THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 30, Issue of July 25, pp. 27789–27795, 2003
Printed in U.S.A.

Senescent Phenotype Can Be Reversed by Reduction of Caveolin Status*

Received for publication, August 8, 2002, and in revised form, April 17, 2003
Published, JBC Papers in Press, May 1, 2003, DOI 10.1074/jbc.M208105200

Kyung A. Cho‡, Sung Jin Ryu‡, Jeong Soo Park§, Ik Soon Jang‡, Jeong Soo Ahn‡, Kyung Tae Kim‡, and Sang Chul Park‡¶

From the ‡The Aging and Apoptosis Research Center, Department of Biochemistry and Molecular Biology, Seoul National University College of Medicine, Seoul 110-799, Korea and §Department of Biochemistry, College of Medicine, Dankook University, Cheonan, Korea

Hyporesponsiveness to growth factors is one of the fundamental characteristics of senescent cells. We previously reported that the up-regulation of caveolin attenuates the growth factor response and the subsequent downstream signal cascades in senescent human diploid fibroblasts. Therefore, in the present experiment, we investigated the modulation of caveolin status in senescent cells to determine the effect of caveolin on mitogenic signaling efficiency and cell cycling. We reduced the level of caveolin-1 in senescent human diploid fibroblasts using its antisense oligonucleotides and small interfering RNA, and this resulted in the restoration of normal growth factor responses such as the increased phosphorylation of Erk, the nuclear translocation of p-Erk, and the subsequent activation of p-Elk upon epidermal growth factor stimulation. Moreover, DNA synthesis and the re-entry of senescent cells into cell cycle were resumed upon epidermal growth factor stimulation concomitantly with decreases in p53 and p21. Taken together, we conclude that the loss of mitogenic signaling in senescent cells is strongly related to their elevated levels of caveolin-1 and that the functional recovery of senescent cells at least in the terms of growth factor responsiveness and cell cycle entry might be achieved simply by lowering the caveolin level.

Caveolae are vesicular organelles that represent a subdivision of the plasma membrane (1, 2). They are most abundant in terminally differentiated cell types, *i.e.* adipocytes, endothelial cells, and muscle cells. Moreover, it has been suggested that caveolae may function as subcellular compartments for the storage of inactive signaling molecules to regulate activation and to facilitate cross-talk between distinct signaling cascades (3, 4).

Caveolin, a 21–24-kDa integral membrane protein, is the principal component of caveolae, and the caveolin gene family consists of caveolin-1, -2, and -3. Caveolin-1 and -2 are co-expressed, form a hetero-oligomer in the plasma membrane, and exist in many cell types (5–7), whereas the expression of caveolin-3 is muscle-specific (8). Moreover, recent studies have suggested a regulatory role for caveolin in addition to its structural function. For example, the stable expressions of the caveolin-1 or -3 genes in caveolin-deficient mammalian cells induced the formation of caveolae structures (9).

The caveolin functions as scaffolding protein within the caveolae membrane and interacts with signaling proteins such as EGFR,[1] G-proteins, Src-like kinases, Ha-Ras, protein kinase C, endothelial nitric-oxide synthase, and integrin (10–15). In addition, a short cytosolic N-terminal region of caveolin is involved in the formation of oligomers and mediates the interaction with signaling molecules, which result in the their inactivation (4). The targeted down-regulation of caveolin-1 is sufficient to drive cell transformation and hyperactivates the Erk kinase cascade (16). And caveolin levels in most tumor tissues are significantly lower than in normal tissues, suggesting the circumvention of signal suppression by caveolin. Moreover, the co-expression of EGFR, Raf, MEK-1, or Erk-2 with caveolin-1 dramatically inhibits signaling from the cytoplasm to the nucleus *in vivo* (3).

Many cell types, such as human diploid fibroblast, show cellular senescence *in vitro*. Senescent cells enter into irreversible growth arrest when they contain increased levels of cell cycle inhibitory molecules (17–19), and in this state, the cells have a large and flat morphology and show senescence-associated β-galactosidase activity (21). Senescent human diploid fibroblast (HDF) cells do not show mitogen-activated protein kinase activation upon EGF stimulation (20–22). Moreover, accumulated cytosolic p-Erk-1/2 failed to translocate to the nuclei upon EGF stimulation (23–25). In consequence, senescent cells show lower levels of p-Erk in the nucleus, despite its high level in the cytosol, and lack transcription factor (Elk-1) activation (9).

Previously, we reported that the hyporesponsiveness of senescent fibroblasts to EGF stimulation might be related to the up-regulation of caveolins with aging and that the overexpression of caveolin-1 in young HDF cells could down-regulate EGF signaling (21). However, it is not known whether the reduced caveolin-1 in senescent cells might restore the signaling cascades and cellular cycling in response to growth factors. Therefore, in this study, we attempted to test the possibility of recovery of growth factor responsiveness by reducing the level of caveolin-1 in senescent HDFs by use of antisense oligonucleotide and small interfering RNA (siRNA).

* This work has been supported by the grants from the Aging and Apoptosis Research Center (RII-2002-001-01-001), the Korea Research Foundation for Health Science, and the Virtual Research Institute of Aging, Nippon Boehringer Ingelheim. 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.

¶ To whom correspondence should be addressed. Tel.: 82-2-740-8244; Fax: 82-2-744-4534; E-mail: scpark@snu.ac.kr.

[1] The abbreviations used are: EGFR, epidermal growth factor receptor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; HDF, human diploid fibroblast; Erk, extracellular signal-regulated kinase; siRNA, small interfering RNA; BrdUrd, bromodeoxyuridine; AS-ON, antisense oligonucleotide; S-ON, sense oligonucleotide; CDK, cyclin-dependent kinase; EGF, epidermal growth factor; DAPI, 4,6-diamidino-2-phenylindole.

EXPERIMENTAL PROCEDURES

Reagents—Human diploid fibroblasts were isolated from newborn foreskin (48). Monoclonal anti-caveolin-1 antibody (C43420), monoclonal anti-caveolin-2 antibody (C57820), and monoclonal anti-caveolin-3 antibody (C38320) were purchased from Transduction Laboratories. Polyclonal anti-EGFR antibody (sc-03), polyclonal anti-phospho-Erk antibody (sc-7383), polyclonal anti-Erk-1/2 antibody (sc-94), anti-p53 antibody (sc-126), and anti-p21 antibody (sc-6246) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were purchased from Jackson Immunochemicals. Other biochemical reagents were purchased from Sigma and Invitrogen.

Cell Culture—Human diploid fibroblasts were kept in 10-cm plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics and subculture at a ratio of 1:4. We defined young cells as those resulting from <25 population doublings, and old cells were from >60 population doublings. Cellular senescence of all of the old cells was confirmed by their delayed population doubling times and by a senescence-associated β-galactosidase activity assay as described by Dimri *et al.* (26). After being grown in a semi-confluent state, senescence-associated β-galactosidase, pH 6.0, activity was examined. Cells were washed with phosphate-buffered saline and fixed with 2% paraformaldehyde containing 0.2% glutaraldehyde in phosphate-buffered saline for 5 min at room temperature. After washing with phosphate-buffered saline, cells were incubated with β-galactosidase reagent (1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), 40 mM citric acid/sodium phosphate buffer, pH 6.0, 5 mM potassium ferrocyanide/potassium ferricyanide, 150 mM NaCl, 2 mM $MgCl_2$) at 37 °C.

Down-regulation of Caveolin-1 by Antisense Oligonucleotide and siRNA—HDF cells were seeded at a density of 1×10^6 cells/100-mm dish. Twenty-four hours later, 100 nM each of antisense caveolin-1 oligonucleotide (TTTGCCCCCAGACAT, complementary to the 15-base initiation sequence of human caveolin-1) was transfected into HDF cells using LipofectAMINE PLUS (Invitrogen). Three hours later, the medium was discarded and replaced with complete Dulbecco's modified Eagle's medium. A sense caveolin-1 oligonucleotide (ATGTCTGGGGGCAAA) corresponding to 15-bases of the initiation sequence of caveolin-1 was used as a control (27). For monitoring purposes, we conjugated fluorescein isothiocyanate in the N-terminal of oligonucleotides.

Double-stranded siRNA molecules were designed to target the human caveolin-1 mRNA. 21-nucleotide siRNAs were obtained from Dharmacon Research (Lafayette, CO) as duplexed 2′-unprotected, desalted, and purified siRNA. We targeted 5 regions of caveolin-1 mRNA. The target sequence of C1 is AACACCTCAACGATGACGTGG; that of C2 is AACCAGAAGGGACACACAGTT; that of C3 is AATACTGGTTTTACCGCTTGC; that of C4 is AACAGGGCAACATCTACAAGC; and that of C5 is AACAUCUACAAGCCCAACAAC. A GL2 luciferase oligonucleotide was used as a negative control. Senescent HDF cells were grown to 40% confluence in 10-cm dishes and transfected with annealed siRNA using OligofectAMINE (Invitrogen) for 6 h as recommended by the manufacturer. Dulbecco's modified Eagle's medium containing 30% fetal bovine serum and 3% penicillin-streptomycin was added to each dish to a final concentration of 10% fetal bovine serum and 1% penicillin-streptomycin. Cells were harvested 48 h later for further assays.

Western Blot and Erk Activation Analysis—Whole cell extracts were separated using SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filters (Protran, Schleicher & Schuell) using standard techniques. The following antibodies were used: mouse monoclonal anti-caveolin-1 (Transduction Laboratories), mouse monoclonal anti-p-Erk (Santa Cruz Biotechnology), rabbit polyclonal anti-Erk (Santa Cruz Biotechnology), rabbit polyclonal anti-p-Elk (Cell Signaling Technology), rabbit polyclonal anti-Elk (Cell Signaling Technology), rabbit polyclonal anti-EGFR (Santa Cruz Biotechnology), goat polyclonal anti-actin (Santa Cruz Biotechnology), mouse monoclonal anti-p53 (Santa Cruz Biotechnology), and mouse monoclonal anti-p21 (Oncogene). The immune complexes were incubated with a peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody and finally visualized using an enhanced chemiluminescence detection kit (ECL kit, Amersham Biotechnology).

Electron Microscopic Analysis—Sense and antisense oligonucleotide-treated senescent HDFs were pelleted and fixed with 3% glutaraldehyde/phosphate-buffered saline at pH 7.4. After washing with 0.2 M sodium cacodylate buffer, pH 7.4, the cell pellets were treated with 1% osmium tetroxide in cacodylate buffer for 1 h. Cells were then dehydrated in graded ethanol through propylene oxide and embedded in epoxy resin (Polyscience Co.). Ultrathin sections were cut and stained with uranyl acetate and lead citrate. Sections were observed using a transmission electron microscope (H-600, Hitachi).

Subcellular Fractionation—To prepare nuclear and cytoplasmic extracts, $1–2 \times 10^8$ cells were harvested by trypsin, washed three times with calcium-deficient phosphate-buffered saline, and resuspended to 2.5×10^8 cells/ml in a buffer containing 10 mM Tris, pH 7.4, 2 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitors. Resuspended cells were incubated at room temperature for 1 min and then transferred into a tube in ice for 5 min with Triton X-100 added to a final concentration of 0.5% and incubated on ice for 5 min. Cell lysates were separated by several passages through a 260-gauge needle and examined under a phase-contrast microscope. The nuclei were isolated from the cytosol by centrifugation at $300 \times g$ at 4 °C for 10 min in a Sorvall RC-5B centrifuge using a SS-34 rotor. Isolated nuclei were washed with 10 ml of buffer and resuspended in a nuclear extraction buffer containing 20 mM of Hepes, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 25% (v/v) glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, and protease inhibitors. Resuspended nuclei were incubated on ice for 30 min with occasional shaking to extract the nuclear proteins and finally were spun down in a microcentrifuge for 5 min.

Immunocytochemistry and Quantification—Each of the transfected senescent HDFs was cultured on coverslips in 24-well plates and treated with EGF and BrdUrd for 24 h. Cultures were processed for immunocytochemistry as described previously (23). The following antibodies were used: BrdUrd (1:100, mouse; Roche Applied Science), p-Elk (1:200, rabbit; Cell Signaling Technology, Inc), caveolin-1 (1:500, rabbit; Transduction Laboratories), phalloidin (1:1000; Molecular Probes), and DAPI (1:1000; Sigma). Detection was performed using a Bio-Rad confocal system at a magnification of ×40 with individual filter sets for each channel, and images were assembled using Adobe PhotoShop.

Fluorescent-labeled cells were visualized by confocal microscopy. Positive cells were quantified in at least 10 fields systematically across the coverslips from three independent experiments conducted in parallel. Statistical analysis was performed using Microsoft Excel.

RESULTS

Down-regulation of Caveolin Expression—Senescent HDF cells showed high level of caveolin expression and decreased response upon EGF stimulation. The overexpression of caveolin-1 level in young HDF cells had been found to reduce Erk phosphorylation (21). Therefore, we reasoned that the signal attenuating effect of caveolin in senescent cells could be primarily responsible for their hyporesponsiveness to EGF stimulation and that the modulation of caveolin-1 level in these cells could modify Erk signaling effects.

To modulate the intracellular caveolin level of senescent cells, an antisense oligonucleotide (AS-ON) complementary to the 15-base initiation sequence of human caveolin-1 was prepared, whereas a sense oligonucleotide (S-ON) corresponding to the 15 bases of the initiation sequence was used as a control (27). To monitor oligonucleotides efficiency, we used conjugated fluorescent oligonucleotides.

We found that the expression of caveolin-1 was abnormally high (Fig. 1*A*) and that was primarily localized to the membrane domain of senescent HDF cells. To identify the effect of antisense oligonucleotide, we examined the dose responsiveness by immunocytochemistry and Western blotting (Fig. 1*B*). Sense and antisense oligonucleotides could effectively penetrate senescent HDF cells in the presence of LipofectAMINE reagent (Fig 1*B*). Interestingly, the antisense oligonucleotide of caveolin-1 was localized to the nucleus and could inhibit caveolin-1 expression level in senescent HDFs, whereas the sense oligonucleotide was localized mainly to the cytosol and had no effect. The expression of caveolin-1 was dose-dependently reduced by antisense oligonucleotide in senescent cells (Fig 1*B*). Therefore, the expressions of caveolin-1 were found to significantly decrease by AS-ON in senescent HDFs.

Caveolin-1, a key constituent of caveolae structures, forms caveolae structures in senescent HDFs. Electron microscopic experiments upon the plasmalemmal profiles of senescent (S-

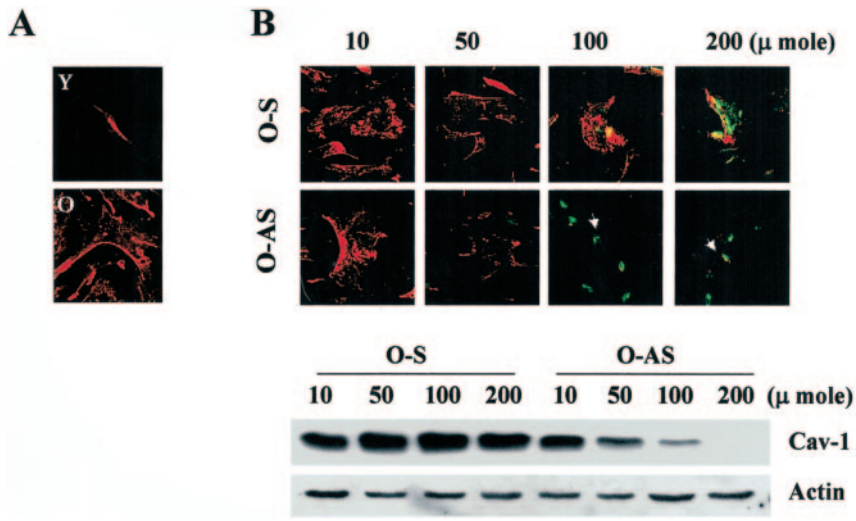

FIG. 1. **Down-regulation of caveolin-1 in senescent HDFs.** Senescent HDFs were transfected with 100 μmol of oligonucleotides by using LipofectAMINE Plus reagent. *A*, the basal expression of caveolin-1 in young (*Y*) and senescent HDFs. Each cell was stained with polyclonal anti-caveolin-1 antibody and rhodamine-conjugated secondary antibody. *O*, old. *B*, the expression level of caveolin was determined with immunocytochemistry (*upper panel*) and Western blotting (*lower panel*) after dose-dependent sense and antisense oligonucleotide transfection. For immunocytochemistry, polyclonal anti-caveolin-1 antibody was used, whereas for Western blotting, monoclonal anti-caveolin-1 antibody was used. The *arrow* indicates reduction of caveolin-1 expression in the membrane. *Red*, caveolin-1; *green*, oligonucleotides. *C*, total cell lysates from each of the cells were analyzed by Western blotting using monoclonal anti-caveoin-1 antibody and polyclonal anti-actin antibody. *Cav*, caveolin.

ON-transfected cells) HDFs revealed the presence of numerous non-clathrin-coated vesicular structures 50–100 nm in diameter that were juxtaposed to the plasma membrane and morphologically indistinguishable from caveolae (Fig. 2*A*). In contrast, profiles of AS-ON-transfected cells contained significantly fewer caveolae-like structures. Quantitative analysis of multiple plasmalemmal profile revealed that AS-ON-treated senescent HDF cells contained over five times less caveolae-like structure than S-ON-treated senescent HDF cells (Fig. 2*B*). From these results, the antisense oligonucleotide to caveolin-1 was found to be active not only in terms of caveolin reduction but also in terms of the inhibition of caveolae structures in senescent HDFs.

To confirm the specificity of AS-ON effect, we designed five siRNA for caveolin-1 and tested their efficiency. As shown in Fig. 4*A*, C2 and C5 of siRNA for caveolin-1 significantly reduced the level of caveolin-1 in senescent HDFs compared with the control. For further experiment, we used C2 siRNA and we observed that siRNA effect could maintain until 72 h (Fig. 4*B*).

Recovery of Downstream Signal Transduction Cascade in Senescent HDF Cells—Despite the elevated p-Erk level in cytosol, the translocation of p-Erk and the phosphorylation of Elk failed upon EGF stimulation in senescent HDFs (24, 25). However, the expression levels of EGFR and Erk were not significantly different in young and senescent HDFs (Fig. 3*A*). Despite the high basal level of p-Erk, no mitogenic response was observed upon EGF stimulation in senescent HDFs (Fig. 3*B*). As a consequence of adjusting the caveolin-1 levels in senescent cells, basal p-Erk was decreased by AS-ON treatment but not by S-ON treatment (Fig. 3*C*). We further tested the functional recovery of the Erk system in response to EGF stimulation by modulating the caveolin-1 level in senescent HDFs. No significant changes in the level of Erk phosphorylation by EGF stimulation in S-ON-treated senescent HDFs were observed, whereas Erk activation significantly resumed in AS-ON-treated senescent HDFs, and this was similar to that observed in young cells (Fig. 3*D*). To confirm this recovery of Erk activation by down-regulation of caveolin-1 using AS-ON, we used siRNA for this experiment. No significant changes in the level of Erk phosphorylation by EGF stimulation in control-transfected senescent HDFs were observed, whereas Erk activation

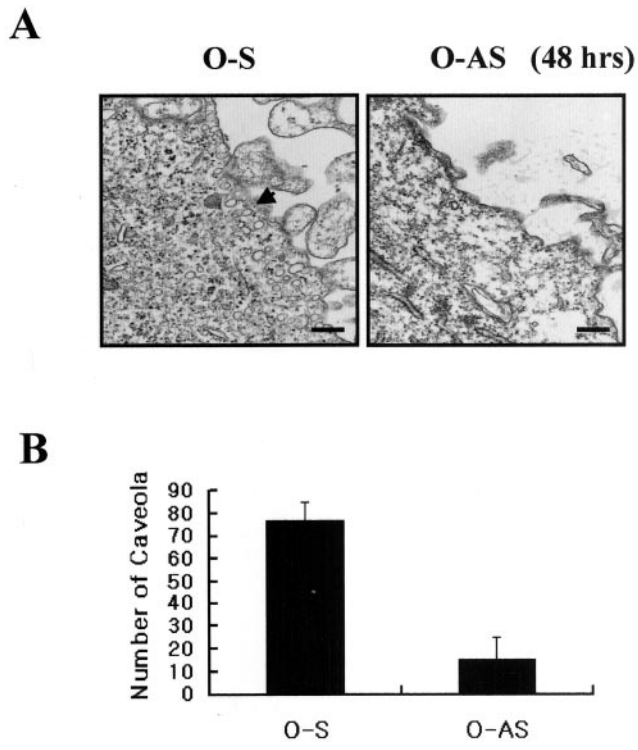

FIG. 2. **Ultrastructural analysis of caveolae structures in senescent HDFs.** *A*, electron microscopic analysis of senescent HDFs after antisense oligonucleotides treatment. The *arrow* indicates caveolae structures. Bar = 100 nm. *B*, numeric counts of caveolae-like vesicles were statistically analyzed in 10 independent cells (O-S denotes that sense-oligonucleotide for caveolin-1 was treated, whereas O-AS denotes that antisense-oligonucleotide for caveolin-1 was treatment).

was resumed in C2-transfected senescent HDFs (Fig. 4*C*). Therefore, these data show that the recovery of EGF signaling in senescent HDF cells was due to reduction of the caveolin-1 expression level by siRNA as well as antisense oligonucleotide.

Activated Erk translocates into the nucleus and subsequently activates several target transcriptional factors, including members of the transcription factor family (25–28). It is

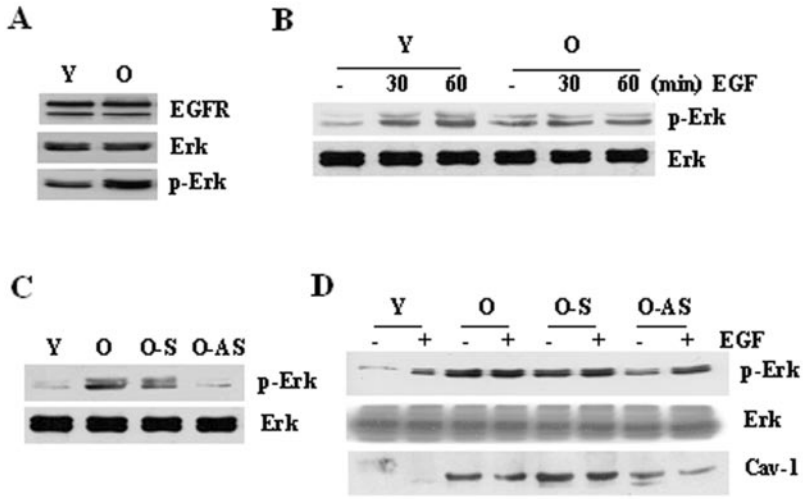

FIG. 3. **Restoration of Erk activation by down-regulation of caveolin-1 in senescent HDFs.** *A*, total cell lysates from young and senescent cells were analyzed by Western blot using polyclonal anti-EGFR antibody, monoclonal anti-phospho-Erk antibody, and polyclonal anti-Erk antibody, respectively. *B*, young and senescent HDFs were starved of fetal bovine serum for >24 h and then treated with EGF at 100 ng/ml for the indicated times. Each of the samples was analyzed using anti-phospho-Erk antibody and anti-Erk antibody. *C*, young, senescent, and transfected HDF cells were prepared, and similar amount of protein in the total cell lysates were analyzed by Western blotting using anti-phospho-Erk antibody and anti-Erk antibody. *D*, young, senescent, and transfected senescent HDFs were starved of fetal bovine serum and treated with EGF at 100 ng/ml for 30 min and subjected for Western blotting with anti-phoshpo-Erk antibody, anti-Erk antibody, and anti-caveolin-1 antibody, respectively.

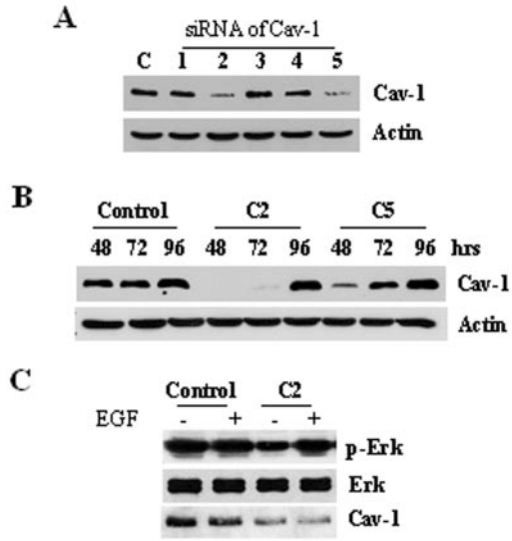

FIG. 4. **The Erk activation was recovered by down-regulation of caveolin-1 using siRNA in senescent HDF cells.** Senescent cells were transfected with 0.5 nmol of five different siRNAs of caveolin-1 (*Cav-1*) and control siRNA of GL2 luciferase using by OligofectAMINE. *A*, after 48 h, the proteins were harvested and analyzed by Western blotting. *B*, the transfected cells were harvested with time dependently after C2 and C5 siRNA treatment and were determined with mouse monoclonal anti-caveolin-1 antibody and goat polyclonal anti-actin antibody, respectively *C*, after transfection of C2 siRNA for 48 h, the cells were treated with EGF (100 ng/ml) and harvested within 10 min. The results were analyzed by Western blotting with rabbit polyclonal anti-phospho-Erk antibody and rabbit polyclonal anti-Erk antibody.

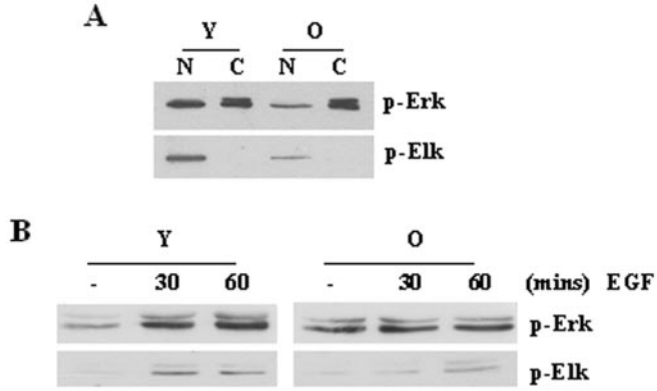

FIG. 5. **The phosphorylation of Erk and Elk upon EGF stimulation in young and senescent HDF cells.** *A*, nuclear and cytosol fractions were isolated using Triton X-100 from young and senescent HDFs. Each of the fractions was analyzed by Western blot using monoclonal anti-phospho-Erk antibody and polyclonal anti-phospho-Elk antibody. *B*, young and senescent HDFs were starved of fetal bovine serum and treated with EGF at 100 ng/ml for the indicated times and subjected for Western blotting using polyclonal anti-phospho-Erk and polyclonal anti-phospho-Elk antibodies. *N*, nucleus; *C*, cytosol; *Y*, young cells; and *O*, senescent cells.

known that the abundance of serine-phosphorylated Elk is reduced in senescent cells (25). Therefore, in our experiment, we checked the mode of Elk activation in response to EGF in caveolin-reduced senescent HDF cells and determined the basal levels of p-Erk and p-Elk by subcellular fractionation in young and senescent HDFs. Despite the high level of p-Erk, Elk was very weakly phosphorylated in nuclei, suggesting that it would be inactive in senescent cells (Fig. 5*A*). To confirm this result, we determined the phosphorylation status of Erk and Elk in young and senescent HDFs and found that EGF stimulation strongly phosphorylated Elk within 30 min in young HDFs but weakly in senescent HDFs (Fig. 5*B*). We also examined Elk phosphorylation in senescent HDFs upon EGF simulation after S-ON or AS-ON treatment (Fig. 6*A*). Interestingly, the treatment of senescent HDFs with AS-ON restored EGF-induced Elk phosphorylation, although partially and slowly, it reached maximum activity only after 60 min. Moreover, immunocytochemical analysis showed that Elk was phosphorylated only in young and AS-ON-treated senescent HDFs and very weakly in senescent and S-ON-treated cells (Fig. 6*B*).

Modulation of Cell Cycle Regulatory System—The restoration of EGF-induced signal transduction by adjusting caveolin status in senescent cells led us to question the impact of recovery on cell cycle, because replicative senescence implied irreversible growth arrest despite growth factor stimuli. Therefore, we examined the cell cycle-associated molecules. Certain members of the cyclin-dependent kinase (CDK)/cyclin family, which

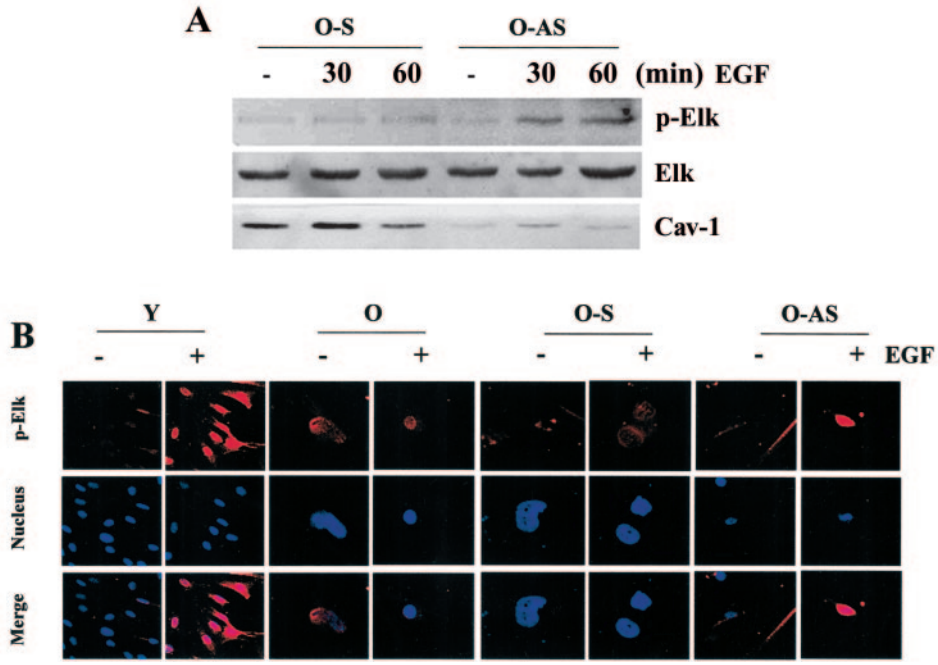

FIG. 6. **Recovery of Elk activation in the nucleus of senescent HDFs.** Young, senescent, and transfected HDFs were starved of fetal bovine serum and treated with EGF at 100 ng/ml for 30 and 60 min. *A*, cells were treated with EGF at 100 ng/ml for indicated time and analyzed by Western blotting using polyclonal anti-phospho-Elk, polyclonal anti-Elk antibody, and anti-caveolin-1 (*Cav-1*) antibody, respectively. *B*, for staining, cells were cultured on coverslips and stained with polyclonal anti-phospho-Elk and DAPI after EGF treatment. *C*, numeric counts of phospho-Elk-positive cells.

are responsible for cell cycle progress, are expressed but are inactive in the senescent state (29). This lack of CDK activity and arrest in the G_1 phase of the cell cycle is attributed to the induction of the G_1-S CDK/cyclin inhibitors p21 (WAF1) (30) and p16 (INK4a) (31, 33–35). Therefore, we determined the levels of p53 and p21 in AS-ON- and S-ON-treated senescent HDFs and found that the levels of p53 and p21 are significantly attenuated in AS-ON-treated senescent HDFs (Fig. 7*A*). To monitor the G_1-S transition, we examined changes in the BrdUrd incorporation rate in response to EGF stimulation. Since the BrdUrd index is a measure of the DNA synthesis rate, it can be used to monitor S phase activity. As a results, almost 60% AS-ON-transfected senescent HDFs showed DNA synthesis in contrast to the negligible activity shown by S-ON-transfected HDFs as monitored using anti-BrdUrd antibody (Fig. 7, *B* and *C*).

DISCUSSION

Senescent fibroblasts are generally resistant not only to extrinsic noxious stimuli (36, 37) but also to growth factor stimulation (38–40). Previously, we reported that the hyporesponsiveness of senescent fibroblasts to EGF stimulation might be related to the up-regulation of caveolins with aging (21). Moreover, the expression of the caveolin family has been confirmed *in vivo* to increase in the tissues of aged rat such as in the brain, heart, spleen, and liver. These results suggest that caveolin might play a major role in the aging process *in vivo* (21). In addition, the high level of caveolins in the brain tissues of old animals might provide a clue to the susceptibility of elderly people to Alzheimer's disease (41, 42).

In this study, we set out to show that the down-regulation of caveolin-1 expression could recover the attenuated Erk signaling and the consequent cell cycling of senescent HDFs. Accordingly, we reduced the expression of caveolin-1 (Fig. 1) and the formation of caveolae structures (Fig. 2) successfully using the antisense oligonucleotide of caveolin-1 in senescent HDFs. We found that the down-regulation of caveolin-1 in senescent HDFs could recover the signaling from EGF to Erk-1/2 in the cytosol with respect to Erk phosphorylation to a considerable degree (Fig. 3*D*).

Over the last few years, RNA interference has been recognized as a major mechanism of posttranscriptional gene silencing in various systems (43, 44). To verify the effect of AS-ON, we used siRNA of 21 nucleotides for down-regulation of caveolin-1 in senescent HDFs. As seen in Fig. 4, the effect of recovery of Erk activation by siRNA of caveolin-1 was correlated well with the effect of AS-ON. Our results suggest that the down-regulation of caveolin-1 by AS-ON as well as siRNA can lead to the recovery of signaling in senescent HDFs.

Senescent HDF cells and Ha-Ras mutant-expressed HDFs did not respond to EGF stimulation in the MAPK activation system (20, 22), and the accumulated cytosolic p-Erk-1/2 failed to translocate to nuclei on EGF stimulation (23, 24). Our data also confirmed that the translocation of p-Erk was diminished in senescent HDFs, regardless of the elevated level of p-Erk in the cytosol (Fig. 5*A*). Interestingly, we found that the down-regulation of caveolin-1 by antisense oligonucleotide and siRNA treatment reduced the basal p-Erk level (Figs. 3*C* and 4*C*) and resumed Erk activation upon EGF stimulation (Figs. 3*D* and 4*C*), which resulted in Elk phosphorylation in senescent HDFs (Fig. 6). These results suggest that a simple reduction of the caveolin level in senescent HDF cells can induce the restoration of the Erk signaling system upon EGF stimulation, not only in terms of its phosphorylation but also its translocation into and activation of transcriptional factors in the nuclei.

Recently, it was reported that caveolin-1-mediated cell cycle arrest occurs through a p53/p21-dependent pathway (35). p53 is directly involved in G_1 phase arrest through the induction of p21 (42). p21 mediates p53-dependent G_1 arrest by inhibiting the activity of CDKs, which phosphorylate the retinoblastoma gene product as well as other substrates. p21 also induces growth arrest by preventing proliferating cell nuclear antigen from activating DNA polymerase σ, which is essential for DNA

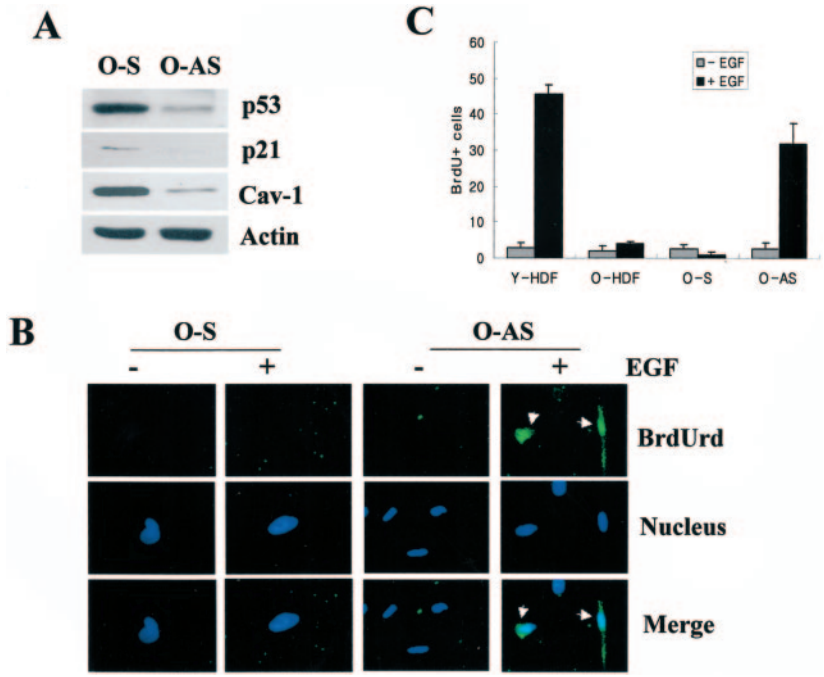

FIG. 7. **Decrease of p53 and p21 and re-initiation of DNA synthesis in senescent HDFs.** *A*, total cell lysates from transfected cells were analyzed by Western blotting using monoclonal anti-p53 antibody, monoclonal anti-p21 antibody, monoclonal anti-caveolin-1 (*Cav-1*) antibody, and polyclonal anti-actin (*Actin*) antibody. *B*, senescent HDFs were cultured on coverslips and transfected with sense and antisense oligonucleotides. After 48 h, each sample was treated with BrdUrd and EGF at 100 ng/ml for 24 h and stained with monoclonal anti-BrdUrd antibody and DAPI. *C*, numeric counts of BrdUrd-positive cells.

replication (45). Moreover, p21-deficient fibroblasts show impairment of G_1 arrest (46), confirming a key role for p21 in cell cycle arrest. Our experiment showed that the down-regulation of caveolin-1 in senescent HDF also reduced the level of the cell cycle inhibitors p53 and p21 (Fig. 7*A*). The reduction of p53 and p21 by antisense oligonucleotide to caveolin-1 gene in senescent HDF cells indicated the strong possibility of deregulation of the senescence-associated cell cycle inhibition. Therefore, the cell cycle reentry of the senescent cells was examined by monitoring BrdUrd incorporation in response to EGF stimuli after reduction of caveolin status. Because BrdUrd index is the measure of DNA synthesis rate, it indicates the S phase activity. Actually, in our experiment, almost 60% antisense-oligonucleotide-treated senescent HDF cells showed the BrdUrd incorporation in contrast to the negligible activity in the control senescent HDF cells (Fig. 7, *B* and *C*). These data strongly suggest that the cell cycle reentry into S phase can be induced by the reduction of caveolin status in the senescent cells.

These results indicate that a simple adjustment of the caveolin level in senescent cells can profoundly influence the aging phenotype. Moreover, evidence of the role of caveolin in cancer cells have been well documented (47), wherein caveolin was suggested to be a tumor suppressor gene. Therefore, it is apparent that caveolin plays an important role in the regulation of both aging and cancer.

Taken together, we suggest that the growth factor responsiveness of senescent cells can be resumed to a considerable degree by reducing caveolin levels. Therefore, we cannot exclude the possibility that the accepted nature of the senescent phenotype, *i.e.* irreversible growth arrest and irresponsiveness to growth factors, may be at the point of redefinition. These results imply that the functional recovery of senescent cells can be probably achieved, which may extendable to the functional restoration of aged organisms.

REFERENCES

1. Severs, N. J. (1988) *J. Cell Sci.* **90,** 341–348
2. Anderson, R. G. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 10909–10913
3. Engelman, J. A., Chu, C., Lin, A., Jo, H., Ikezu, T., Okamoto, T., Kohtz, D. S., and Lisanti, M. P. (1998) *FEBS Lett.* **428,** 205–211
4. Lisanti, M. P., Scherer, P. E., Vidugiriene, J., Tang, Z., Hermanowski-Vosatka, A., Tu, Y. H., Cook, R. F., and Sargiacomo, M. (1994) *J. Cell Biol.* **126,** 111–126
5. Scherer, P. E., Okamoto, T., Chun, M., Nishimoto, I., Lodish, H. F., and Lisanti, M. P. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 131–135
6. Scheiffele, P., Verkade, P., Fra, A. M., Virta, H., Simons, K., and Ikonen, E. (1998) *J. Cell Biol.* **140,** 795–806
7. Li, S., Galbiati, F., Volonté, D., Sargiacomo, M., Engelman, J. A., Das, K., Scherer, P. E., and Lisanti, M. P. (1998) *FEBS Lett.* **434,** 127–134
8. Tang, Z., Scherer, P. E., Okamoto, T., Song, K., Chu, C., Kohtz, D. S., Nishimoto, I., Lodish, H. F., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271,** 2255–2261
9. Lipardi, C., Mora, R., Colomer, V., Paladino, S., Nitsch, L., Rodriguez-Boulan, E., and Zurzolo, C. (1998) *J. Cell Biol.* **140,** 617–626
10. Li, S., Couet, J., and Lisanti, M. P. (1996) *J. Biol. Chem.* **271,** 29182–29291
11. Ko, Y.-G., Lee, J.-S., Kang, Y.-S., Ahn, J.-H., and Seo, J.-S. (1999) *J. Immunol.* **162,** 7217–7223
12. Ko, Y.-G., Liu, P., Pathak, R. K., Craig, L. C., and Anderson, R. G. (1998) *J. Cell. Biochem.* **71,** 524–535
13. Garcia-Cardena, G., Martasek, P., Masters, B. S., Skidd, P. M., Couet, J., Li, S., Lisanti, M. P., and Sessa, W. C. (1997) *J. Biol. Chem.* **272,** 25437–25440
14. Couet, J., Sargiacomo, M., and Lisanti, M. P. (1997) *J. Biol. Chem.* **272,** 30429–30438
15. Okamoto, T., Schlegel, A., Scherer, P. E., and Lisanti, M. P. (1998) *J. Biol. Chem.* **273,** 5419–5422
16. Galbiati, F., Volonte, D., Engelman, J. A., Watanabe, G., Burk, R., Pestell, R. G., and Lisanti, M. P. (1998) *EMBO J.* **17,** 6633–6648
17. Chang, B. D., Xuan, Y., Broude, E. V., Zhu, H., Schott, B., Fang, J., and Roninson, I. B. (1999) *Oncogene* **26,** 4808–4818
18. Kagawa, S., Fujiwara, T., Kadowaki, Y., Fukazawa, T., Sok-Joo, R., Roth, J. A., and Tanaka, N. (1999) *Cell Death Differ.* **6,** 765–772
19. Ohkusu-Tsukada, K., Tsukada, T., and Isobe, K. (1999) *J. Immunol.* **15,** 1966–1972
20. Liu, Y., Guyton, K. Z., Gorospe, M., Xu, Q., Kokkonen, G. C., Mock, Y. D., Roth, G. S., and Holbrook, N. J. (1996) *J. Biol. Chem.* **16,** 3604–3607
21. Park, W. Y., Park, J. S., Cho, K. A., Kim, D. I., Ko, Y. G., Seo, J. S., and Park, S. C. (2000) *J. Biol. Chem.* **275,** 20847–20852
22. Ishigami, A., Reed, T. D., and Roth, G. S. (1993) *Biochem. Biophys. Res. Commun.* **196,** 181–186
23. Lim, I. K., Won, H. K., Kwak, I. H., Yoon, G., and Park, S. C. (2000) *Mech. Ageing Dev.* **119,** 113–130
24. Lim, I. K., Hong, K. W., Kwak, I. H., Yoon, G., and Park, S. C. (2001) *Ann. N. Y. Acad. Sci.* **928,** 176–181
25. Tresin, I. M., Lorenzini, A., Frisoni, L., Allen, R. G., and Cristofalo, V. J. (2001) *Exp. Cell Res.* **269,** 287–300
26. Roa, V. N., Huebner, K., Maria, S. M., Sobieszczuk, P., and Wasylyk, B. (1994) *Genes Dev.* **8,** 1502–1513
27. Gille, R., Sharrocks, A. D., and Shaw, P. E. (1992) *Nature* **358,** 414–417
28. Hill, C. S., Marais, R., John, S., Wynne, J., Dalton, S., and Treisman, R. (1993) *Cell* **73,** 395–406
29. Dulic, V., Drullinger, L. F., Lees, E., Reed, S. I., and Stein, G. H. (1993) *Proc. Natl. Acad. Sci. U. S. A.*, **90,** 11034–11038
30. Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M., and Smith, J. R. (1994) *Exp. Cell Res.* **211,** 90–98
31. Alcorta, D. A., Xiong, Y., Phelps, D., Hannon, G., Beach, D., and Barrett, J. C. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13742–13747
32. Hara, E., Smith, R., Parry, D., Tahara, H., Stone, S., and Peters, G. (1996) *Mol. Cell. Biol.* **16,** 859–867
33. Brown, J. P., Wei, W., and Sedivy. J. M. (1997) *Science* **277,** 831–834
34. Gire, V., and Wynford-Thomas, D. (1998) *Mol. Cell. Biol.* **18,** 1611–1621
35. Galbiati, F., Volonte, D., Liu, J., Capozza, F., Frank, P. G., Zhu, L., Pestell, R. G., and Lisanti, M. P. (2001) *Mol. Biol. Cell* **12,** 2229–2244

36. Wang, J., and Walsh, K. (1996) *Science* **273,** 359–361
37. Sargiacomo, M., Sudol, M., Tang, Z., and Lisanti, M. P. (1993) *J. Cell Biol.* **122,** 789–807
38. Matsuda, T., Okamura, K., Sato, Y., Morimoto, A., Ono, M., Kohno, K., and Kuwano, M. (1992) *J. Cell. Physiol.* **150,** 510–516
39. Seshadri, T., and Campisi, J. (1990) *Science* **247,** 205–209
40. Riabowol, K., Schiff, J., and Gilman, M. Z. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 157–161
41. Ikezu, T., Trapp, B. D., Song, K. S., Schlegel, A., Lisanti, M. P., and Okamoto, T. (1998) *J. Biol. Chem.* **273,** 10485–10495
42. El-Deiry, W. S. (1998) *Curr. Top. Microbiol. Immunol.* **227,** 121–137
43. Xia, H., Mao, Q., Paulson, H. L., and Davidson, B. L. (2002) *Nat. Biotechnol.* **20,** 1006–1010
44. Paul, C. P., Good, P. D., Winer, I., and Engelke, D. R. (2002) *Nat. Biotechnol.* **20,** 505–508
45. Waga, S., Hannon, G. J., Beach, D., and Stillman, B. (1994) *Nature* **369,** 574–578
46. Deng, C., Zhang, P., Harper, J. W., Elledge, S. J., and Leder, P. (1995) *Cell* **82,** 675–684
47. Razani, B., Schlegel, A., Liu, J., and Lisanti, M. P. (2001) *Biochem. Soc. Trans.* **29,** 494–499
48. Park, S. C., Yeo, E.-J., Han, J.-A., Hwang, Y.-C., Choi, J.-Y., Park, J. S., Park, Y.-H., Kim, K.-O., Kim, I.-G., Seong, S.-C., and Kwak, S.-J. (1999) *J. Gerontol.* **54,** B78–B83