin A2 luciferase reporter containing wild-type cyclin A2 promoter. **B**. diagram of cyclin A2 luciferase reporter with deletion of CRE site (cyclin A2Δ).

U2OS cells (4×10^5 cells in 6-well culture plate) were transfected with 2 μg each of CREG1 and p16 expression vectors, alone and in combination as mentioned above, followed by double drug selection at 48h after transfection for 3 days. At day 4 of drug selection, 2 μg of the cyclin A reporter and 0.1 μg TK-Renilla vector were transfected into CREG1 and p16 transfected cells. Forty hours after reporter transfection, cells were harvested for luciferase assay (dual-luciferase reporter assay system, Promega) to measure the activity of luciferase and renilla for normalization. Consistent with a decrease of the protein and mRNA level of cyclin A2, coexpression of CREG1 and p16 showed a significant decrease of transcriptional activity of cyclin A2 wild-type promoter (Figure 34). On the other hand, the activity of the cyclin A2Δ reporter was lower compared to the cyclin A2 wild-type promoter and overexpression of CREG1 and p16 did not decrease the activity of this reporter compared to the vector control (Figure 35). This indicates that the CRE element on cyclin A2 promoter plays a role in the transcriptional repression mediated by CREG1 and p16. However, since the luciferase activity of this reporter is lower than the wild-type promoter, the deletion of the CRE element may affect the overall activity of the cyclin A2 promoter.

Figure 34. Cyclin A2 reporter activity in U2OS cells transfected with CREG1 and p16. The bar graphs show the luciferase activity obtained from luciferase/renilla ratio from three independent experiments. ANOVA analysis was performed to test the differences of promoter activity relative to vector control ($P<0.01$). Student's t-test was performed to compare the promoter activity of p16 with combination of CREG1 and p16. ** $P<0.01$
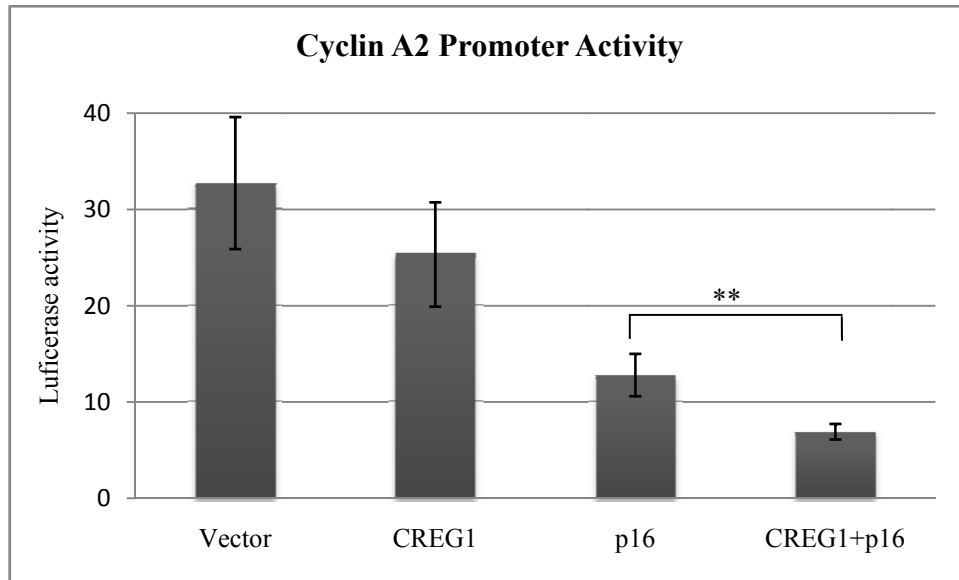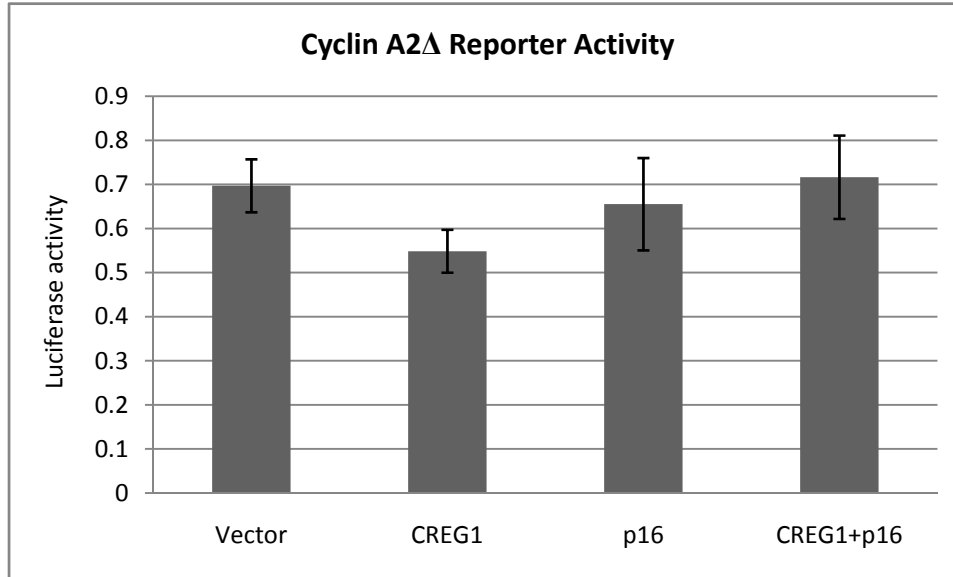
Figure 35. The cyclin A2Δ reporter activity in U2OS cells cotransfected with CREG1 and p16. The bar graphs show the luciferase activity obtained from luciferase/renilla ratio from two independent experiments.

4.6   The effect of CREG1 and p16 on E2F1 transcriptional activity

CREG1 was previously shown to inhibit the transcriptional activity of the E2F transcription factors (67). In this study, we found that coexpression of CREG1 and p16 greatly inhibits the transcriptional activity of the cyclin A promoter which contains an E2F transcription factor binding site. The expression of cyclin A is regulated by binding of E2F (89).To further investigate the function of CREG1 and its enhancement of p16 in transcriptional repression, we examined the activity of the E2F reporter obtained by cloning four E2F binding sites driven by TATA promoter into a luciferase gene reporter as well as the activity of the E2F1 promoter reporter in U2OS cells transfected with CREG1 and p16, alone and in combination. In the E2F-TA (four E2F binding sites driven by TATA promoter) luciferase reporter assay, U2OS cells were transfected with vector control, CREG1 and p16, alone and in combination as described in section 2.2 followed by double drug selection for 3 days. U2OS cells ($4x10^5$ cells) were seeded into 6-well culture plate in tripicate prior to transfection. One μg of E2F-TA reporter plasmid, 0.5 μg of E2F1/CMVexpression vector, and 0.1 μg of TK-renilla plasmid for internal control, were transfected into CREG1 and p16 transfected cells at day 4. The cell lysate for luciferase assay were harvested at 40 h after reporter transfection using dual-luciferase reporter assay system kit (Promega) according to the manufacturer's instruction. However, we did not observe the inhibition of E2F activity from this experiment after repeating three times (Figure 36). Moreover, the activity of the E2F1 promoter reporter that was examined similar to the E2F-TA reporter described above was not significantly different among vector control and CREG1 and p16 combinations (Figure 37).

Figure 36. The E2F-TA reporter activity of U2OS cells transfected with CREG1 and p16. The bar graphs show the average luciferase activity (the ratio of luciferase/renilla) in triplicate from two independent experiments. ANOVA analysis was performed to test the differences of luciferase activity relative to vector control (*P*=0.04).
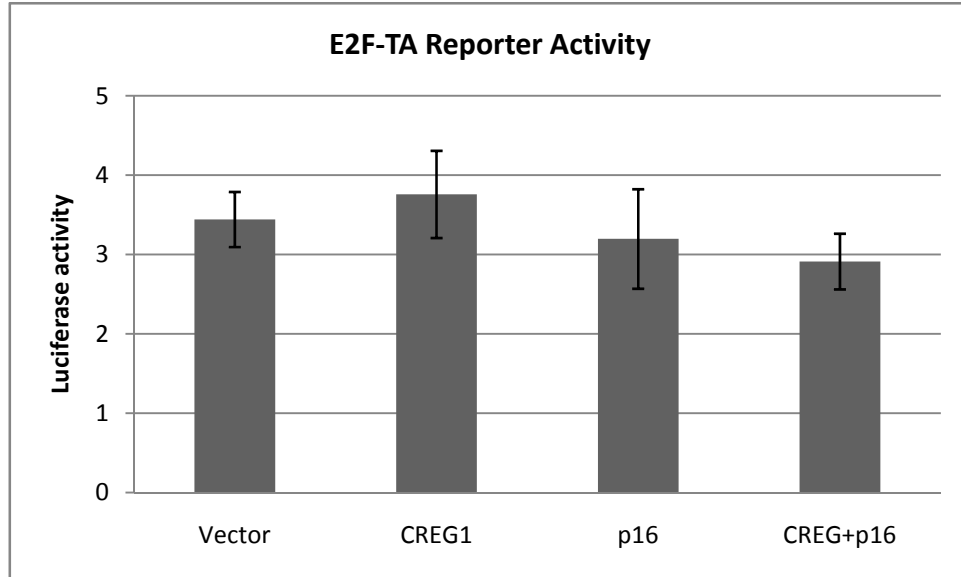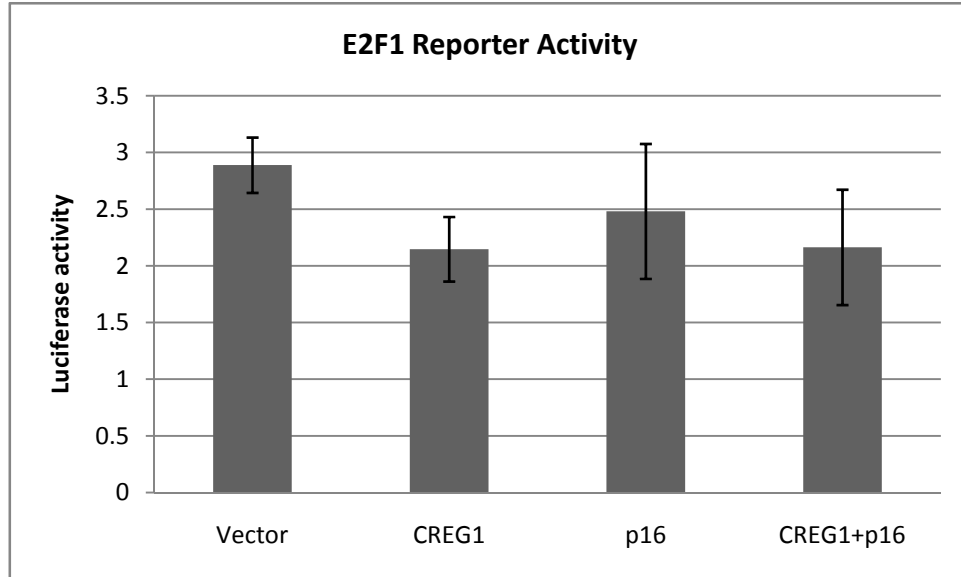
Figure 37. The E2F1 promoter reporter activity in U2OS cells transfected with CREG1 and p16. The bar graphs show the average luciferase activity (the ratio of luciferase/renilla) in triplicate from three independent experiments. ANOVA analysis was performed to test the differences of luciferase activity relative to vector control ($P= 0.2$).

4.7 The effect of Rb family members on cellular senescence induced by CREG1 and p16^{INK4a} coexpression

The retinoblastoma tumor suppressor (pRb/p105) and its related proteins, RbL1/p107 and RbL2/p130, share similar structures (pocket protein) and biological functions. They mainly function to bind to and negatively regulate the transcription of the E2F transcription factor. Different pocket proteins associate with and inhibit the transcriptional activity of different E2F isoforms. While pRb interacts and inhibits the transcriptional activator E2F, including E2F1, E2F2 and E2F3, p107 and p130 interact with the transcriptional repressor E2F4 and E2F5 (79).

To investigate whether the effect of CREG1 on enhancement of p16 requires pRb, p107 or p130, shRNA mediated knockdown of each pocket protein using a lentiviral system was performed in U2OS cells. U2OS cells were infected with lentivirus targeting each of the Rb members or a non-silencing control. The infected U2OS cells were selected with puromycin (0.5 µg/ml). Uninfected U2OS cell, non-silencing and each of the Rb family protein knockdown cells were then transfected with CREG1/pcDNA3.1-hygromycin and p16/pcDNA3.1-neo. To ensure the transfection efficiency, the selection of transfected cells was done using 3 drugs: puromycin for the lentivirus, hygromycin for CREG1 and G418 for p16. Stably transfected cells were analyzed by flow cytometry for cell cycle analysis, detection of senescence by senescence-associated β-galactosidase staining and western blotting after 5 days of selection. The cell morphology and senescence-associated β-galactosidase staining of Rb knockdown U2OS cells transfected with CREG1 and p16 is shown in Figure 38-42. The quantification of SA-β gal staining of non-silencing and each of Rb member knockdown U2OS cells transfected with

CREG1 and p16, alone and in combination, at day 5 after drug selection is shown in Figure 43. We found that while expression of p16 alone and in combination with CREG1 seemed to be able to induce senescence in uninfected U2OS, non-silencing, p107, and p130 knockdown cells, knockdown of Rb showed less of a senescent phenotype than others considering cell morphology and senescence-associated β-galactosidase staining. We detected the expression of cell cycle regulator proteins by western blot analysis of lentiviral-mediated Rb knockdown, non-silencing and U2OS cells transfected with CREG1 and p16. Stably transfected cells were harvested for western blotting at day 5 (Figure 44) and day 10 (Figure 45) after three drug selection. The knockdown efficiency of each Rb family member was confirmed. Consistent with the cell morphology observed, we found that pRb knockdown U2OS cells, when overexpressed with p16 alone and in combination with CREG1, showed less decrease of cyclin A and cyclin B compared with non-silencing control, p107 and p130 knockdown cells. In addition, whereas the protein levels of p107 were shown to be decreased in uninfected U2OS and non-silencing cells transfected with CREG1 and p16, which was observed previously in senescent cells, the levels of p107 and p130 in pRb knockdown cells seemed to be increased. This might indicate the compensation of p107 and p130 in Rb knockdown cells. However, while we observed the effect of Rb-family knockdown U2OS cells transfected with CREG1 and p16 from cell morphology changes and analysis of protein expression levels, cell cycle analysis by flow cytometry did not reveal conclusive results obtained from three experiments (data not shown).

Non-silencing_vector
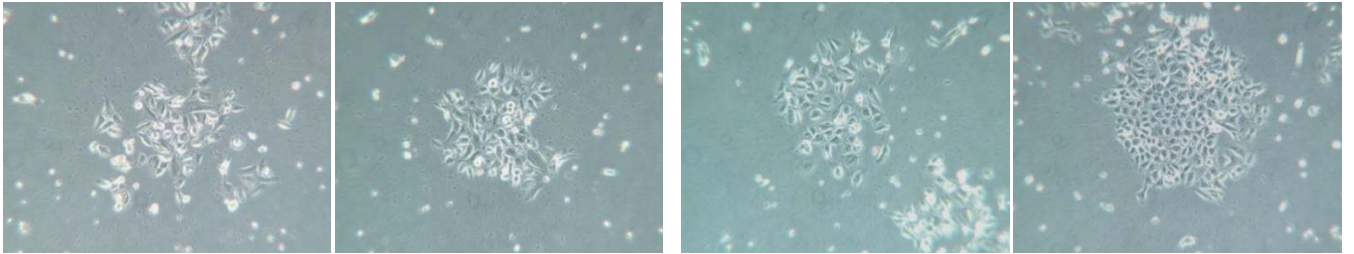
Non-silencing_CREG1
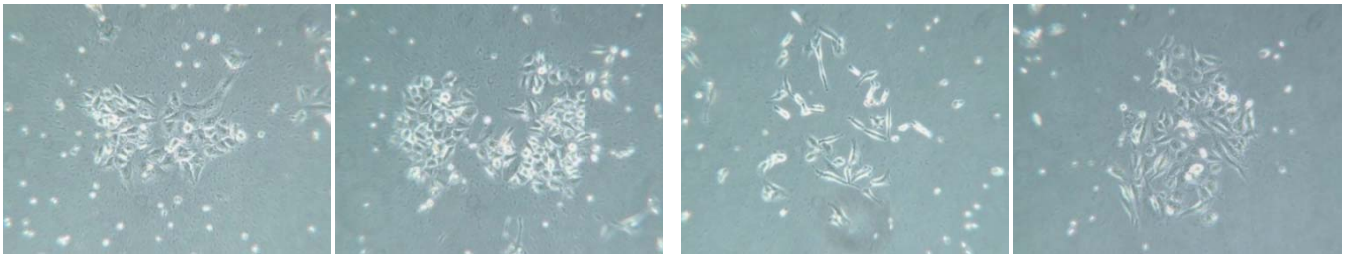
Non-silencing_p16
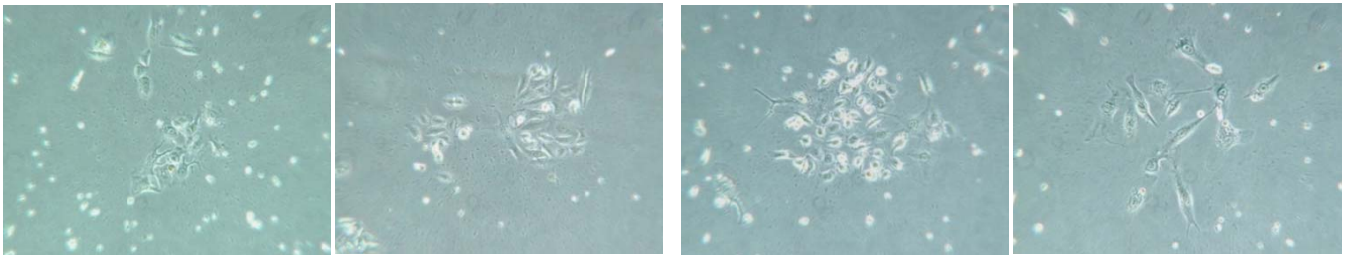
Non-silencing_CREG1+p16
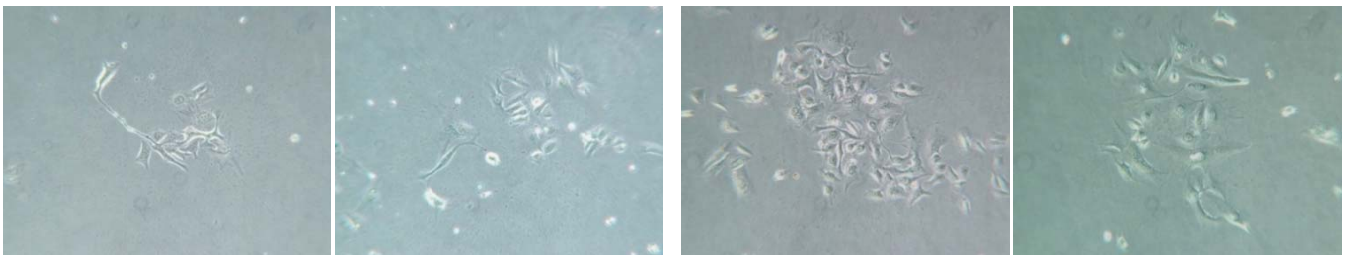
Exp#65   **Exp#67**

Figure 38. Cell morphology and senescence-associated β-galactosidase staining of lentivirus mediated-non-silencing knockdown U2OS cells transfected with CREG1 and p16 at day 5 after selection. The results are from two independent experiments.

Rb-KD_vector
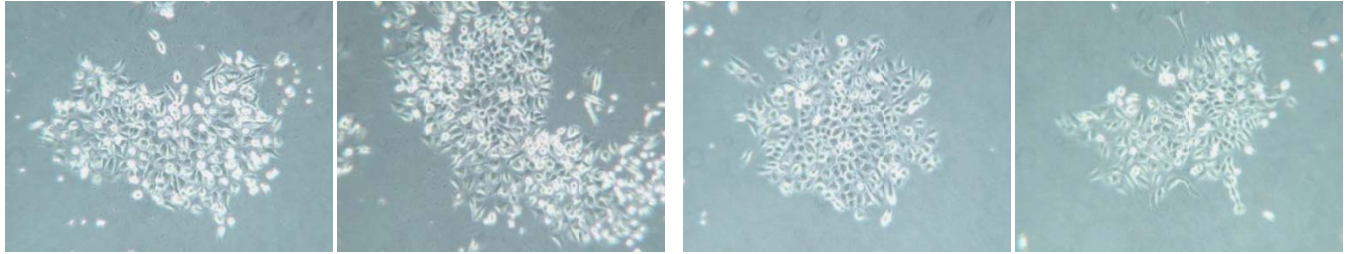
Rb-KD_CREG1
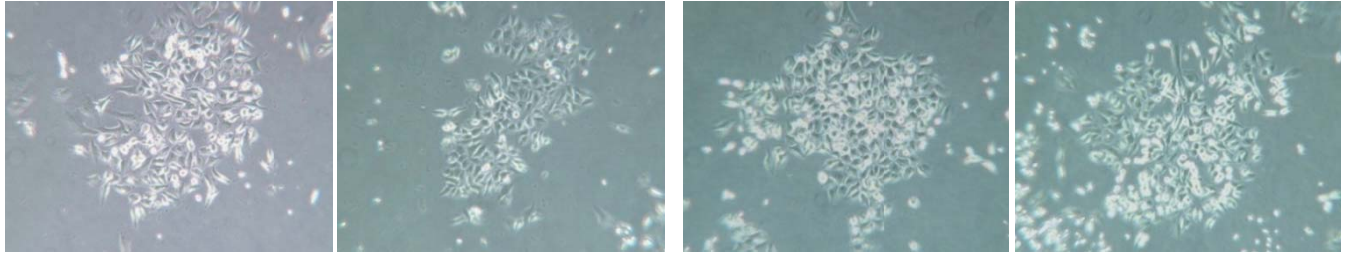
Rb-KD_p16
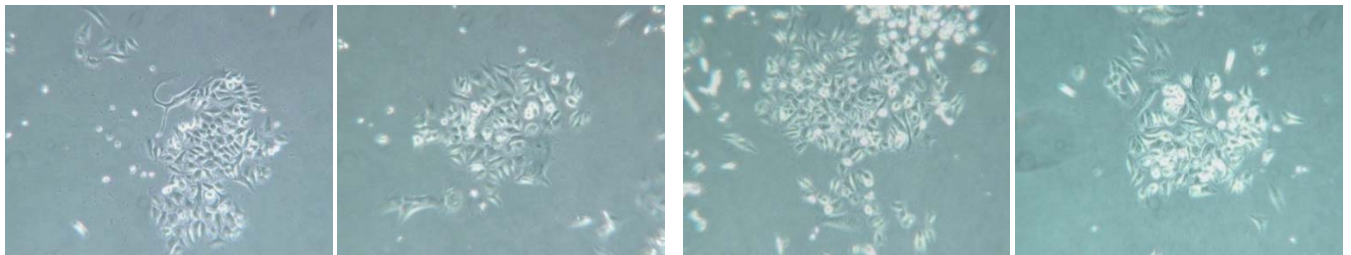
Rb-KD_CREG1+p16
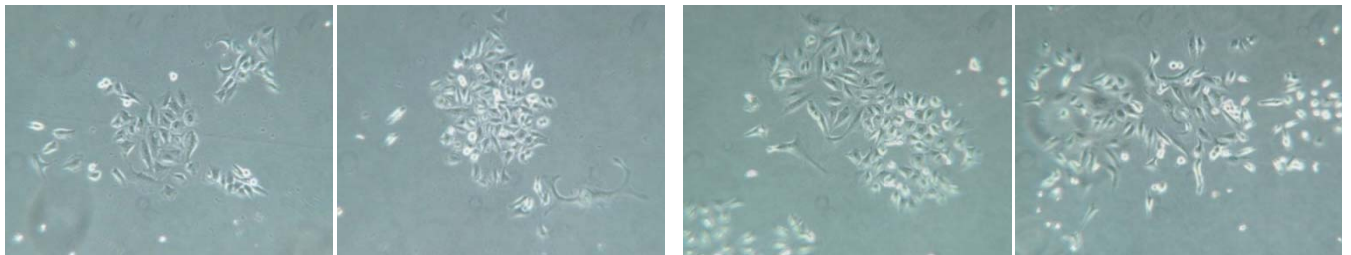
Exp#65   **Exp#67**

Figure 39. Cell morphology and senescence-associated β-galactosidase staining of lentivirus mediated-Rb knockdown U2OS cells transfected with CREG1 and p16 at day 5 after selection. The results are from independent fields from independent experiments.

p107-KD_vector

p107-KD_CREG1

p107-KD_p16

p107-KD_CREG1+p16

Exp#65   **Exp#67**
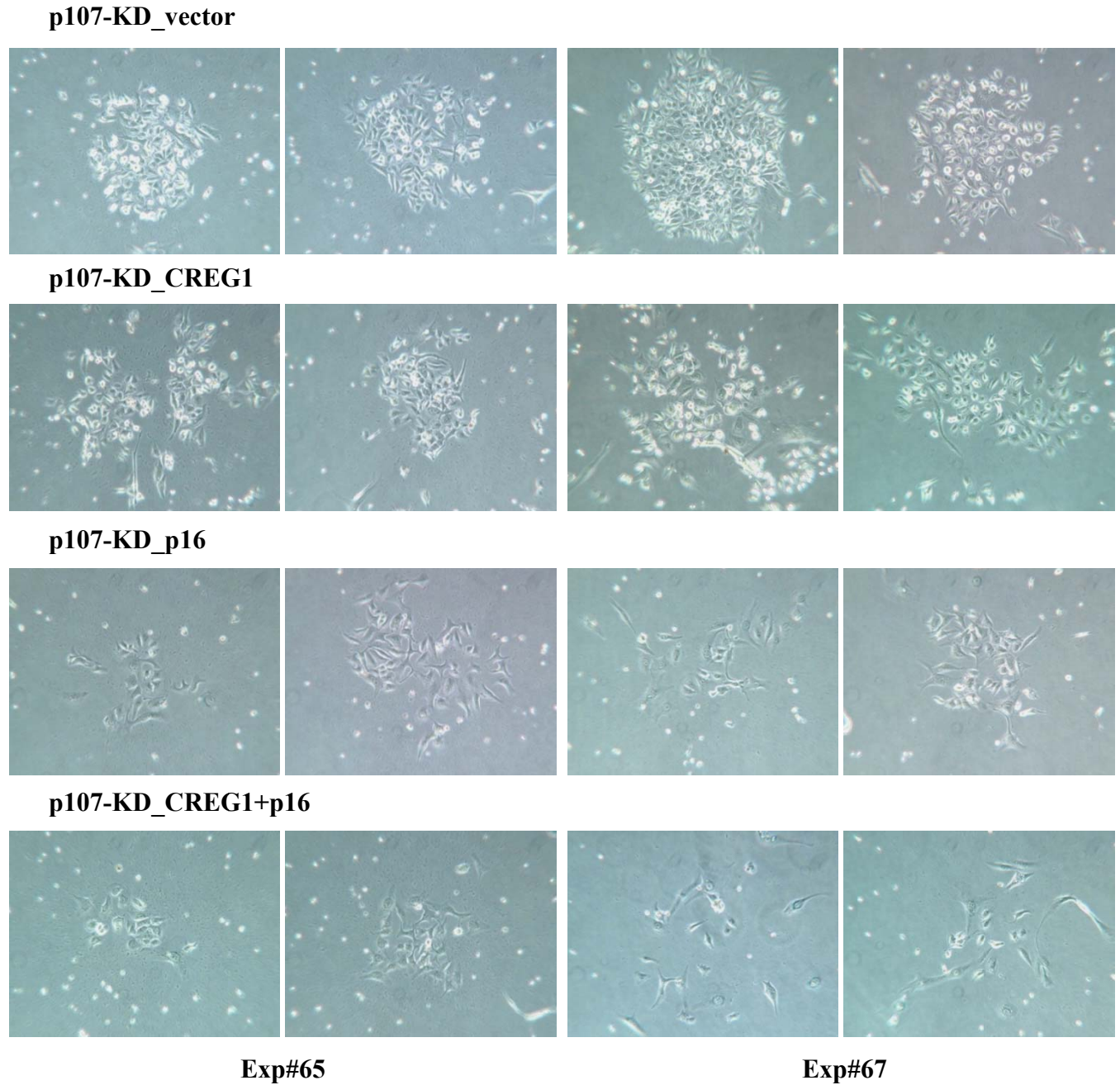
Figure 40. Cell morphology and senescence-associated β-galactosidase staining of lentivirus mediated-p107 knockdown U2OS cells transfected with CREG1 and p16 at day 5 after selection. The results are from independent fields from independent experiments.

p130-KD_vector

p130-KD_CREG1

p130-KD_p16

p130-KD_CREG1+p16

Exp#65   **Exp#67**
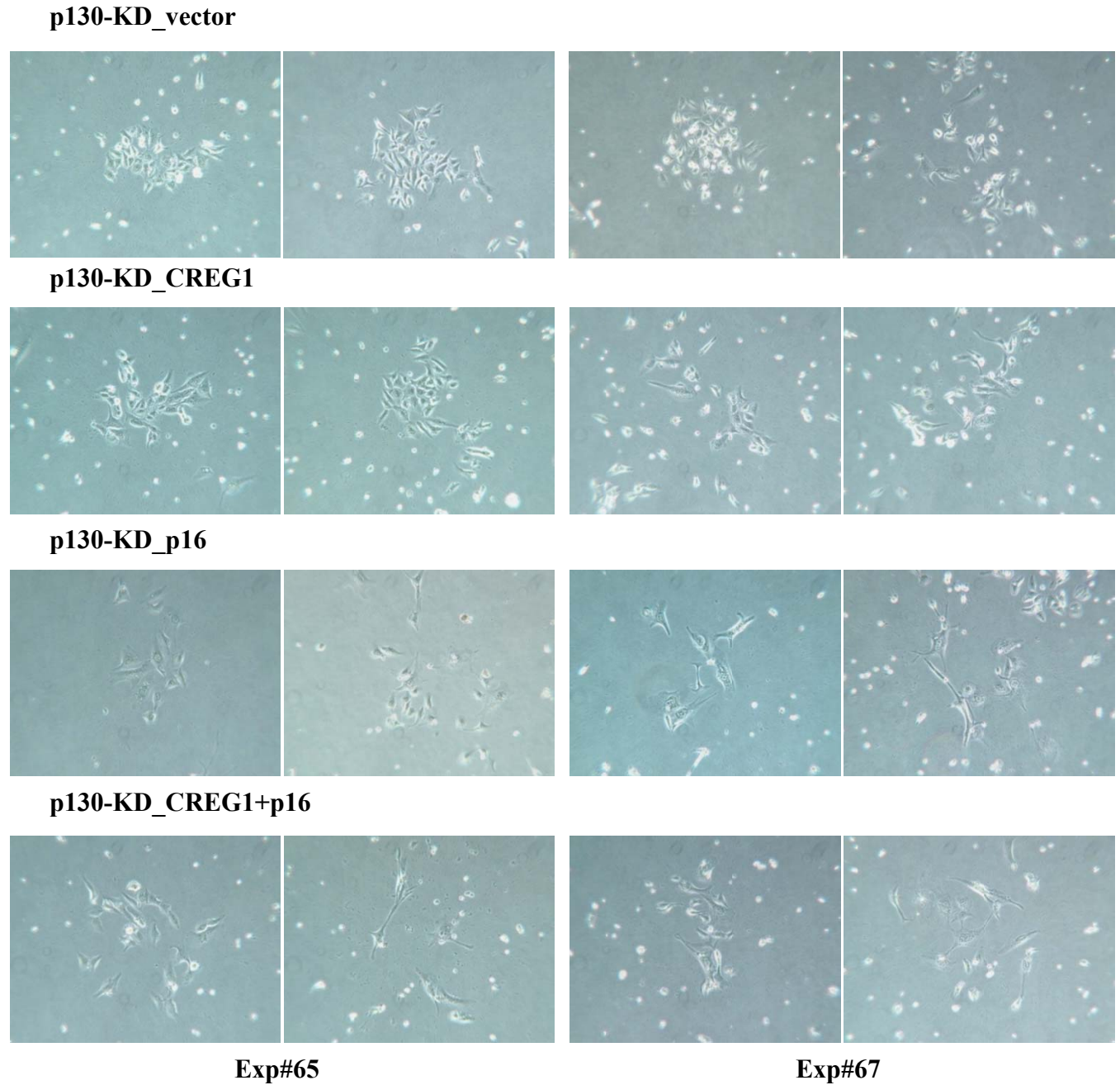
Figure 41. Cell morphology and senescence-associated β-galactosidase staining of lentivirus mediated-p130 knockdown U2OS cells transfected with CREG1 and p16 at day 5 after selection. The results are from independent fields from independent experiments.

U2OS_vector
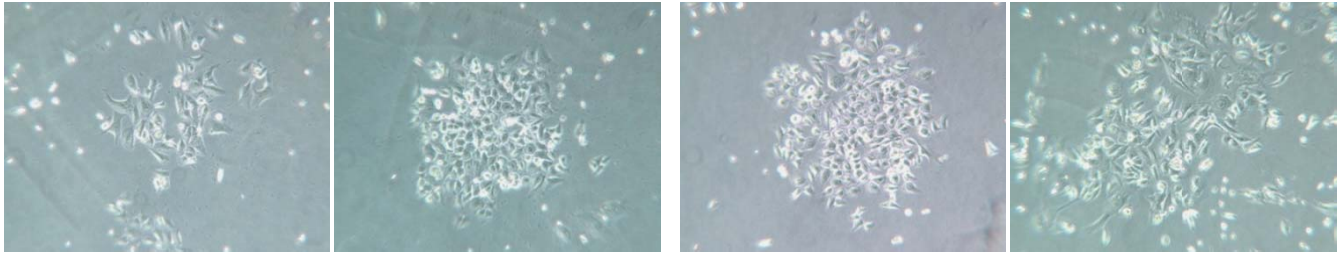
U2OS_CREG1
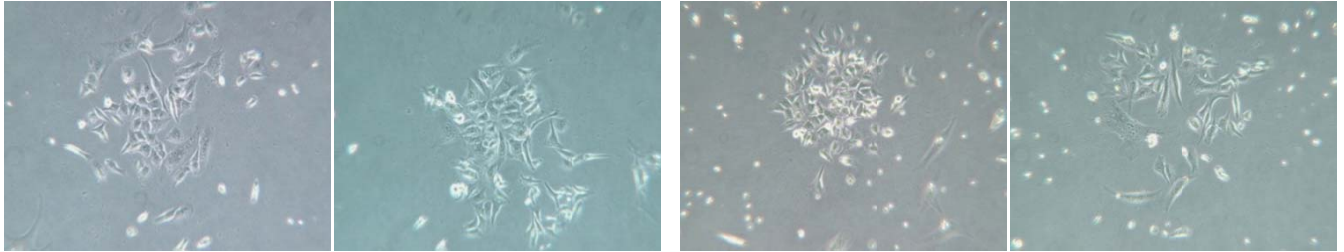
U2OS_p16
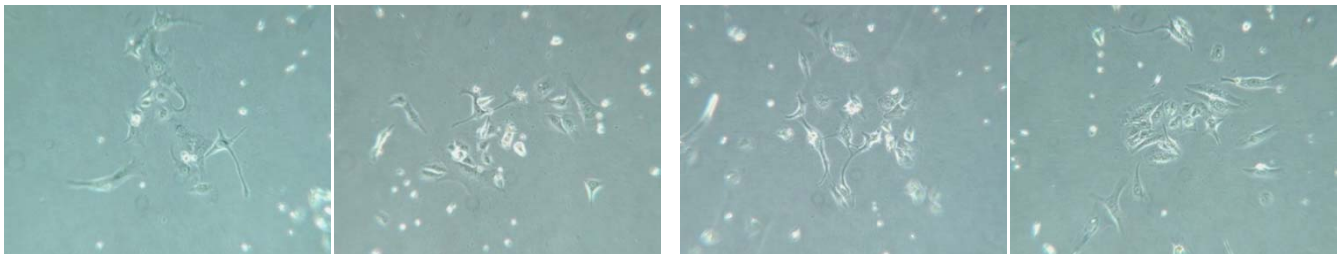
U2OS_CREG1+p16

<div align="center">
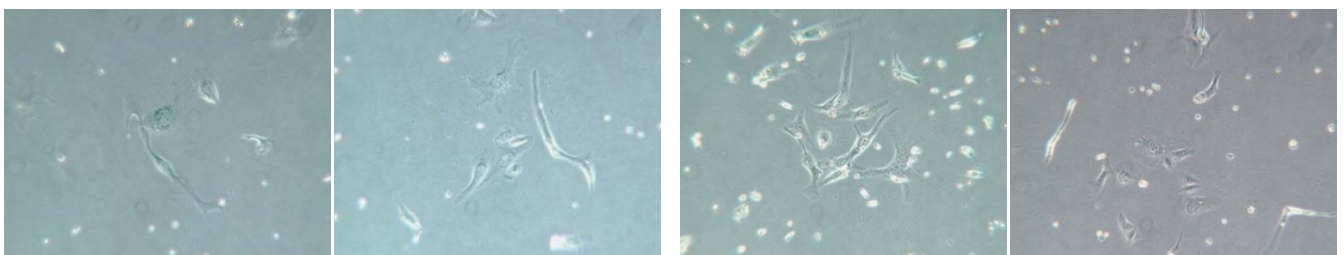
Exp#65   **Exp#67**

</div>

Figure 42. Cell morphology and senescence-associated β-galactosidase staining of uninfected U2OS cells transfected with CREG1 and p16 at day 5 after selection. The results are from independent fields from independent experiments.
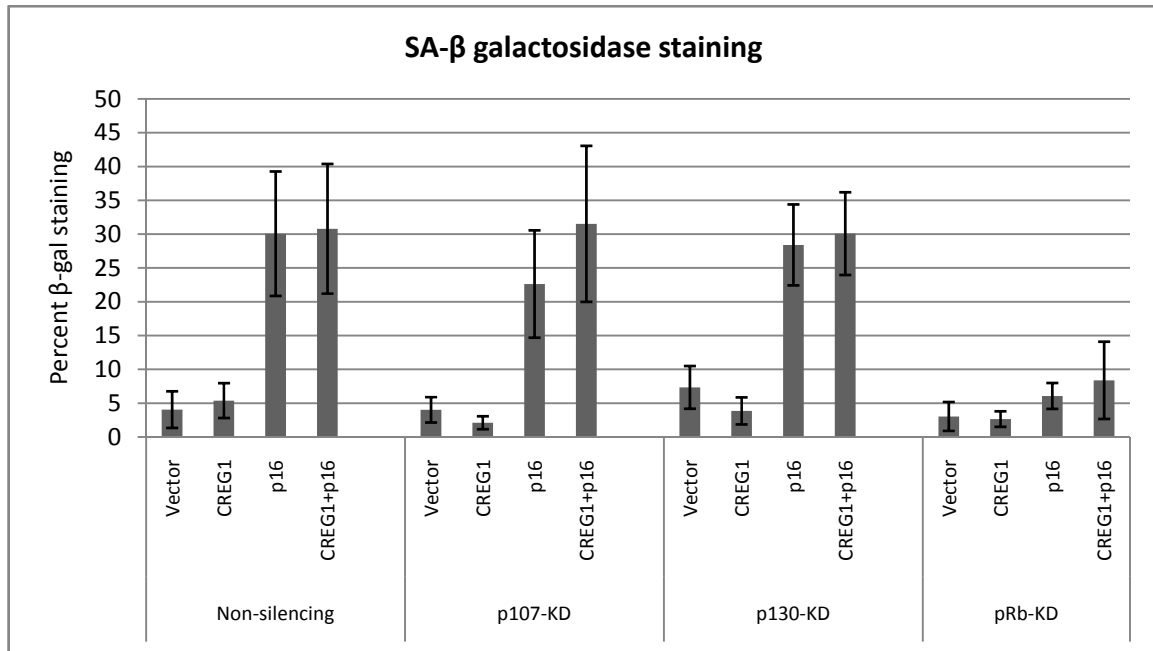
Figure 43. Senescence-associated β-galactosidase staining of lentivirus-mediated non-silencing and Rb member knockdown U2OS cells transfected with CREG1 and p16. Cells were stained for SA-β gal activity at day 5 after drug selection. The bar graphs show the average percent of stained cells counted from 5 different fields on cell culture plates from two independent experiments.
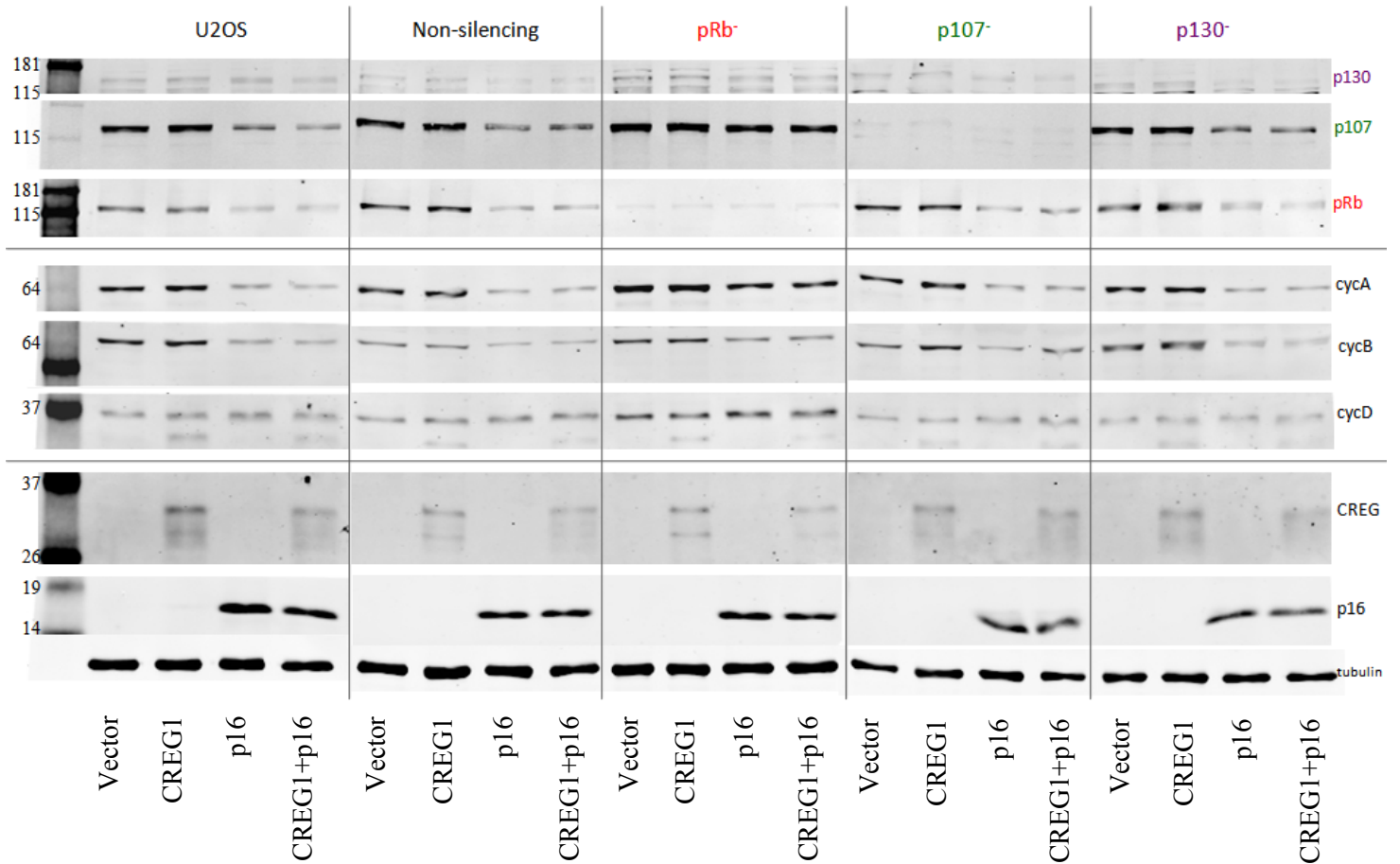
Figure 44. Western blot analysis of lentivirus-mediated Rb members knockdown U2OS cells transfected with CREG1 and p16, alone and in combination. Cells lysates were harvested at day 5 after selection. Cyclin A expression are less decreased in pRb-knockdown cells when transfected with p16 and combination of CREG1 and p16.
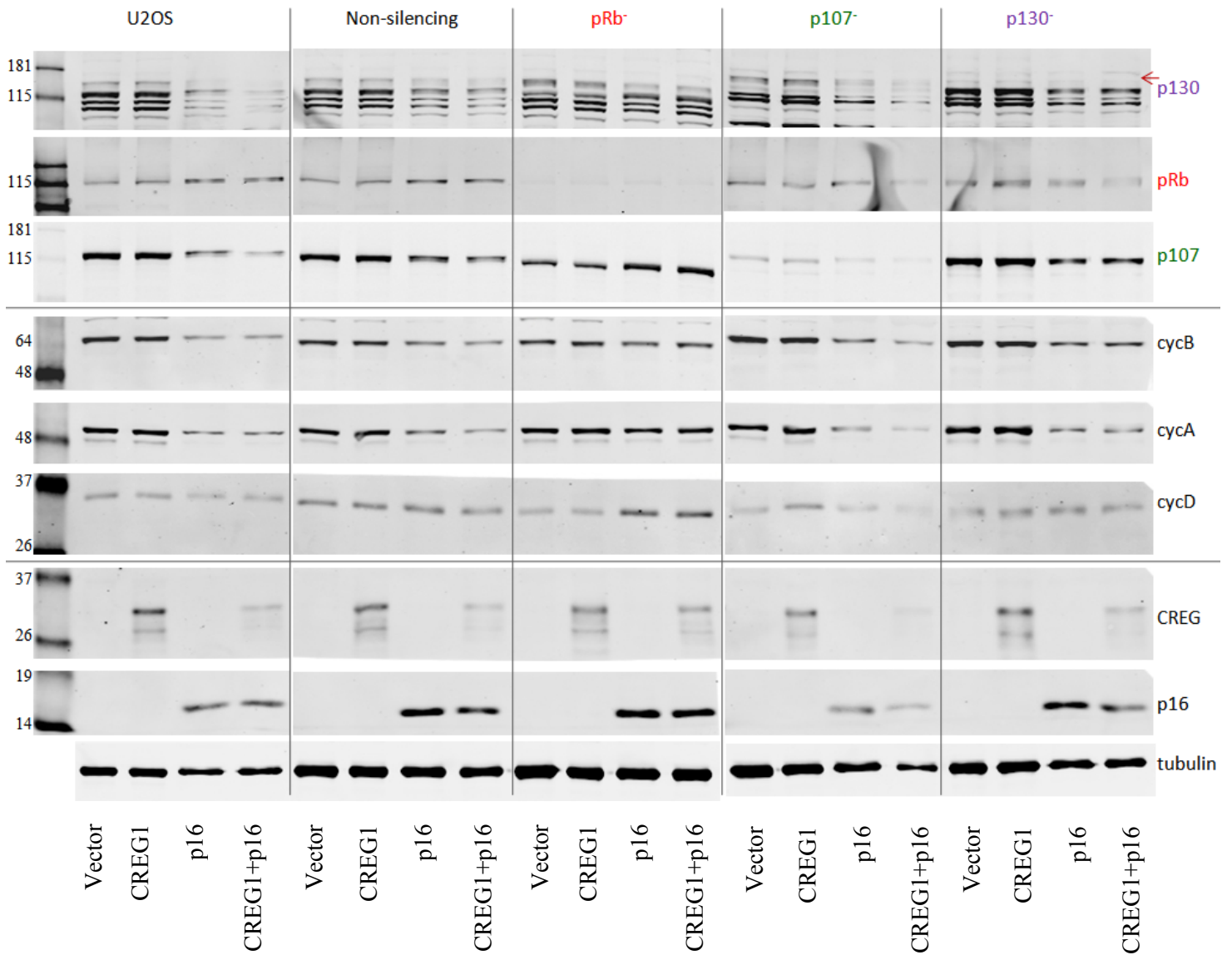
Figure 45. Western blot analysis of lentivirus-mediated Rb members knockdown U2OS cells transfected with CREG1 and p16, alone and in combination. Cells lysates were harvested at day 10 after selection. Cyclin A and cyclin B expressions are less decreased in pRb-knockdown cells when transfected with p16 and combination of CREG1 and p16.

4.8   Discussion

Abrogation of genes and pathways regulating cellular senescence can cause cells to become immortal leading to additional genetic and epigenetic changes to develop in cancers. Cellular senescence is one of the tumor suppressor mechanisms, besides apoptosis, which is responsible for preventing cells from uncontrolled propagations. The senescence can occur by telomere dependent (replicative senescence) and telomere-independent (oncogene- or stress-induced senescence) mechanisms.

Several studies were pursued to understand more knowledge of senescence mechanism in order to provide additional benefits for better management of human cancers. An attempt to identify new regulators of cellular senescence has been done in the Tainsky lab using spontaneously immortalized fibroblasts from Li-Fraumeni Syndrome patients as a study model. Taking advantage of these spontaneously immortal cell lines as opposed to other immortal cells induced by either viral oncoprotein such as SV40 large T antigen or chemicals may provide fewer side effects that may cause by those factors.

In this thesis research, CREG1 was chosen as a candidate gene for investigation because its involvement in cellular senescence appeared to be due to its function in cell cycle regulation regulating E2F transcription activity (67) and its negative growth effect in cancer (69) and vascular (71) cells. The method that we used to examine the role of CREG1 in cellular senescence was stable expression of CREG1 in immortal LFS and cancer cell lines followed by investigating growth and lifespan. We found that overexpression of CREG1 alone in immortal LFS and cancer cell lines did not induce cellular senescence but was able to slow cell proliferation. Because the cells we used have lost the function of p16INK4a and because CREG1 was shown to interact with Rb

family pocket proteins thus inhibiting the transcription of E2F transcription factor, we then hypothesized that CREG1 might be involved in a p16INK4a-pRb-dependent senescence pathway. p16INK4a, a CDK inhibitor of pRb phosphorylation, is one of the tumor suppressor genes that is commonly disrupted in immortal cells and some cancer cells. p16INK4a expression is also upregulated in senescent fibroblasts (63, 91, 92). Several studies showed that ectopic expression of p16INK4a caused cell cycle arrest and senescence in immortal LFS (54) and human cancer cells including U2OS cell line (80, 93-95) and these effects depended on the normal function of pRb.

Interestingly, we discovered that coexpression of CREG1 and p16 in U2OS cells (pRb+/p16-/p53+) had a greater effect on cell growth inhibition, induction of cell cycle arrest, inhibition of colony formation, and the induction of senescence than expression of CREG1 or p16 alone. Consistent with its activity to induce cell cycle arrest, cells cotransfected with CREG1 and p16 showed a marked decrease of cyclin A and B expression. The decrease expression of cyclin A was regulated at the transcriptional level. We also found that expression of pRb and p107 decreased in p16 and CREG1+p16 transfected cells, but p130 expression was unchanged. The changes of Rb pocket proteins in our study were consistent with those shown by Helmbold et al who showed that p130 accumulates and is important to maintain senescence and that pRb and p107 decrease in DNA damage and p16-induced senescence (96, 97). The decreased expression of pRb and p107 in senescent cells that we observed upon overexpression of p16 and combination of CREG1 and p16 were similar to those from other studies (12, 96, 98). However, the mechanism of pRb and p107 downregulation during senescence is poorly understood. It may be partly due to the unnecessary of pRb and p107 upon fully engaged

senescence. Moreover, cyclin A expression was shown to be downregulated in cellular senescence by p130 recruitment to the cyclin A promoter to repress transcription (96, 99, 100). The enhancement of p16-induce senescence by CREG1, resulted in an increase of senescence-associated β-gal activity and other changes in cell cycle regulator proteins, was also observed in the HT1080 fibrosarcoma cell line, which is p53 deficient indicating that the additive effect of CREG1 in p16^{INK4a}-induced senescence may be independent of p53. Because both p16-pRb and p53-p21 signaling pathways have been shown to play an important role in cellular senescence, this study demonstrated that cellular senescence can be engaged by p16 overexpression in p53-deficient cells indicating the independent role of these two pathways in senescence mechanism. Study by Vogt et al. also demonstrated that overexpression of p16 or p21 were sufficient to induce senescence-like phenotype in two of immortal LFS cell lines, MDAH041 and MDAH87 which harbor p53 mutation (54). The effect of p16- or p21-induced senescence was independent with each other, in other words, induction of senescence by p16 overexpression did not alter p21 expression and p21 induced senescence without the upregulation of p16 (54).

Rb family pocket proteins, pRb, and its related protein p107 and p130, play an important role in suppressing cell growth by controlling cell cycle progression and chromatin condensation (101). p130 maintains cells in G0 and early G1 by binding and inactivating E2F4 and E2F4 which are repressor E2Fs. When cells progress through cell cycle, p130 is then replaced by p107 in mid to late G1 to bind to and inactivate E2F4 and E2F5. In late G1 and S phase, pRb is replaced to interact with and inhibit activator E2Fs, E2F1, E2F2, and E2F3 (reviewed in Macaluso M et al. (102)). Because different pocket proteins bind to and inhibit different isoforms of E2Fs thus modulate different phases in

cell cycle progression, we asked if the enhancement of p16-induced senescence by CREG1 involved particular Rb-family members. We used lentivirus-mediated gene knockdown to target each of Rb member in U2OS cells expressing all three pocket proteins. Cells stably infected with lentivirus-mediated knockdown were then transfected with CREG1 and p16, alone and in combination, to determine the effect of CREG1 and p16 coexpression in senescence induction. We found that expression of p16 and combination of CREG1 and p16 in p107- and p130-knockdown cells showed the senescence-like phenotype, as judged by cell morphology and senescence-associated β gal staining, similar to non-silencing and uninfected U2OS cells. However, the level of CREG1 and p16 combination or p16 alone-induced senescence in pRb knockdown cells was decreased. We observed more colonies of transfected cells formed after drug selection and fewer senescent cells in pRb knockdown cells with p16 and coexpression of CREG1 and p16. These results indicated that pRb but not p107 and p130 is required for the cellular senescence mechanism and that the enhancement of p16 induced senescence by CREG1 is dependent on the pRb signaling pathway. This observation is consistent with the study from Chicas et al. and Talluri et al. demonstrating that Rb has a unique role in cellular senescence that is nonredundant with other pocket proteins, p107 and p130, by repressing DNA replication as cells exit the cell cycle into senescence (103, 104). That knockdown of p107 and p130 in cells used in our study (U2OS cells) resulted in induction of senescence-like phenotype by p16 and combination of CREG1 and p16 expressions may indicate the redundant role of p107 and p130 because these two pocket proteins bind to and inhibit the same subset of repressor E2Fs. This finding is consistent

with study from Jacson et al. demonstrating the compensation of p107 with p130 loss upon DNA damaging agent-induced senescence (99).

The senescent cells that were induced by the combination of CREG1 and p16 overexpression were not insignificantly different compared with p16 expression alone in the experiments of lentivirus-mediated non-silencing and Rb member knockdown U2OS cells considering the senescence-associated β galactosidase staining,. This can be due to the complexity of lentivirus-mediated knockdown and cell culture stresses of using three-drug selection. We also observed less transfection efficiency (more cell death after selection in all transfection conditions) in this experiment compared with the cotransfection of CREG1 and p16 in U2OS cells in early experiment.

We demonstrated that coexpression of CREG1 and p16INK4a significantly inhibited cyclin A and B expression at the transcriptional level. We specifically investigated cyclin A2 (protein, mRNA, and promoter) and B1 (protein) because they are expressed in proliferating cells. Cyclin A and cyclin B are essential for S phase entry and G2 phase, respectively (105-107). Decreased expression of cyclin A and B in senescent cells was observed from other studies including senescence induced by DNA damaging agent and replicative senescence (96, 108), although it is not certain whether this downregulation is a cause or a consequence of growth arrest in cellular senescence. However, we did not observe downregulation of cyclin D in coexpression of CREG1 and p16INK4a-induced senescence. On the other hand, cyclin D1 was shown to be upregulated in senescent cells, and its expression has also been used as a marker of senescence (109-113) which was also observed in our study. An attempt to investigate the effect of CREG1 and p16 overexpression, alone and combination, on the transcriptional

activity of E2F using two reporter plasmids, E2F-TATA (four E2F binding sites driven by TATA promoter) and E2F1 promoter reporter, did not detect transcriptional repression by p16 or by coexpression of CREG1 and p16. This might be due to the complexity of the balanced amount of E2F transcription factor in the cell studies (U2OS cells). The activity of the E2F-TA reporter was undetectable without overexpression of E2F1 transcription factor in the reporter assay. This overexpression of E2F1 may influence the transcriptional repression effects of CREG1 and p16 expression.

Nevertheless, we cannot exclude the possibilities that the additive effect of CREG1 and p16 that was observed in this study may be due to the p16INK4a-pRb signaling pathway and that CREG1 is part of this mechanism, or that CREG1 functions through other pathway(s) such as its regulation of IGF-II signaling (74) which may promote the p16INK4a-pRb signaling-induced senescence. More studies are needed to further investigate whether involvement of CREG1 in p16INK4a-induced senescence is due to its transcriptional repressor activity. Chromatin immunoprecipitation method would be able to address that question. In addition, to further elucidate the role of CREG1 in cellular senescence involved in p16-pRb signaling pathway, depletion of CREG1 and p16 expression in normal diploid fibroblast or precrisis fibroblasts by lentivirus-mediategd gene knockdown following the analysis of lifespan can provide more evidences on the necessity of these two genes in cellular senescence.

In summary, we demonstrated that CREG1 is regulated by epigenetic mechanisms and its downregulation may be one of the important steps in cellular immortalization. Ectopic expression of CREG1 in immortal and cancer cells lacking endogenous p16INK4a results in decreased cell proliferation but does not induce senescence.

Importantly, CREG1 enhances p16INK4a-induced senescence. Analysis of how CREG1 and its enhancement of p16INK4a-induced senescence may provide better understanding of cellular immortalization, an early step in human tumorigenesis, and could improve treatment or prevention of cancer at early stage.

APPENDIX

Flow cytometry
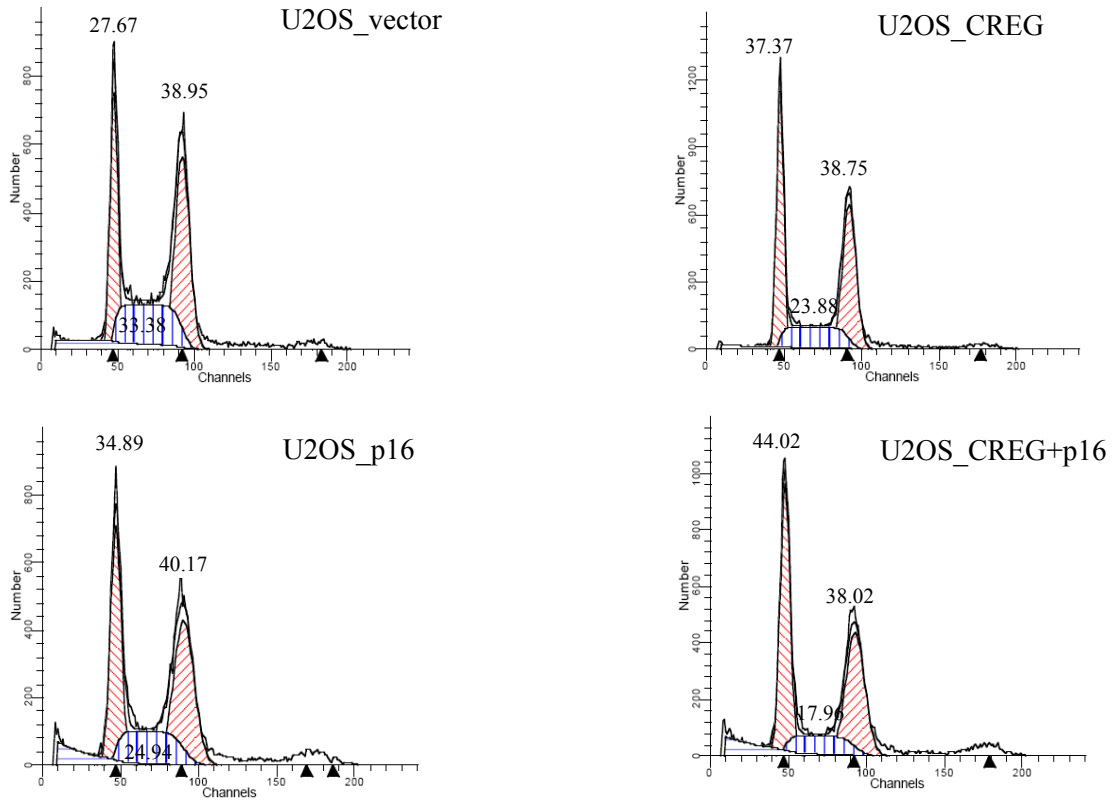
Appendix 1. Histograms of cell cycle analysis by flow cytometry of U2OS cells transfected with CREG1 and p16. Cells were analyzed at day 5 after drug selection. The numbers indicate the percent of cell population in G0-G1, S and G2-M phases.
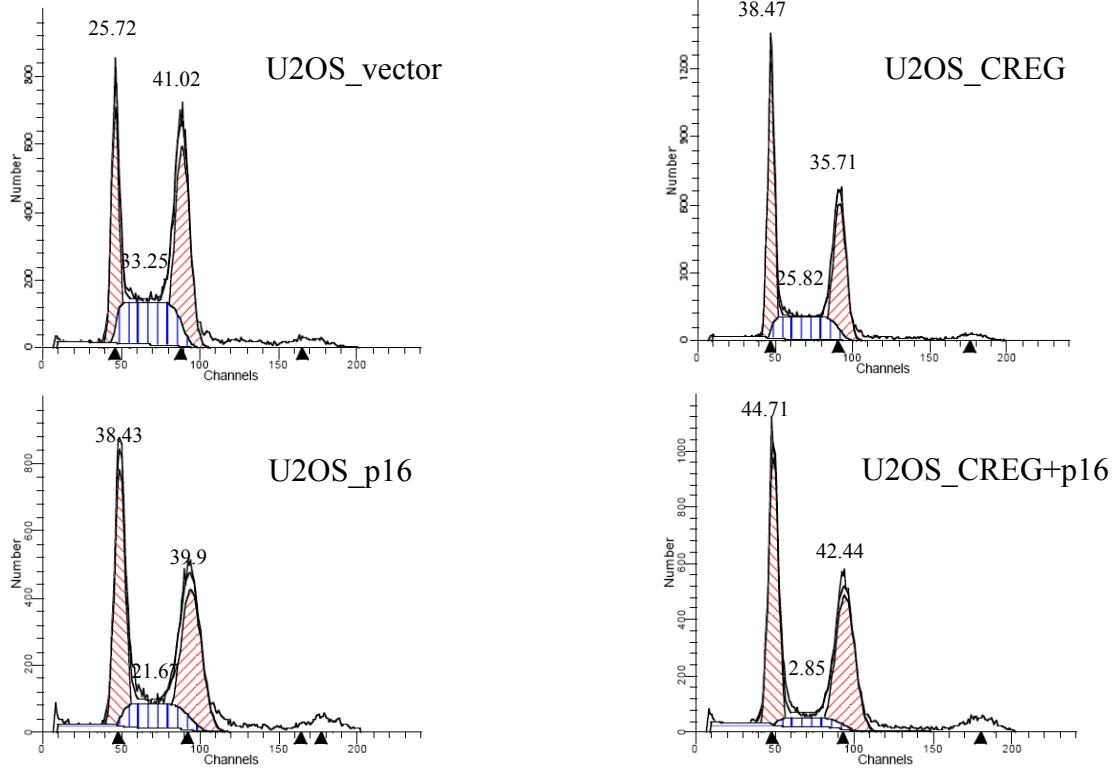
Appendix 2. Histograms of cell cycle analysis by flow cytometry of U2OS cells transfected with CREG1 and p16. Cells were analyzed at day 5 after drug selection. The numbers indicate the percent of cell population in G0-G1, S and G2-M phases.

Western blot analysis
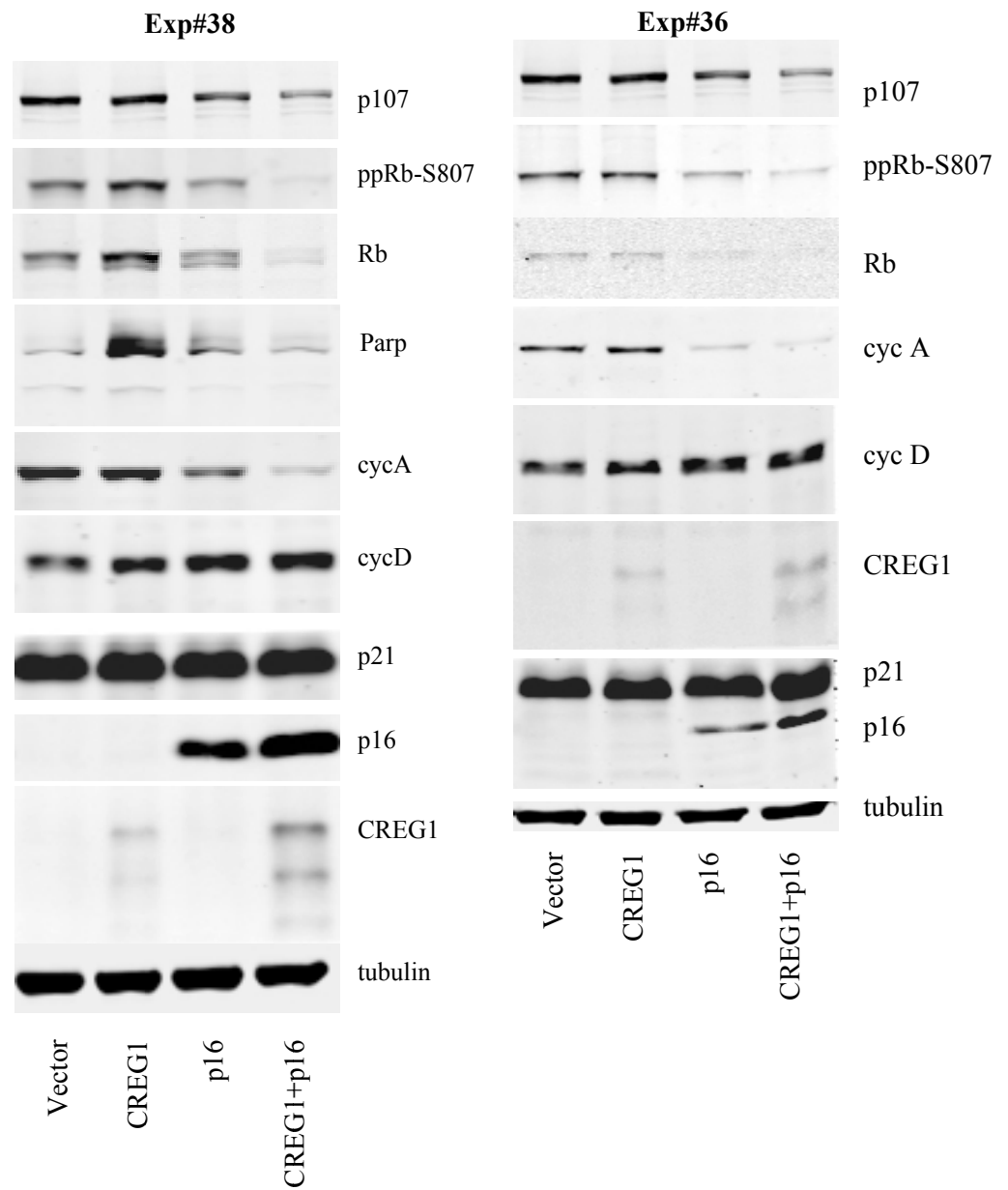
Appendix 3. Western blot analysis of U2OS cells stably transfected with CREG1 and p16 from independent experiments. Cells were harvested at day 5 after selection.

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ABSTRACT

CREG1 AND ITS ENHANCEMENT OF P16INK4A-INDUCED SENESCENCE

by

BENCHAMART MOOLMUANG

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Advisor: Dr. Michael A. Tainsky

Major: Cancer Biology

Degree: Doctor of Philosophy

Bypassing cellular senescence, an irreversible growth arrest of cells that is activated in normal cells to become immortal is one of the prerequisites for cancer development. Cellular senescence can be triggered by shortening of telomeres and certain cellular stresses. Using spontaneously immortalized Li-Fraumeni Syndrome (LFS) fibroblasts, we found that CREG1 (Cellular Repressor of E1A-stimulated Genes1) is one of genes whose expression fit the criteria of senescence-associated genes, decreased expression during immortalization and increased in senescence. Moreover, we found that epigenetic mechanisms regulate CREG1 expression in LFS fibroblasts. CREG1 is a secreted glycoprotein that was shown to bind Rb-family pocket proteins and inhibit E2F transactivation activity. Therefore, we hypothesize that CREG1 plays a role in cellular senescence involving the $p16^{INK4a}$-Rb pathway. Ectopic expression of CREG1 in the immortal LFS cell lines decreases cell proliferation but does not directly induce senescence. We confirmed this in osteosarcoma and fibrosarcoma cancer cell lines,

similar to those seen in patients with Li-Fraumeni Syndrome. Because CREG1 was shown to interact with Rb pocket proteins *in vitro*, we tested whether growth suppression by CREG1 may depend on the phosphorylation status of Rb. We found that p16^{INK4a}, an inhibitor of CDK and Rb phosphorylation, is also downregulated in immortal cells and that coexpression of CREG1 and p16^{INK4a}, has a greater effect than CREG1 or p16^{INK4a} alone to reduce cell growth, to induce cell cycle arrest and cellular senescence in immortal LFS, osteosarcoma and fibrosarcoma cell lines. Moreover, cooperation of CREG1 and p16^{INK4a} inhibits the expression of cyclin A and cyclin B by inhibiting promoter activity thereby decreasing mRNA and protein levels; these proteins are required for S-phase entry and G2/M transition. In conclusion, we are the first to find that CREG1 enhances p16^{INK4a}–induced senescence by transcriptional repression of cell cycle mediated genes.

AUTOBIOGRAPHICAL STATEMENT

BENCHAMART MOOLMUANG

Education

- Graduate Program in Cancer Biology, Ph.D.

  Wayne State University, Detroit, MI   2005-2010

- Molecular Genetics and Genetic Engineering, Ms.C.

  Institute of Molecular Biology and Genetics,

  Mahidol University, Thailand   2002-2004

- Department of Biology, B.Sc.

  Khon Kaen University, Thailand   1998-2001

Publication

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