ECRG1, a Novel Candidate of Tumor Suppressor Gene in the Esophageal Carcinoma, Triggers a Senescent Program in NIH3T3 Cells

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Esophageal cancer–related gene 1 (ECRG1) is a novel tumor-suppressor gene candidate identified from the human esophagus. Previous studies showed that ECRG1 overexpression could inhibit cell growth and induce G1 cell cycle arrest and $p15^{INK4b}$ expression by interacting with Miz-1 (Myc-interacting zinc finger protein). Such evidence suggests the alterations in ECRG1 may play an important role in tumorigenesis. To further study the biological function of the ECRG1 gene, we transfected ECRG1 into NIH3T3 cells. Expression of ECRG1 in these cells caused senescence-like changes characterized in terms of altered cell morphology, cell cycle arrest at the G1/S phase, and significantly impaired cell proliferation ($P < 0.01$). Moreover, NIH3T3 cells transfected with ECRG1 stained positive for SA-β-gal staining (pH 6.0), which is a specific marker of cellular senescence. We also studied changes in telomerase activity and the related senescence genes, such as p21 and p16. The results indicated that when ECRG1 induced a senescence-like state, telomerase activity was markedly decreased ($P < 0.05$), and expression of p21 was distinctly increased, whereas no changes were detected in p16 and telomerase-component RNA levels. These findings suggest that ECRG1 may be of importance in murine cell senescence, promoting senescence by regulating expression of p21. Exp Biol Med 231:84–90, 2006

Key words: ECRG1; esophageal; carcinoma; G1 cell cycle arrest; senescence; telomerase

Introduction

When propagated in culture, normal human somatic cells withdraw from a finite number of divisions and enter an irreversible arrest, designated replicative senescence (1–5). During the process, senescence is partially mediated by the loss of telomere length, which occurs with progressive divisions of mortal cells (6). Cellular senescence can also be induced prematurely by a variety of stimuli, including DNA, perturbations of chromatin structure, and overexpression of mitogenic signals such as E2F1, oncogenic Ha-ras, Raf, or mitogenic activated protein (MEK; Refs. 7, 8). Current studies show that inactivation of the p16/pRb and p53 pathways is necessary to bypass senescence, although it is insufficient for immortalization (9). In addition, genetic analysis of immortalized prostate epithelial and other cell types revealed that a number of consistent genetic alterations were required, including the amplification of chromosome 20p and 8q (10). On the basis of these findings, it has been proposed that alterations in multiple pathways are required for the acquisition of the immortal phenotype (11). Because replicative senescence was believed to provide a barrier against the unlimited cellular proliferation and formation of cancer (12–14), the identification of human genes involved in senescence could provide a set of genes products for use in cancer protection and therapy.

Esophageal cancer–related gene 1 (ECRG1) (Genbank accession no. AF071882.1), a novel tumor-suppressor gene candidate, was cloned by the effective technique of messenger RNA differential display by comparing the differential gene expression between normal esophageal epithelia and esophageal cancer (15, 16). Previous studies showed that ECRG1 was expressed in normal esophagus, liver, colon, and lung tissues, but was down-regulated in adjacent and cancerous tissues, with especially low frequency in esophageal cancer (16). *In vitro* and *in vivo* assays indicated that ECRG1 inhibits tumor cell proliferation (17), suggesting that ECRG1 overexpression might serve in antitumor gene therapy. Using a yeast two-hybrid

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screen system, we identified that ECRG1 interacted with Miz-1 (Myc-interacting zinc finger protein) (18). Because Miz-1 is involved in cell senescence (19), we considered that ECRG1 might be involved in this process as well. In this study, we examine the role of ECRG1 on murine cellular senescence. Our results indicate that in NIH3T3 cells, expression of ECRG1 could cause senescence-like changes, marked G1 cell cycle arrest, and up-regulation of p21 expression.

Material and Methods

Plasmid Construction. The amplified ECRG1 complementary DNA (cDNA) fragment and plasmid were disgested by *Eco*RI and *Bam*HI, then ligated by ligase, and inserted into the *Eco*RI-*Bam*HI (blunted) site of pHA. The recombinant vector pHA-ECRG1 was identified by restriction digest and sequencing.

Cell Culture and Transfection. NIH3T3 murine fibroblast cells were cultured in Dulbecco modified Eagle medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), 100 μg/ml penicillin, and 100 μg/ml streptomycin at 37°C in an incubator with 5% CO_2. Transfection of the recombinant vector was performed by company protocol (Invitrogen Life Technologies). Briefly, before transfection, cells were transferred to DMEM without serum or antibiotics. Then, a mixture of vector and lipofectamine was added to the plates. After being cultured for 6 hrs, cells were incubated with fresh medium for the desired time period.

MTT Assay. NIH3T3 cells were seeded in 96-well plates (each condition has six parallel samples). Two group samples were transfected with pHA and pHA-ECRG1, respectively; then cell viability was examined every other day (total, 6 days). Then, 10 μl (3-[4, 5-dimethylthiazol-2-y]-2, 5-diphenylterazolium bromide [MTT], 5 mg/ml) was added and cells were incubated for an additional 4 hrs at 37°C. Subsequently, 100 μl of dimethyl sulfoxide was added, and the plate was incubated for 5 mins at 37°C to dissolve the formazan crystals. The optical density (O.D.) was measured at a wavelength of 570 nm. Each experiment was repeated at least three times.

Cell Lysates and Western Blot Analysis. Cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in a solubilization buffer (pH 8.0, 1% Triton X-100, 20 m*M* Tris-HCl, 150 m*M* NaCl, 5 m*M* EDT, 100 IU aprotinin) on ice for 30 mins. Supernatants were generated by centrifugation at 13,000 *g* for 10 mins. Protein concentration was measured by the Bradford method and 30 μg of total protein was loaded onto a 12% sodium dodecyl sulfate–polyacrylamide gel, followed by transfer to a nitrocellulose membrane. Membranes were blocked overnight with 5% nonfat milk in Tris-buffered saline (0.1% Tween-20 in PBS), and then probed, respectively, with mouse monoclonal anti-HA antibody (1:2000), mouse monoclonal anti-p21, and mouse monoclonal anti-p16 (1:500; Santa Cruz Biotech, Santa Cruz, CA) for 2 hrs at 4°C. After several washings, membranes were incubated with anti-mouse IgG conjugated with horseradish peroxidase, washed, and analyzed with enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ). Densitometric assessment of bands was conducted using National Institutes of Health image 1.62 software. Band intensity was qualified by measuring the volume of the band in the lane profile.

Senescence-Associated X-gal (SA-β-gal) Staining. Cells were washed twice with PBS, fixed in 2% formaldehyde for 3–5 mins at room temperature, washed twice with PBS, and incubated at 37°C (without CO_2) with fresh senescence-associated x-gal stain solution (1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactosidase [x-gal] per milliliter [stock = 20 mg of dimethlformamide per milliliter]/40 m*M* citric acid/sodium phosphate, pH 6.0/5 m*M* potassium ferrocyanide/5 m*M* potassium ferricyanide/150 m*M* NaCl/2 m*M* $MgCl_2$) as described previously (20).

Cell Cycle Assay. Cells were harvested by trypsinization and were suspended in 0.5 ml of PBS (pH 7.2), then fixed in ice-cold ethanol overnight at 4°C. Fixed samples were washed, resuspended in PBS containing 50 μg/ml propidium iodide and 50 μg/ml RNase, and incubated at 37°C for 30 mins. Change in cell cycle was assayed by flow cytometry.

Telomerase Activity and Telomerase-Component RNA Transcription. The direct effect of ECRG1 on the transcription of the telomerase RNA component (hTR) was examined by reverse transcription–polymerase chain reaction (RT-PCR). For PCR reactions, hTR sequence-specific TRC3 primers, described by Feng *et al.* (21) (TRC3 primers: 5′-CTAACCCTATCTGAGTTGGGCGTA-3′ and 5′-CAACGGACAGACAGCAGCTGACAT-3′), were used to amplify telomerase-component RNA complementary DNA. β-actin was amplified as an internal standard using 5′-TCGCACCAATATCGAAAT-3′ as the antisense primer and 5′-ACCAACAAGAAGAACAAT-3′ as the sense primer. Moreover, the change in telomerase activity was examined by telomeric repeat amplification protocol–enzyme-linked immunosorbent assay (TRAP-ELISA) according to the kit protocol.

Electron Microscopy. Cells were pelleted and then fixed with 3% glutaraldehyde/PBS (pH 7.4). After one wash with 0.2 sodium cacodylate buffer (pH 7.4), the pellet was treated with 1% osmium tetroxide in cacodylate buffer for 1 hr. Cells were then dehydrated in graded steps of ethanol through propylene oxide and embedded in Embed 812 (Electron Microscope Sciences, Hatfield, PA). One-micrometer sections were cut and stained with methylene blue and azure B, observed via light microscopy, and representative areas were chosen for ultrathin sectioning. Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Sections were observed with a JEM 100CX transmission electron microscope.

Statistical Analysis. Statistical significance was

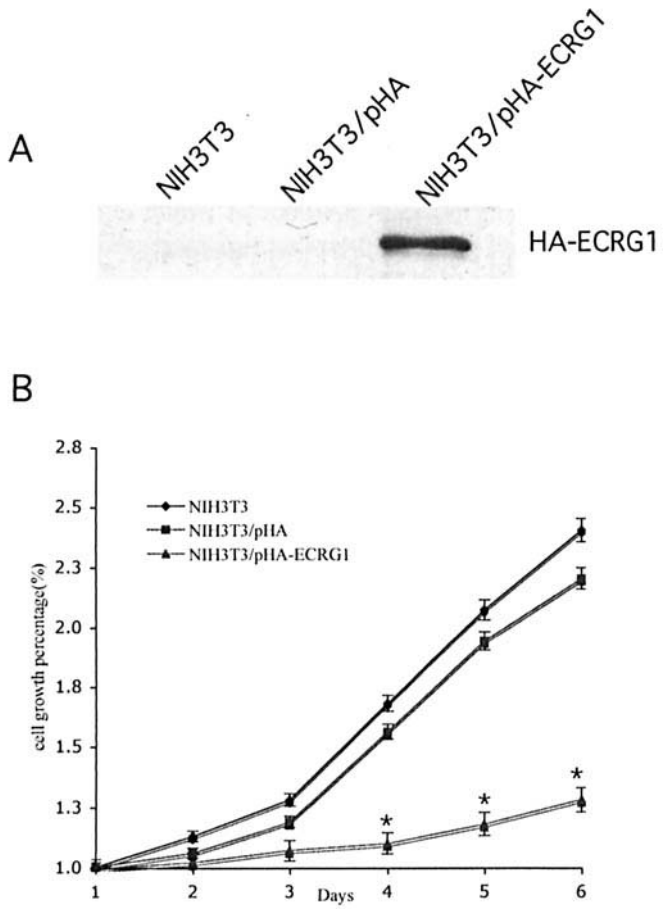

Figure 1. (A) Identification of ECRG1 expression in NIH3T3 cells transfected with pHA-ECRG1. (B) Effect of ECRG1 on NIH3T3 cell proliferation. At 1, 2, 3, 4 5, and 6 days after transfection cells were collected and assessed by MTT assay. Transfection of ECRG1 significantly inhibited cell proliferation more than it did the transfection of empty-vector pHA (*$P < 0.01$; Students t test, significantly different from controls). Bars, ± SD. Each point is the average of six parallel samples.

determined by the Students t test. Results were considered significant if probability of the difference occurring by chance was less than 5 in 100 ($P < 0.05$). Statistical analysis was performed using Microsoft Office Excel software (Redmond, WA).

Results

Expression of ECRG1 in NIH3T3 Cells. To study the biological effect induced by ECRG1, we constructed the recombinant vector of ECRG1 by using vector pHA, and transfected it into NIH3T3 cells. Untreated NIH3T3 cells transfected with pHA could be used as controls. After 48 hrs of transfection, expression of ECRG1 in transfected cells was identified by monoclonal anti-HA antibody (Fig. 1A).

ECRG1-Induced Cell Growth Inhibition. Previous studies showed that ECRG1 could inhibit tumor cell growth. Hence, we researched the effect of ECRG1 on the growth of NIH3T3 cells. We examined the state of cell growth after NIH3T3 cells had been transfected with ECRG1 and control cells had been cultured for 1, 2, 3, 4, 5, or 6 days. NIH3T3 cells transfected with ECRG1 grew markedly more slowly than control cells. The MTT assay showed that ECRG1 markedly inhibited the rate of cell proliferation, compared with that of the empty vector pHA ($P < 0.01$, Fig. 1B).

Induction of Senescence-like Changes by Expression of ECRG1. It has been reported that senescent (but not presenescent), quiescent, or terminally differentiated cells express a senescence-associated β-gal (SA-β -gal) that can be detected by incubating cells at pH 6.0 with x-gal senescent-specific marker, so we stained control cells and cells transfected with the ECRG1 plasmid with SA-β-gal. The results showed that about 80% of NIH3T3 cells/ pHA-ECRG1 were stained positive after the 48-hr trans-

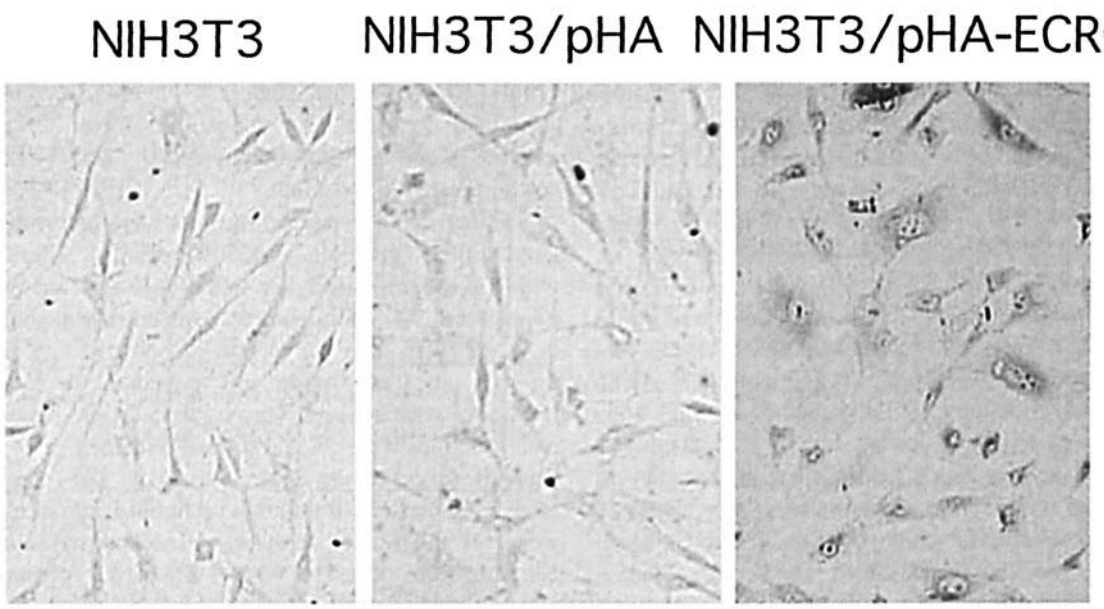

Figure 2. Cell staining positive for acidic β-galactosidase.

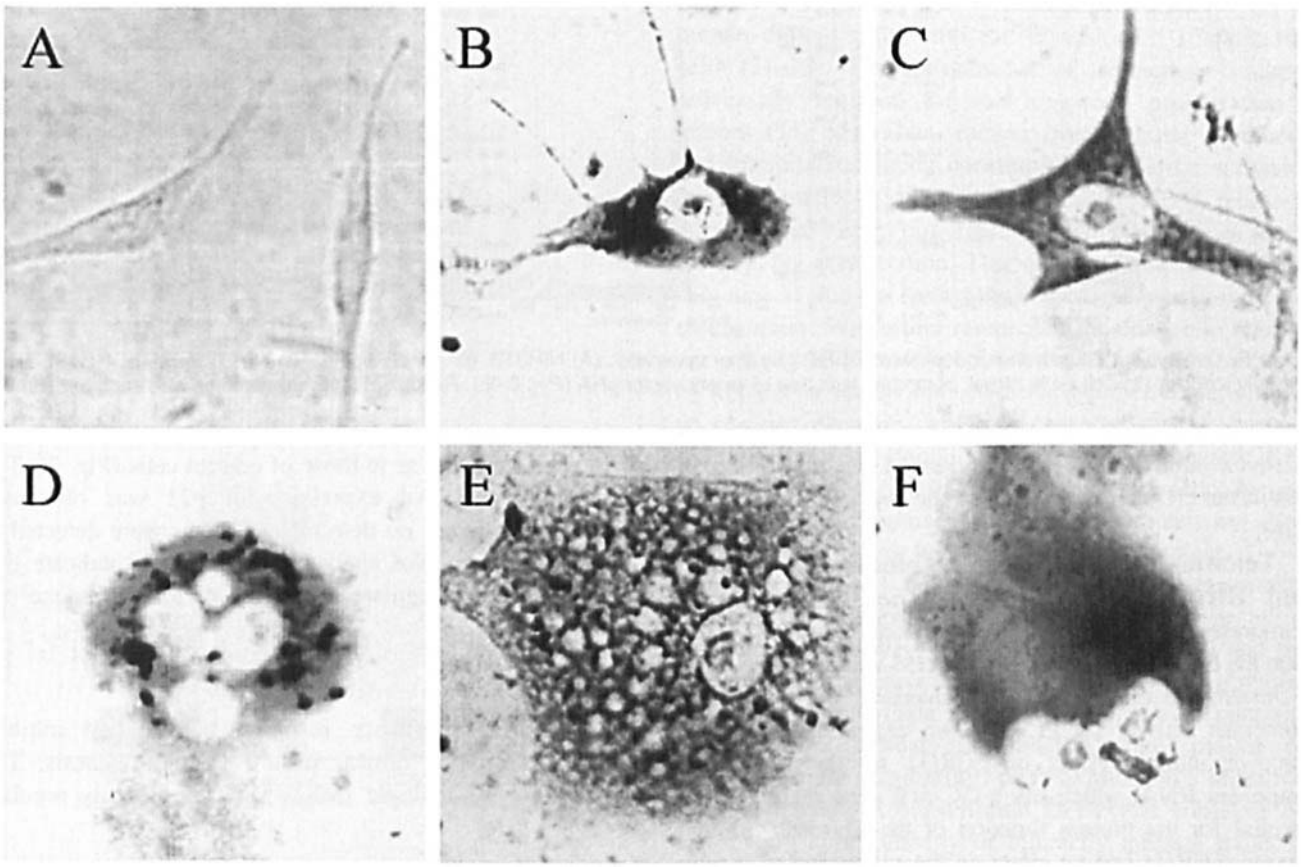

Figure 3. Morphological change in senescent NIH3T3 cells by ECRG1 action. (A) Normal NIH3T3 cells. (B–F) The senescent process of NIH3T3 cells by the action of ECRG1. (B) Cell and nucleus were enlarged and flattened. (C) Cells formed a dendriform protuberance. (D) Nuclei appeared in some cells. (E) Cytoplasmic vacuoles formed. (F) Cell nucleus moved to the margin of the cell and was ejected. The change process was consistent with that of senescent cells.

fection, whereas control cells showed no staining (Fig. 2). Moreover, the positive cells exhibited clear morphological changes, compared with that of control cells. First, cells were enlarged and flattened (Fig. 3B), then the extended cells formed a dendriform protuberance (Fig. 3C). Sequentially, positive cells displayed nuclear division, and some cells appeared to have a smaller nucleus (Fig. 3D). Then, the cytoplasm appeared to form vacuoles (Fig. 3E). Last, the cell nucleus moved to the margin of cell and was ejected from the cell (Fig. 3F). This change process was consistent with that of senescent cells (22). Using transmission electron microscopy to analyze esophageal cancer (EC) 9706 cells, we also found, in contrast to control cells, that cells transfected with ECRG1 had more large vacuoles, mitochondria appeared to shrink, and the karyotheca was irregular and had a lower electron density (Fig. 4). This demonstrated that ECRG1 could induce senescence-like changes in cancer cells.

Induction of G1 Cell Cycle Arrest by Expression of ECRG1. Because G1 cell cycle arrest is a characteristic of senescent cells, we examined the cell cycle changes by flow cytometry. After 2 days of induction, as shown in Figure 5, for pHA-ECRG1 transfected cells, the percentages of G1 and S phases were 75.6% and 23.2%, respectively, for control cells; about 55.8% of cells were at the G1 phase and 38.9% were at the S phase. These results indicated that ectopic overexpression of ECRG1 induced G1

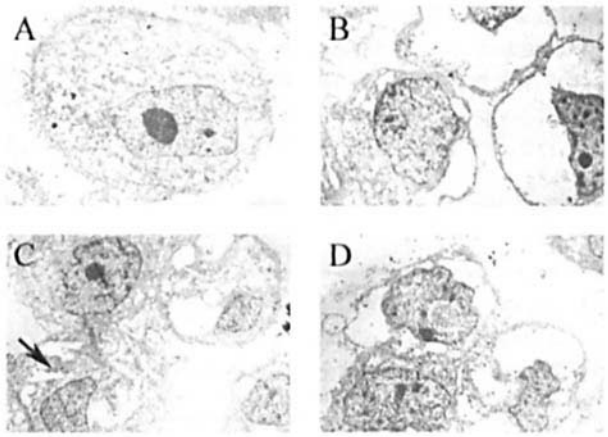

Figure 4. Electron micrographs of EC9706 cells and EC9706/pHA-ECRG1 cells analyzed using transmission electron microscopy. (A) EC9706 cells. (B–D) EC9706 cells/pHA-ECRG1. Arrow shows mitochondria.

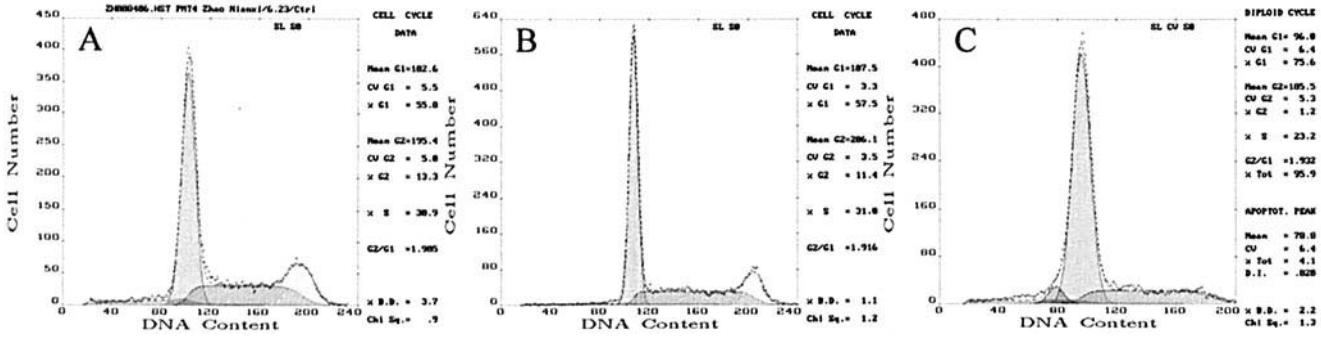

Figure 5. Cell cycle changes after induction of ECRG1 by flow cytometry. (A) NIH3T3. (B) NIN3T3/pHA. (C) NIH3T3/pHA-ECRG1. ECRG1 markedly induced G1 cell cycle arrest, compared with that of empty vector pHA ($P < 0.05$). Results of triplicate samples with each experiment.

cell cycle arrest significantly increased the proportion of cells in the G1 phase and reduced the fraction of those in S phase.

Telomerase Activity and Telomerase-Component RNA Transcription. As shown in Table 1, telomerase activity was markedly decreased ($P < 0.05$) when the NIH3T3 cells were transfected with pHA-ECRG1. To investigate the inhibitory mechanisms of ECRG1 on telomerase activity by ECRG1, we examined a possible direct or indirect effect of ECRG1 on the telomerase component RNA, which has a GC-rich gene region that is essential for the protein function of the ribonucleoprotein enzyme. ECRG1 had no effect on the expression of β-actin cDNA, which was used as an internal standard. Using hTR-component specific primers, approximately 400 base pairs of TRC3 cDNA were amplified. When treated with ECRG1 for 2, 3, 4, and 5 days, no apparent change in TRC3 transcription was detected (Fig. 6). These results showed that ECRG1 did not perturb the telomerase component of RNA transcription, suggesting that ECRG1 may post-translationally modulate telomerase activity.

Effect of ECRG1 on the Related Senescence Gene. Because p21 and p16 were believed to be functionally relevant for the establishment and maintenance of a senescence state, we further researched the effect of ECRG1 on p21 and p16 expression. In cells transfected with pHA-ECRG1, the relative amounts of p21 protein at 2, 3, and 4 days after transfection were 287%, 645%, and 520%, respectively, of that in control cells. Expression levels of p16 remained similar to those of control cells (Fig. 7). The results showed that expression of p21 was obviously increased, whereas no detectable changes were detected in p16 by Western blot analysis. These results indicate that ECRG1 may up-regulate expression of p21 to induce cell senescence.

Table 1. Effect of ECRG1 on Telomerase Activity by TRAP-ELISA[a]

	Negative control	NIH3T3	NIH3T3/ ECRG1
A (OD450) ($n = 4$)	0.037 ± 0.03	0.157 ± 0.05	0.107 ± 0.01*

[a] When the O.D. value of the sample >2 of the O.D. value of the negative sample, telomerase activity is considered to be positive. Values are mean ± SD, four samples were examined in each group. *$P < 0.05$ compared with NIH3T3 sample.

Discussion

Cellular senescence forms a barrier that inhibits immortalization, a critical feature in tumorigenesis. The inactivation of multiple pathways that positively regulate

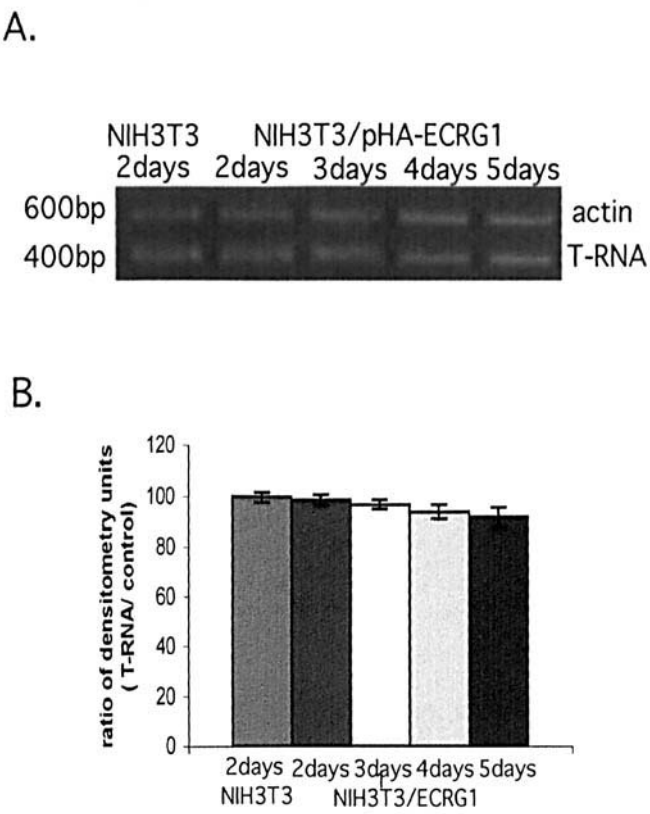

Figure 6. Changes in the telomerase component of RNA transcription by RT-PCR. (A) T-RNA determined by RT-PCR. (B) The band quantified. Values are means ± SD, each time point was the result of triplicate experiments.

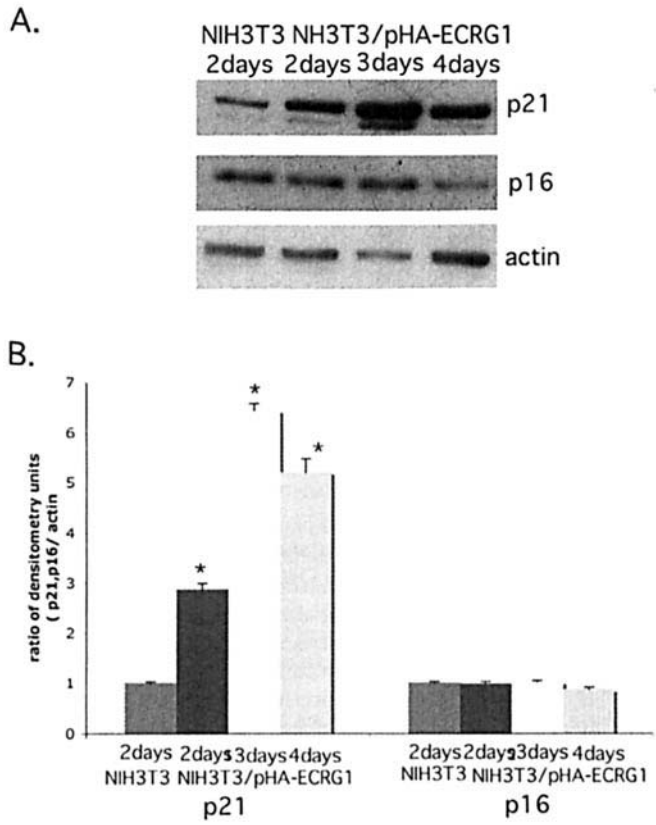

Figure 7. Effect of ECRG1 on expression of p21 and p16. β-actin controlled the expression level. Cells were harvested at the indicated time to determine p21 and p16 expression levels. (A) p21 and p16 expression by Western blotting. (B) The band quantified. Values are mean ± SD, each time point was the result of a triplicate experiment. $^*P < 0.001$, compared with NIH3T3 cells not transfected with ECRG1.

senescence are required for immortalization, thus it is necessary to identify the genes involved in these pathways. Presently, many tumor suppressor genes such as p16, p53, p21, p15, p27 (23–30), and so on, were believed to have a close correlation with senescence. Very little is known about the onset and maintenance of senescence, and to further clarify the mechanism of senescence, more senescence genes need to be identified. ECRG1 was a novel tumor-suppressor gene candidate associated with esophageal carcinoma, which could interact with Miz-1, and which induced senescence in NIH3T3 cells (24). We reason that ECRG1 may be involved in the senescence process. Here, we transfected ECRG1 into NIH3T3 cells. NIH3T3 cells/pHA and NIH3T3 cells were used as controls. Expression of ECRG1 in these cells caused senescence-like changes that were characterized by altered cell morphology, G1 cell cycle arrest, and significantly impaired cell proliferation. In ECRG1 overexpressing cells, we also observed positive staining (pH 6.0), which is a specific marker of cellular senescence.

Telomerase is present in human germ line cells, cancer cells, immortal cells, and tumor-derived cell lines, but it has not been detected in normal adult somatic tissues or cultured human diploid cells, with the exception of somatic stem cells (31–33). The maintenance of telomerase is almost universally required for the long-term proliferation of tumors (34, 35). Thus, escape from cellular senescence and immortalization by constitutive telomerase activation, or an alternative telomere maintenance (36, 37), represents an additional step in tumorigenesis that most tumors require for ongoing proliferation. This makes telomerase a significant area of study to further the understanding of senescence mechanisms. We further researched the change in telomerase activity and transcription of the telomerase component of RNA. Our results indicated that telomerase activity had an obvious decrease, which is characteristic of senescent cells. Because we found no change in telomerase RNA levels, we propose that ECRG1 induced senescence by post-translationally modulating telomerase either by direct protein interaction or by indirect protein signaling.

At present, p21 and p16 are believed to be functionally relevant for the establishment and maintenance of the senescence state. We also studied changes in p21 and p16. Western blotting showed that p21 levels were obviously increased, whereas there were no changes in p16 expression. These results indicate that ECRG1 may induce cell senescence by regulating p21 expression. Moreover, ECRG1 may be a transcription factor that enhances p21 expression, either directly or indirectly through molecular intermediates. Further studies on ECRG1 will shed light on the regulatory mechanism of the p21 pathway; and inactivation of ECRG1 is likely to bypass senescence of normal cells during tumorigenesis, but to elucidate the function of ECRG1 more precisely, further investigation is necessary.

It is well known that immortal cells are different from tumor cells. Tumor cells often acquire many genetic changes, often losing their correct protein function in the process, so we further transfected ECRG1 into EC9706 cells. The result showed that the transfected cells were not stained by SA-β-gal. Using transmission electron microscopy, we found EC9706 cells transfected with ECRG1 exhibited large vacuoles, mitochondrial shrinkage, lower electron density, and irregular karyotheca. Changes in mitochondrial dynamics are significant signs of cellular aging and can serve as precursors of programmed cell death or necrosis. Thus, such signs of cellular aging may also indicate senescence.

Multicellular organisms have evolved mechanisms to prevent the unregulated growth and malignant transformation of proliferating cells. One such mechanism is cellular senescence, which arrests proliferation—essentially irreversibly—in response to potentially oncogenic events. Cellular senescence appears to be a major barrier that cells must overcome to progress to immortalization and malignancy. Data indicate that cellular senescence suppresses tumorigenesis *in vivo* (38–40). In summary, replicative senescence is a powerful tumor suppressive mechanism. Therefore, further research on the pathways that regulate senescence

will undoubtedly shed light on how cells may bypass senescence during tumorigenesis.

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