Regulation of Cellular Senescence and p16\(^{\text{INK4a}}\) Expression by Id1 and E47 Proteins in Human Diploid Fibroblast*  

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Id1, a member of Id family of helix-loop-helix transcriptional regulatory proteins, is implicated in cellular senescence by repressing p16\(^{\text{INK4a}}\) expression, but the mechanisms and cellular effects in human diploid fibroblasts remain unknown. Here we analyzed the patterns of p16\(^{\text{INK4a}}\) and Id1 expression during the lifespan of 2BS cells and presented the inverse correlation between these two proteins. Immunoprecipitation assays demonstrated the presence of endogenous interaction of Id1 and E47 proteins that was strong in young 2BS cells and weakened during replicative senescence and, thereby, influenced the transcription activation of p16\(^{\text{INK4a}}\) by E47. Furthermore, we found that E47 protein could bind to the E-box-containing region in p16\(^{\text{INK4a}}\) promoter in senescent cells by chromatin immunoprecipitation analyses, suggesting that E47 is indeed ultimately involved in the regulation of p16\(^{\text{INK4a}}\) transcription in vivo. Silencing Id1 expression in young cells by RNA interference induced an increased p16\(^{\text{INK4a}}\) level and premature cellular senescence, whereas silencing E47 expression inhibited the expression of p16\(^{\text{INK4a}}\) and delayed the onset of senescent phenotype. The present study demonstrated not only the capacity of Id1 to regulate p16\(^{\text{INK4a}}\) gene expression by E47, but also the phenotypic consequence of the regulation on cellular senescence, moreover, raised the possibility of Id1-specific gene silencing for human cancer therapy.  

* This work was supported by grants from the Special Funds for Major State Basis Research of China (G2000057001) and National Natural Science Foundation of China (39930170 and 30271432). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.  

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§ The abbreviations used are: HLH, helix-loop-helix; CDK, cyclin-dependent kinase; Rb, retinoblastoma; PD, population doubling; PBS, phosphate-buffered saline; Ig, rabbit immunoglobulin; siRNA, small interfering RNA; SA, senescence-associated; EMSA, electrophoretic mobility shift analysis; RNAi, RNA interference; siId1, pSilencer-Id1; siE47, pSilencer-E47; ChIP, chromosomal immunoprecipitation.  

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Id1 Regulates Cellular Senescence through E47 Protein

Could delay cellular senescence through regulation of p16\(^\text{INK4a}\) expression. p16\(^\text{INK4a}\) is an important tumor suppressor that is inactivated in a large proportion of human tumors (30). p16\(^\text{INK4a}\) elicits its cellular effects by inhibiting CDK4 and CDK6, which regulate cell cycle progression in G1 phase by contributing to the phosphorylation of the retinoblastoma (Rb) protein (31). Despite the significant function of it in cell senescence, immortalization, and tumorigenesis, the transcriptional control of p16\(^\text{INK4a}\) is poorly understood at the present time but is thought to concern diverse nuclear factors. Understanding the mechanisms of p16\(^\text{INK4a}\) regulation may help us to find a way of controlling its expression and inducing senescence in cancer.

Here we take Id1 as a model to explore how the Id proteins are involved in the regulation of p16 expression and cellular senescence. We report here that Id1 inhibits p16\(^\text{INK4a}\) expression by sequestering E47 protein and blocking the transcriptional activation of p16\(^\text{INK4a}\) and, therefore, maintains the proliferative state of young 2BS cells. The reducing expression of Id1 is an important tumor suppressor that is inactivated in a large proportion of human tumors (30). p16\(^\text{INK4a}\) elicits its cellular effects by inhibiting CDK4 and CDK6, which regulate cell cycle progression in G1 phase by contributing to the phosphorylation of the retinoblastoma (Rb) protein (31). Despite the significant function of it in cell senescence, immortalization, and tumorigenesis, the transcriptional control of p16\(^\text{INK4a}\) is poorly understood at the present time but is thought to concern diverse nuclear factors. Understanding the mechanisms of p16\(^\text{INK4a}\) regulation may help us to find a way of controlling its expression and inducing senescence in cancer.

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As expected, middle-aged and young 2BS cells possessed low level in cells undergoing spontaneous replicative senescence (Fig. 1). Id1 serves as loading control.

RESULTS

Id1 Expression Inversely Correlated with p16INK4a Expression during the Lifespan of 2BS Cells—Due to the potential role of Id1 and E47 in the modulation of p16INK4a expression, their expression patterns during the progression of successive passages are of interest. Western blot analysis was performed on the 2BS cells cultured to PD26 (young), PD42 (middle-aged), and PD60 (senescent), respectively. To exclude the discrepancy of expression levels due to different stages of the cell cycle, cells were synchronized to G1 phase by serum starvation and harvested at 8 h after serum addition. Then endogenous expression of Id1, E47, and p16INK4a were measured. The results of Id1 in various ages of 2BS cells revealed a high Id1 protein level in young cells compared with the relatively low Id1 level in cells undergoing spontaneous replicative senescence (Fig. 1). As expected, middle-aged and young 2BS cells possessed low p16INK4a levels (Fig. 1). However, there was an increase in the p16INK4a level in senescent cells. There was either no change, or minimal change, in the levels of either E47 or β-actin (Fig. 1). These data indicated that young 2BS cells expressed high levels of Id1, however, Id1 expression declined accompanied by an improving level of p16INK4a and the onset of cellular senescence. In these cells, Id1 expression reduced during the lifespan of 2BS cells and inversely correlated with p16INK4a, which gave the opportunity for Id1 to adjust p16INK4a expression by changing itself. The stable level of E47 suggests it would not be a variable in the regulation of p16INK4a expression, but the data did not rule out the possibility that Id1 could repress p16INK4a expression by sequestering E47 and antagonize its function of transcriptional activation.

Id1 and E47 Interact with Each Other in Young 2BS Cells Stronger Than in Senescent Cells—To discover the probable mechanism by which Id1 can affect p16INK4a expression in 2BS cells, the endogenous protein-protein interaction of Id1 and E47 was determined. Total proteins from young (PD26), middle-aged (PD42), and senescent (PD60) 2BS cells were prepared as aforementioned. The proteins were immunoprecipitated with a polyclonal anti-Id1 antiserum or with normal rabbit immunoglobulin (Ig) as a negative control. Crude cell extract from 2BS cells (PD 26) was run in a parallel lane. Immunodetection was done with polyclonal anti-E47 or anti-Id1 antiserum, as indicated (Fig. 2). The data showed that Id1 functionally interacted with E47, and the strength of binding was diminished with cellular senescence. Evidently, the binding of Id1 to E47 in young 2BS cells was the strongest, second only was that in middle-aged cells, and the combination potential was the weakest in senescent cells, corresponding to the low expression of Id1. As mentioned above, the E47 levels kept constant in young, middle-aged, and old cells, so the more E47 bound to Id1, the less E47 were free to activate transcription of target genes, for example, p16INK4a. interestingly, the level of p16INK4a was induced in senescent 2BS cells where the binding strength of E47 to Id1 was weak and the E47 proteins had much more possibilities to function as transcriptional regulators. These data demonstrated the existence of a physical interaction between Id1 and E47 in vivo and established the relationship between amounts of free E47 and p16INK4a expression levels. It was conceivable that p16INK4a might be induced at least partly by the E47 protein which couldn’t bind to Id1 in senescent cells.

E47 Bound to E-Box containing Region of the Endogenous p16INK4a Promoter—Two E-boxes have been identified in the p16INK4a promoter sequence, which locate, respectively, at positions −354 (E1) and −620 (E2) relative to the translation initiation site (31). To seek evidence that E47 can bind to and influence the endogenous p16INK4a promoter, ChIP assays were performed to detect the occupancy of E47 in the two E-boxes in the p16INK4a promoter. The chromatin was isolated in young (PD26) and senescent (PD60) 2BS cells and broken into small fragments via sonication after formaldehyde treatment of the cell cultures (Fig. 3C). An antibody specific for E47 was used to immunoprecipitate E47-cross-linked chromatin fragments.
Following reversal of the cross-links and purification of the genomic DNA fragments, the DNA sequences were analyzed by PCR with specific pairs of primers flanking the E1 or E2 boxes (Fig. 3A). The binding of E47 was observed to both E1 and E2 boxes in senescent cells, compared with a weak amplification in young cells (Fig. 3B). The irrelevant antibody control (β-actin) and no antibody control had no amplification products, which suggested the binding of E47 to E1 and E2 boxes was specific. These results identified the binding of E47 to p16\textsubscript{INK4a} promoter in vivo and implicated the role of E47 in p16\textsubscript{INK4a} transcriptional regulation.

Silencing of the Id1 Gene by RNA Interference (RNAi) Induced p16\textsubscript{INK4a} Expression and Premature Senescence, Whereas Silencing of the E47 Gene Inhibited p16\textsubscript{INK4a} Expression and Extended the Lifespan of 2BS Cells—Normal human diploid fibroblast 2BS cells enter a senescence state at about PD55–60. To determine the effects of Id1 and E47 expression levels on cellular senescence, we used the pSilencer vector system to stably suppress the expression of Id1 or E47 genes. Early passage of 2BS cells (PD26) were transfected with the pSilencer-Id1 (siId1), pSilencer-E47 (siE47), or pSilencer NC vector, and transfectants were selected with G418. The siRNA-transfected cells were analyzed for the relative senescence markers compared with the untransfected 2BS cell control, all of which came from the same batch of early passage cells.

The Efficiency and Specificity of Id1- or E47-specific RNAi and the Impact on p16\textsubscript{INK4a} Expression—To ensure the specificity of Id1 and E47 siRNA, we compared their potential sequences with the human genome data base using BLAST, and eliminated the sequences with more than 16–17 bp of homology to other coding sequences. We compared the mRNA sequences of the Id family, from Id1 through Id4, at the target domain of siId1 and the surrounding region, and discovered that the target sequence of siId1 had 11 bp of homology to Id2 mRNA, 9 bp of homology to Id3 mRNA, and 7 bp of homology to Id4 mRNA (Fig. 4A), which were all lower than 16–17 bp. Simultaneously, the sequences comparison of E proteins indicated that the target sequence of siE47 had 13 bp of homology to E12 mRNA and 11 bp of homology to E2-2 and HEB mRNAs (Fig. 5A). Hence the used sequences of siId1 and siE47 had less homology to the related proteins, which aided to avoid the impact on the expression of these proteins.

To further determine the specificity and efficiency of siId1 and siE47, we detected the expression of related proteins in siId1 and siE47 transfected cells by Western blotting. Because the siId1 cells ceased cell division at PD42–45 (Table I), these pools of resistant clones to G418 were used (Fig. 4B). While the lifespan of siE47-transfected cells was prolonged (Table I), the cells both at PD42–45 and PD65–70 were used to detect the stable knockdown of E47 expression during extended cell growth (Fig. 5B). NC cells were used at same PDs. We found that in siId1-transfected cells the Id1 level reduced 75% compared with the NC cells (Fig. 4, B and C), establishing the efficiency of the Id1 siRNA. However, the levels of Id2, Id3, and Id4 lessened 22%, 8%, and 6%, respectively, which were extremely trifling compared with the level of Id1 knockdown (Fig. 4, B and C). Besides, E47 level decreased 73% in siE47 transfected cells at PD42–45, compared with NC cells at same PD (Fig. 5, B and C). The knockdown of E47 sustained during the extended lifespan and even had a minimal rise in cells at PD65–70. The levels of E12, E2-2, and HEB depressed 13%, 9%, and 12%, respectively, in siE47-transfected cells at PD42–45, but the losses of expression seemed to be minimal and temporary, because in cells at PD65–70, their expressions were restored. Moreover, E12 and HEB levels advanced a little compared with NC cells at same PD (Fig. 5, B and C). These data identified the efficiency and specificity of siId1 and siE47.

In addition, Western blot assays revealed an increased p16\textsubscript{INK4a} expression in the siId1-transfected cells compared with the NC cells (Fig. 4, B and C), which provided the in vivo significance of Id1 repression of p16\textsubscript{INK4a}. The expression of p16\textsubscript{INK4a} in siE47-transfected cells markedly reduced at PD42–45 (Fig. 5, B and C), however, when cells entered irreversible growth arrest at PD65–70, the p16\textsubscript{INK4a} level increased to some extent, although the knockdown of E47 still kept stable. The results identified E47 as a latent activator of p16\textsubscript{INK4a}, yet there may be some other mechanisms stimulated when E47 expression was inhibited.
siE47 Promoted Cell Growth, Whereas siId1 Led to Growth Inhibition—To observe the impact of Id1 and E47 gene-specific silencing on the cell proliferation, the growth curves for siRNA transfected and untransfected 2BS cells were compared. The curve of siId1-transfected cells was approaching that of senescent cells, showing nearly complete growth inhibition (Fig. 6A); however, the growth curve of siE47 advanced quickly, displaying its strong proliferation potential similar to young cells (Fig. 6B). As a control, NC cells had almost the same growth rate or growth potential as middle-aged 2BS cells without transfection. siId1 Brought G1 Cell Cycle Arrest, and siE47 Postponed It—To clarify the mechanisms of growth rate inhibition described above, the cell cycle profile of siRNA transfected and untransfected 2BS cells was analyzed by flow cytometry. Each experiment was performed at least three times, and representative data are shown in Fig. 7A. In contrast to the irreversible G1 arrest imposed by siId1, siE47 postponed the irreversible growth arrest. As a control, NC cells had a parallel percentage of cells in G1 phase to middle-aged 2BS cells (Fig. 7B). Thus, silencing the Id1 expression led to premature senescence by initiating the G1 arrest, whereas silencing the E47 expression efficiently delayed the onset of cellular senescence by postponing it.

SA-β-galactosidase Staining—A common marker for cellular senescence, SA-β-galactosidase staining was also checked with siRNA transfected and untransfected 2BS cells. Nearly all of the siId1-transfected cells were strongly stained blue, with gross enlargement and flattened morphology as with senescent cells (Fig. 8). siE47 cells and young cells retained a refractive cytoplasm with thin and long projections, and there were a few dispersed cells that were SA-β-Gal-stained. For NC cells, the positive ratio was comparable with middle-aged cells (Fig. 8).

All these outcomes manifested that RNAi-mediated silencing of Id1 gene could induce premature senescence accompanied with increased p16INK4a levels, however, doing the same thing to E47 could cut down the p16INK4a expression, extend the proliferative lifespan, and finally delay the onset of senescence of 2BS cells.

DISCUSSION

p16INK4a, the inhibitor of D-type CDK4 and CDK6, is implicated in cell immortalization and senescence (31). Previous studies have demonstrated that it is the accumulation of p16INK4a that triggers the onset of cellular senescence (34). So how is p16INK4a expression raised during cellular senescence? p16INK4a expression is more likely to be ruled by physiological stress than by telomere shortening, because in rodent senescent cells where telomere shortening can’t be detected the accumulation of p16INK4a is also observed. We have been studying p16INK4a transcription modulation for years and found a novel negative regulatory element, the INK4a transcription silence element, in the p16INK4a promoter (35). Moreover, the GC-rich region of the p16INK4a promoter is a positive transcription regulatory element (35). Based on these findings we propose that p16INK4a transcription control may deal with a number of known and unknown nuclear factors, such as members of the Sp family.

To find clues of the transcriptional regulators we searched the p16INK4a promoter sequence and identified two E-boxes at the positions −354 (E1) and −620 (E2) upstream to the translation initiation site. In addition, one recent report showed that Id1-null primary mouse embryo fibroblasts underwent premature senescence and accompanied increased p16INK4a expression (29). There is a view that the primary mode through which Id proteins function is the antagonism of basic HLH transcriptional regulators. Subtle changes in the equilibrium of heterodimeric interactions between basic HLH and Id proteins cause dramatic cellular effects (41). The mammalian Id proteins (Id1 through Id4) preferentially target the ubiquitously expressed E proteins, which belong to the Class I basic HLH proteins (E47, E12, E2-2, and HEB) (42). As a member of the E proteins, E47 has the DNA-binding specificity that is limited to the consensus DNA sequence CANNTG, known as E-box (43). E47 may bind to the E-box as homodimers or heterodimers with MyoD. The two E-boxes in the p16INK4a promoter with a consensus sequence of CAGGTG belong to the
Group I E-boxes with high affinity to E47 homodimers (7). It has been reported recently that E47 transcriptionally activates p16INK4a promoter in an E-box-dependent manner (26). On the basis of these findings, we reason that Id1 may participate in cellular senescence by blocking E47-mediated transcriptional activation of the key senescence effector, p16INK4a.

In the present study, we detected endogenous expression patterns of p16INK4a and Id1 during the lifespan of human diploid fibroblasts, setting up the relationship between p16INK4a and Id1 expression as well as the correlation between Id1 expression and the age of cells. The high levels in young cells and the strikingly decrease in senescent cells of Id1 expression were consistent with the postulated role of Id1 in the regulation of cell senescence (Fig. 1). The function of constitutively expressed E47 protein may be modulated by altering the level of Id1 protein at a definite stage of cell growth. In general, quiescent cells express low or absent levels of Id genes, whereas the expression of Id is high in proliferating cells (44, 45). Id1 expression changes in diverse phases of the cell cycle, which is rapidly induced following the stimulation of quiescent fibroblasts with serum. After an initial decline, Id1 expression is further up-regulated as cells progress through G1 and enter S phase of the cell cycle (22). To ensure that the expression variations originated from the senescence process other than cell cycle, we performed Western blot assays with synchronized 2BS cells.

Id1 belongs to Class V HLH proteins that lack a basic region, thus the heterodimers cannot bind to DNA when Id1 combines to E proteins, therefore, blocking the transcriptional activation of E proteins to target genes. There are many evidences for the interaction of Id1 and E47. For example, in vitro translated Id1 protein communoprecipitated with E47 protein and inhibited DNA binding by E47 very efficiently (9). Sun et al. (12) have shown that Id1 can form complexes with E47 by glutathione S-transferase-pull-down, thus provide a molecular basis for inhibition of DNA binding by Id proteins through formation of heterodimers. Moreover, dimerization of Id1 protein with E47
Id1 is tested in a quantitative yeast two-hybrid assay, finding mammalian two-hybrid analyses (46). We suppose observations were confirmed by co-immunoprecipitation and following the population divisions, which provided evidence for ascertained that binding strength of the two proteins changed of examined by immunoprecipitation. We identified the presence this hypothesis, the endogenous interaction of nevertheless, due to the evidence for direct dimerization described in our laboratory.

It was reported that ectopic expression of Id1 immortalized primary human keratinocytes and activated telomerase activity (27). Not long after, Nickoloff et al. (28) observed that Id1 extended the lifespan of human keratinocytes but could not immortalize them. A key unresolved issue is the cellular effects of Id1 on human diploid fibroblasts. Our studies try to address this question through specific silencing of Id1 or E47 expression by RNAi in 2BS cells. To ensure the specificity of RNAi, we compared the mRNA sequences of members of Id family and E proteins, respectively, then selected the target sequences of siId1 and siE47 out of the HLH domain in which the sequences contain multiple protein factors. Never-
dimerize with each other, because the surroundings in vivo contact with each other in vivo. There are four probable patterns for proteins bonding to DNA as follows. (a) A transcription factor directly contacts with either a consensus or nonconsensus binding site. (b) Two or more factors bind to separated sites that are drawn near by looping of the DNA by way of protein-protein interactions. (c) A protein factor binding to a low affinity site is stabilized by contact with another protein that is recruited by a factor bound to a different site. (d) Transcription factors are brought to sites through interaction with other DNA-binding proteins. According to the first pattern, E47 and Ets1 may both function through specific binding sites in the p16
INK4a promoter, and they also can interact directly through the second way. The precise mechanism by which E47 and Ets1 function is currently under investigation in our laboratory.
ingly different from that of E12. The chosen siId1 and siE47 sequences have less homology to related proteins.

Introduction of siId1 or siE47 efficiently inhibited the expression of target genes, because the level of Id1 was significantly reduced by 75% in siId1 cells (Fig. 4, B and C), and E47 expression was reduced by 73% in siE47 cells (Fig. 5, B and C). As expected, the introduction of siId1 could barely impact on the expressions of Id2, Id3, and Id4, which have only minimal changes compared with those in NC cells. There were analogous effects on the expression of E12, E2-2, and HEB in siE47-transfected cells at PD42–45, implying the nonspecific inhibition of siE47 was insignificant. When siE47 cells were cultured to senescence, the knockdown of E47 was still maintained stable, whereas the expression of E12, E2-2, and HEB was restored to the levels of NC cells. It seems that certain factors in vivo arouse these genes to return the normal levels at senescence, thereby compensating for the lost function of E47. The expression patterns of p16INK4a in siE47 cells also confirmed this view. In siE47 cells at PD42–45, the p16INK4a level dropped off remarkably. However, it rose at PD65–70 and exceeded the level in siE47 cells at PD42–45, although the absolute value was lower than that of senescent NC cells. On the basis of these data, we conclude that E47 gene silencing decreased the expression of p16INK4a, yet there are other pathways through which p16INK4a is motivated for the senescence progression. The members of E proteins other than E47 may be involved in the process.

siE47 cells have a finite elongation in lifespan and a consid-

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**Fig. 7.** Flow cytometry analysis of siId1, siE47, and NC cells compared with young (PD26), middle-aged (PD42), and senescent (PD60) 2BS cells. Each experiment was performed at least three times. A showed the representative data. B, the graph depicted data from three independent experiments (means ± S.E.).

**Fig. 8.** Morphology and SA-β-gal staining for siRNA-transfected and untransfected cells. A, NC cells; B, siE47 cells; C, siId1 cells; D, young cells (PD26); E, middle-aged cells (PD42); F, senescent cells (PD60). Photographs are at ×400 magnification.
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Id1 expression in senescent 2BS cells, which in turn contributes to cellular senescence in human cells, the inactivation of the Id1 pathway would result in momentous cellular effects that incite senescence in human cells, the inactivation of the Id family of proteins may be effective candidates.

Acknowledgment—We are very grateful to Prof. Yong-Feng Shang for the kind gift of pSilencer NC plasmid.

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