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The Role of Caveolin-1 in Senescence and Ototoxicity of Differentiated Cochlear Hair Cell Line (UB/OC-1)

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Abstract

Caveolin may be a molecular target for modulation of aging process in cochlear hair cells and have association with oxotoxicity. First we investigated the basal expression of caveolin-1, caveolin-2, caveolin-3, nitric oxide synthase, and superoxide dismutase in UB/OC-1 cochlear hair cell line. By using a RNA interference technique, we investigated whether down-regulation of caveolin influenced telomerase activity and reactive oxygen species (ROS) production in cochlear hair cells. In addition, cisplatin and gentamycin, known ototoxic drugs, were administered to the cochlear cells to determine their impact on caveolin expression. Further attempts at elucidating cellular aging mechanism with caveolin and ototoxic drugs were carried out. The main discoveries were the presence of caveolin-1 in UB/OC-1 cells and that down-regulation of caveolin-1 reduced protein kinase A activity. Telomerase was activated by caveolin down-regulation and caveolin down-regulation inhibited oxidative stress at the mitochondrial level. When cisplatin and gentamycin were administered to the cochlear hair cells during a caveolin expression state, a decrease in telomerase activity and increase ROS activity was observed. Caveolin-1 may modulate the senescent mechanisms in cochlear cells. An increase in caveolin-1 levels can lead to ROS production in the mitochondria which may cause ototoxicity.

Keywords: Caveolin, Gentamycin, Ototoxicity, Protein Kinase A

Although many theories and hypotheses have been presented to explain aging processes, specific mechanisms underlying the aging phenomenon remain largely undetermined. Production of free radicals and the loss of antioxidant protection mechanism are thought to contribute to the aging phenomenon. These studies have been investigated by several research groups. According to some studies, free oxygen radicals are not always harmful and may actually be essential to maintaining life[1]. Given these results, many researchers have started to investigate other possible molecular targets that could explain the aging process[1]. Caveolin is a protein which is believed to play a role in the aging process. When caveolin accumulates in cells, interactions between cells decrease. If the amount of caveolin decreases in the cell, not only do interactions between the cells normalize, but the structures of the cells also recovered. These findings have been confirmed by several studies[2].

Caveolae are defined as membrane-invaginated vesicles which have a size of 50-100 nm in diameter and were first discovered by the electron microscope[3,4]. However, caveolae do not only exist as vesicular forms linked to plasma membranes but also as rosette, grape-like clusters, and tubular structures (Figure 1).

Caveolin is one of the structural protein components of caveolae, and it has a critical role in vesicle formation and insertion of caveolae, and thus intracellular vesicular transport[4]. Caveolae/caveolin are known to regulate various signaling systems. The caveolae/caveolin act as signal transduction molecules and have coupling mechanisms which bind signal molecules to transduction proteins[4]. For example, caveolae interacts with signaling proteins such as H-ras, G-proteins, Src family tyrosine kinase, Neu, and endothelial NOS (eNOS). Caveolin, the structural protein of caveolae, suppresses signaling by binding to another signaling protein called motif[5]. Because of these functional characteristics, the roles of caveolae/caveolin in the aging process[1,2], tumors[6,7], age-related diseases[3], and nervous-system disorders[8] have been investigated.

Aminoglycosides are the most efficient and cost-effective antibiotics that target gram negative bacteria and are popularly used world-wide[9]. However, this

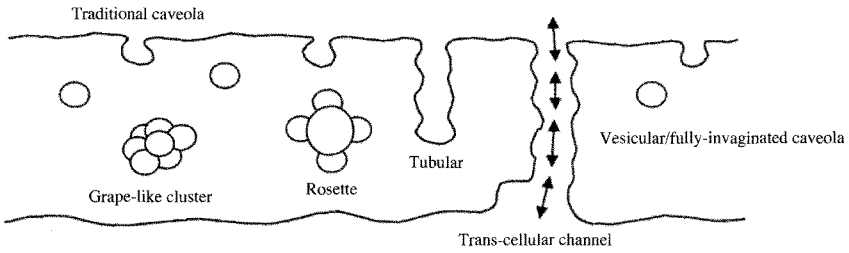

Figure 1. Caveolae can exist in many forms besides the traditional invaginated membrane form. They can be found in vesicular forms or in aggregates of grape-like clusters, rosettes, or even elongated tubules.

family of drugs can have the adverse side effect of severe sensory neural hearing loss and balancing disorders[10]. The mechanism responsible for these side effects has not yet been discovered. However, the creation of reactive oxygen species[11], over stimulation of N-methyl D-aspartate (NMDA) receptors[12], and the mitochondrial dysfunction[13] are thought to play important roles in the ototoxicity that induces hearing impairment and vestibular dysfunctions.

Cisplatin, a platinum agent, is the first anticancer drug that was used in clinical trials, and it was recognized as an effective anti-cancer drug in 1971[9]. Although it is known to have no adverse effects on vestibular cells[14], it has the same ototoxicity against cochlear hair cell as aminoglycosides[9]. The ototoxicity of cisplatin is known to be induced by oxidative injury[15,16]. Like aminoglycosides, the toxicity advances from outer hair cell to inner hair cell, resulting in cellular apoptosis which in turn causes hearing loss[10,16].

The aim of this research is to evaluate intracellular changes associated with the aging phenomenon by suppressing caveolin in UB/OC-1, a cochlear sensory cell line. Furthermore, we investigate the association of caveolin with gentamycin and cisplatin ototoxicity.

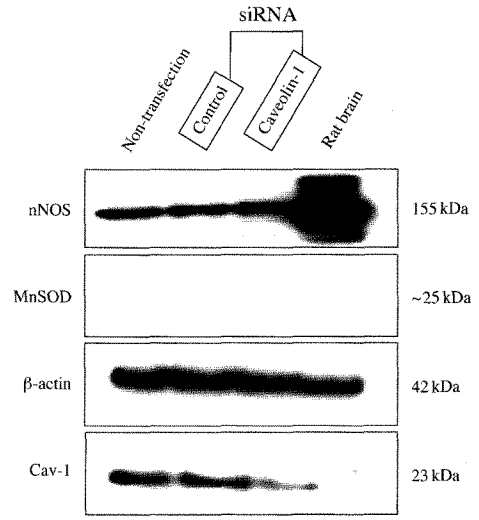

Figure 2. Basal expression of nNOS, MnSOD, and caveolin-1 in the UB/OC-1 hair cell line. Caveolin-1 was selectively down-regulated by siRNA. Down-regulation of caveolin-1 did not affect the expression of nNOS or MnSOD as assessed by western blot.

Differentiation of UB/OC-1 Cells

The UB/OC-1 cells cultured at 33°C, 95% CO_2 for 2 days appeared spindle-shaped. The cells cultured at 39°C at atmosphere with 95% CO_2 for 24 hours were flat. These results correlate with those published by Dr. Matthew Holley, proving that incubation of UB/UE-1 cells at 39°C results in differentiation into acoustic sensory cells. Therefore, for the purpose of this study, we consider the UB/OC-1 cells grown at 39°C in a 95% CO_2 incubator for 24 hours to be vestibular sensory cells.

Expression of Caveolin-1 in UB/OC-1 Cells

Among the three types of caveolin (-1,-2,-3), only basal expression of caveolin-1 was observed in UB/OC-1 cells by western blot. Among the three types of nitric oxide synthases (NOS; iNOS, eNOS, nNOS), only basal expression of neuronal NOS (nNOS) was detected and among the three types of superoxide dismutases (SOD; MnSOD, CuZnSOD, ECSOD), only basal expression of manganese SOD (MnSOD) was seen. There were no changes in expression of NOS and SOD after caveolin-1 siRNA administration (Figure 2).

Effect of Caveolin-1 on the Activity of PKA

The activity of PKA was measured in three cells groups: caveolin-1 processed with siRNA, caveolin-1 processed with control siRNA, and the non-transfec-

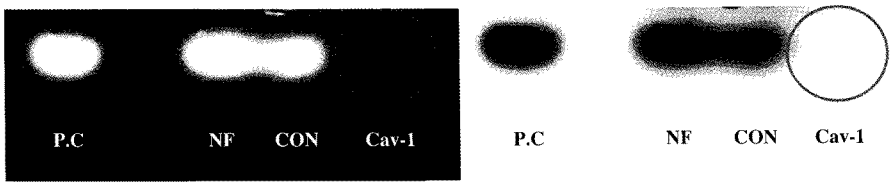

Figure 3. PKA activity was reduced by caveolin-1 siRNA-mediated down-regulation.

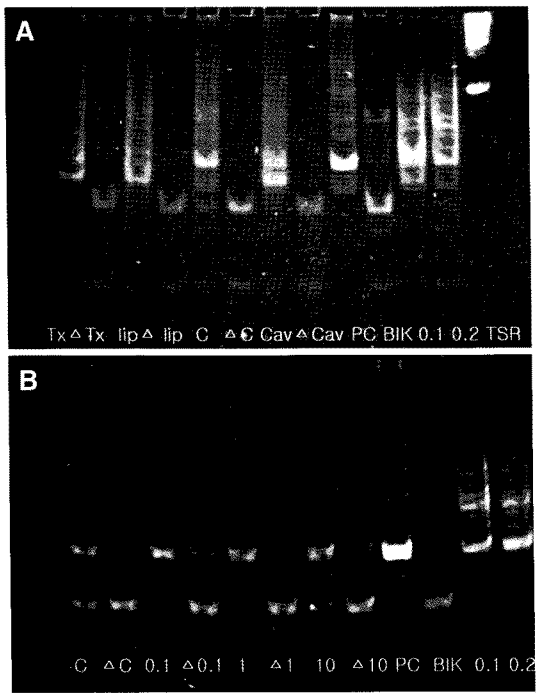

Figure 4. (A) Telomerase activity in relation to caveolin-1 status. Down-regulation of caveolin-1 increased telomerase activity. (B) Telomerase activity with PKA inhibitor H-89. An increase of PKA inhibitor (H-89) increased telomerase activity.

tion group. The two control groups, the control siRNA processed group and the non-transfection group, showed no change in PKA activity. However, the caveolin-1 siRNA group showed a significant decrease in PKA activity (Figure 3).

Effect of Caveolin-1 on Cell Aging

Telomerase activity was measured to determine the effect of caveolin-1 on the cell aging process. Telomerase activity was measured in the non-transfection group, the lipofectamine-only treated group, the control siRNA group, and the caveolin-1 siRNA group. Telomerase activity was increased only in the siRNA processed group (Figure 4A). For comparison, the PKA inhibitor H89 was administered and telomerase activity was measured. Telomerase activity increased in a dose-dependent manner relative to the amount of H89 administered (Figure 4B).

ROS Tracing in Mitochondria

The binding of DHR123 and H_2O_2 results in a florescent green emission and binding of MitoTracker to mitochondria produces an orange fluorescence. DHR123 and MitoTracker were added to the caveolin-1 siRNA group and control siRNA group, and were observed using a LSM510 confocal microscope. In the study group, DHR123 florescence decreased inside the mitochondria (Figure 5).

Effects of Caveolin-1 on Ototoxicity

The caveolin-1 siRNA group and control siRNA group were treated with gentamycin (aminoglycoside; Figure 6A) and cisplatin (Figure 6B). When we compared the two groups, the caveolin-1 siRNA group showed significantly lower DHR123 florescence localized within the mitochondria.

Discussion

Our study showed that caveolin-1 developed spontaneously in the UB/OC-1 cell line, which indicates that caveolae and caveolin protein may play an important role in signal transduction in the inner ear. When caveolin-1 was down-regulated, oxidative-relative substances such as NOS and SOD did not show any expressional changes. However, PKA activity was significantly decreased.

Telomeres are located at each end of the eukaryotic chromosome, which is a specialized chromatin structure, preventing DNA breaks. When cells age, the length of telomere shortens and eventually it disappears. Then, at the end of chromatin, DNA breaks occur and the cell dies[17]. Telomerase is a cellular reverse transcriptase that controls the length of telomere. When

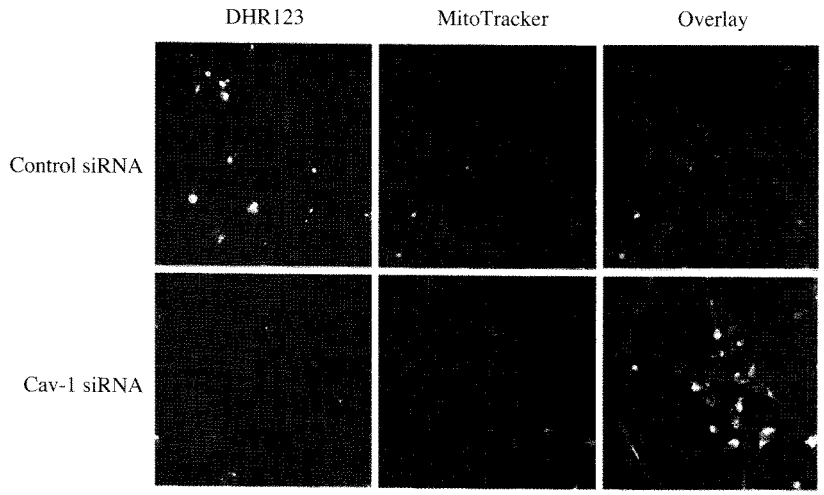

Figure 5. Decreased DHR-123 activity was observed by caveolin-1 siRNA-mediated down-regulation. This phenomenon was observed within mitochondria in UB/OC-1 cells.

the activity of telomerase increases, the telomere is repaired and this maintenance prevents the cell from undergoing apoptosis. In our study, caveolin-1 was selectively down-regulated, causing an increase of intracellular telomerase activity. We then tested with H89, a known PKA inhibitor, and achieved similar results. When the ototoxic drugs gentamycin and cisplatin were administered, the ROS produced by oxidative stress increased when caveolin-1 was not down-regulated and this was clearly seen by confocal microscopy (Figure 7). This phenomenon occurred within mitochondria as observed by confocal microscopy. Our results suggest that up-regulation of caveolin by ototoxic drugs results in oxidative damage to the inner ear cells, thereby inducing sensory neural hearing loss.

There are two main hypotheses to explain the mechanism of aminoglycosides. The first theory, that production of nitric oxide (NO) and an increase of iNOS leads to production of reactive oxidants which have important roles in ototoxicity, was proven in guinea pig studies[18-20]. The second theory, that the cellular stress mediated NMDA receptor is also an important factor for ototoxicity, was proven by DNA analysis of gentamycin-exposed organs of Corti. Cisplatin is not ototoxic to vestibular cells, unlike the aminoglycosides[14]. However, it does cause cochlear hair cell damage, similar to the aminoglycosides. Furthermore, cisplatin damages marginal cells in the stria vascularis earlier before it damages the organ of Corti[9]. The ototoxicity of cisplatin is known to be caused by oxidative injury[15,16]. The damage begins from outer hair cells and progresses to inner hair cells, resulting in eventual acoustic damage[10,16]. Our results indicate that there is a relationship between ototoxicity and caveolin-1. The ototoxic drugs gentamycin and cisplatin do not directly control caveolin-1 expression. We suggest that they change the activity of PKA which controls the caveolin-1 levels indirectly, thereby causing ototoxicity. Furthermore, our study indicates that an increase of caveolin-1 leads to ROS production within mitochondria, which may also result in ototoxicity.

There are few studies that have examined aging in cochlear cells. Our study revealed the possibility that caveolin-1 may modulate the aging process in cochlear cells. Caveolin-1 activates telomerase and regulates the formation of ROS through the PKA pathway in cochlear-neuronal cells. The effect of the ototoxic drugs gentamycin and cisplatin on the inner ear may be due to altered activity of PKA. An increase in caveolin-1 levels can lead to ROS production in the mitochondria which may cause ototoxicity.

Materials & Methods

Cell Culture and Differentiation of UB/OC-1 Cells

The UB/OC-1 cell line used in this study is an immortal cell line derived from the cochlear outer hair cell of 2-day old mice (kindly provided by Dr. Matthew C. Holley, University of Bristol, Bristol, United Kingdom). UB/OC-1 cells were cultured in 100 mm cell culture dishes (tissue culture dish, TPP Trasadingen, Switzerland), using Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 100 U/mL penicillin G. Cells were incubated at 33°C in a 5% CO_2 incubator for 48 hours. The Dulbecco's modified Eagle's medium (DMEM), phosphate buffered

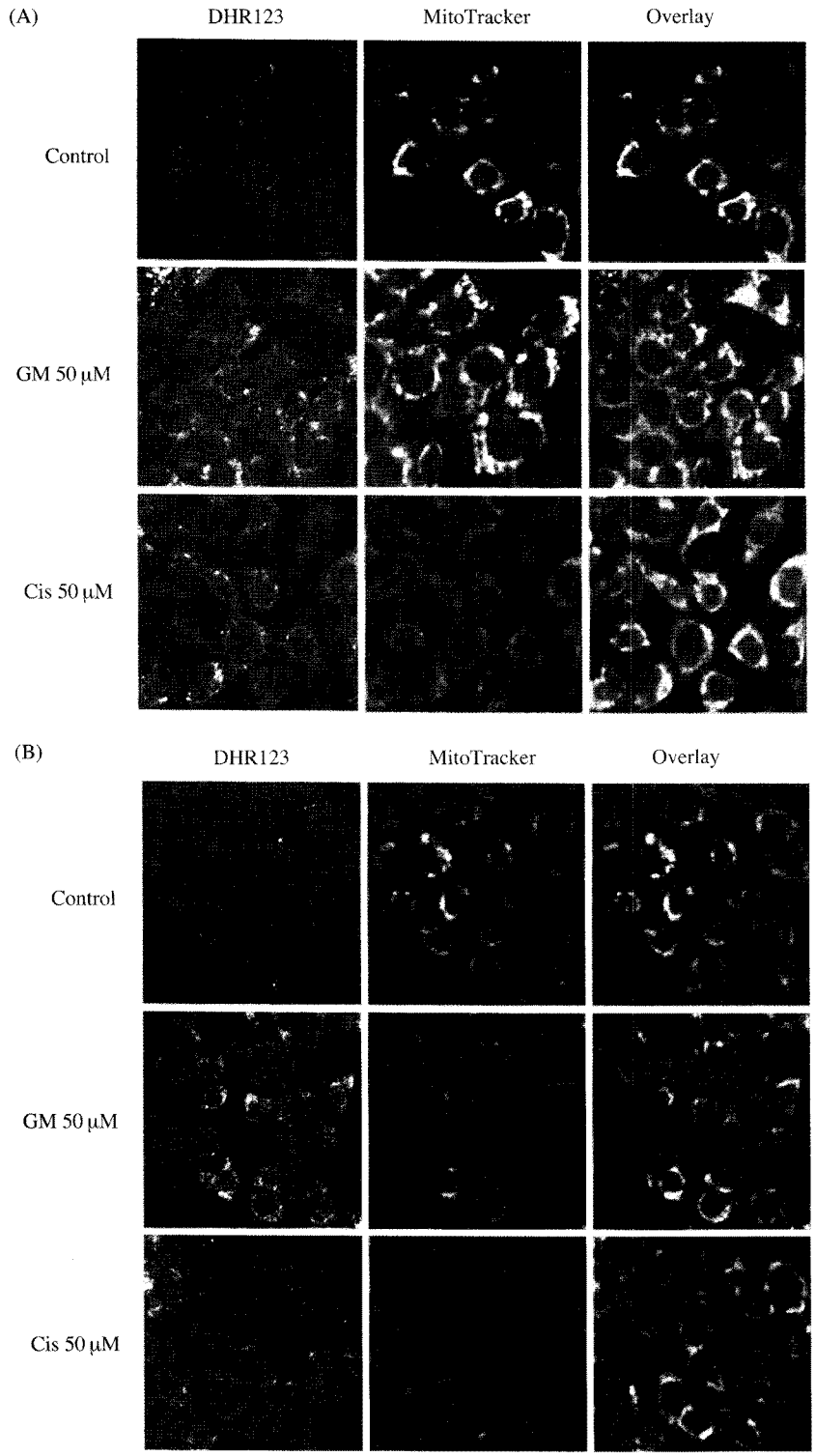

Figure 6. (A) Administration of ototoxic drugs (gentamycin 50 μM, cisplatin 50 μM) revealed an increase of DH123 activity in the control siRNA group. (B) Administration of ototoxic drugs (gentamycin 50 μM, cisplatin 50 μM) revealed an increase of DH123 activity in caveolin siRNA group.

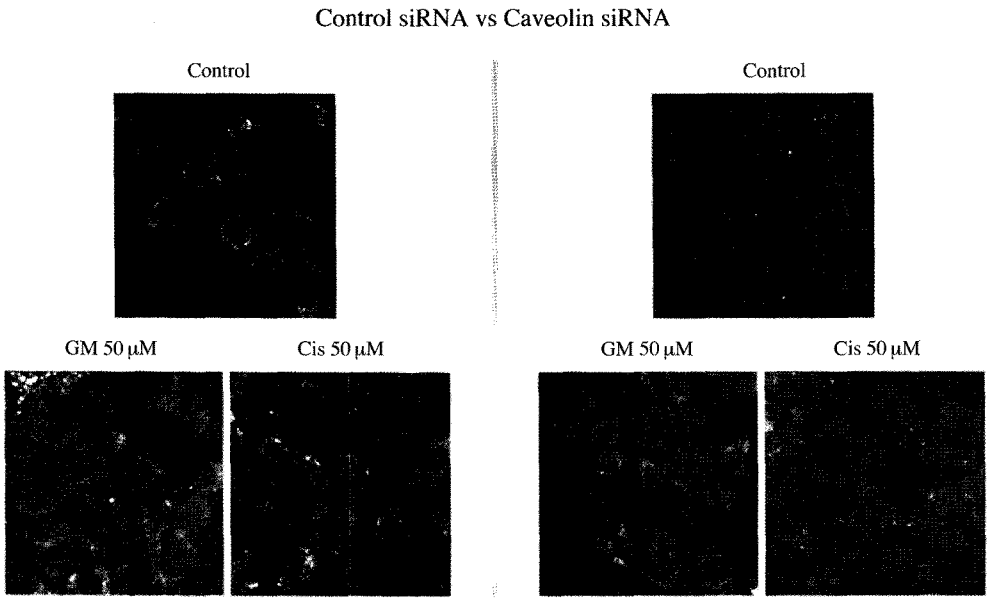

Figure 7. Caveolin-1 siRNA-mediated down-regulation of caveolin-1 may reduce oxidative stress resulting from ototoxic substances.

saline (PBS), and fetal bovine serum (FBS) were bought from Gibco BRL (Gland Island, NY, USA). The penicillin G was purchased from Sigma (Sigma Diagnostics, St. Louis, MO, USA). The cells were cultivated at 39°C for 24 hours in 5% CO_2 incubator. These cells differentiate into hearing sensory cells, and differentiation was confirmed by examination under a light microscope.

Caveolin-1 Down-regulation by siRNA

In this study, we used siRNA to knockdown the expression of caveolin-1. Caveolin-1 and control siRNA were obtained from Santa Cruz® Biotechnology (CA, USA). The cells were stabilized in TPP for 24 hours. Caveolin-1 or control siRNA were introduced to the cells using Lipofectamine 2000 (Invitrogen®, Carlsbad, CA, USA).

After 4-6 hours, the transfection solution was removed and exchanged with fresh media and stabilized for 18 to 24 hours in a 5% CO_2 incubator. After the stabilized cells were collected, we treated the cells with solubilized gentamycin and cisplatin in DMEM without FBS for 18 to 24 hours.

Western Blot Analysis

To determine caveolin-1 protein expression levels in UB/OC-1 cells, western blot analysis was performed. For western blot analysis, the treated cell group was dissolved in lysis buffer (0.3 M sucrose, 200 mM HEPES, 1 mM EDTA, 1 mM DTT, 10 mg/mL leupeptin, 2 μg/mL trypsin inhibitor, 2 mg/mL aprotinin, 1 mM PMSF and 1 g/mL pepstatin) and lysed by sonicator. For quantitative protein analysis, a BCA Protein assay kit (Pierce, Rockford, IL) was used. Protein extracts were mixed with sample buffer (62.5 mM Tris-HCl, pH 6.8, 5% 2-mercaptoethanol, 10% glycerol, 2% sodium dodecyl sulfate (SDS), 0.005% bromophenol blue) and heated at 100°C for 10 minutes. Proteins were then separated by electrophoresis at 80 V for 2 hours in 10% SDS polyacrylamide gels. The proteins were then transferred to a PVDF membrane (Millipore, Bedford, MA) at 30 V and incubated in a 5% non-fat milk containing TBS-T buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween 20) for 1 hour at room temperature to block nonspecific binding.

After blocking, the membrane was washed three times for 10 minutes with the TBS-T buffer, and polyclonal caveolin-1 antibody (Santa Cruz), polyclonal manganese superoxide dismutase (MnSOD, Stressgen), monoclonal neuronal nitric oxide synthase antibody (nNOS, BD Transduction), and monoclonal actin antibody (Sigma) were administered for 1 to 2 hours at room temperature. The membrane was washed three times for 10 minutes with TBS-T buffer, and combin-

ed with horseradish peroxidase-conjugated rabbit-IgG antibody (Pierce, 1 : 5,000 dilution) or horseradish peroxidase-conjugated mouse-IgG antibody (Pierce, 1 : 5,000 dilution) for 1 hour at room temperature. After that, one more washing with an enhanced chemiluminescence (ECL) kit (American, Piscataway, NJ) was used and membranes were then exposed to X-ray film in a dark room.

Measurement of PKA Activity

We measured PKA activity in a non-transfection, control siRNA group, and caveolin-1 siRNA group. We used a cAMP-dependent protein kinase assay system and the reaction took place in 30°C water bath in PKA dilution buffer (PepTag® PKA 5× reaction buffer 5 μL, 0.4 μg/μL peptide 5 μL, PKA 5× activator solution 5 μL) with 5 g protein for 30 minutes. We checked PKA activity by electrophoresis on a 0.8% agarose gel (50 mM Tris-HCl, pH 8.0 solution) after heating the protein at 95°C for 10 minutes.

Measurement of Telomerase Activity

Telomerase activity was monitored using the TRAPEZE detection kit (Chemicon®, USA). Telomerase activity was measured in the non-transfection group, the lipofectamine-treated only group, the control siRNA group, and the caveolin-1 siRNA group, with a variable concentration of H-89 (PKA inhibitor) administered. Cells were incubated in 1x CHAPS buffer with 100 units/mL of RNase inhibitor (Takara), then transferred to ice for 30 minutes. Next, they were purified at 4°C for 10 minutes and pure proteins were collected. Protein (1.5 μg) was mixed with the buffer in the kit, and the solution was incubated at 30°C for 30 minutes. PCR was done in 30-33 cycles of 94°C/30 sec, 59°C/30 sec, 72°C/1 min after processing. The amplification of telomerase was measured by electrophoresis at 60-80 V in a 12.5% non-denaturing PAGE gel.

Ototoxic Drug Administration to the UB-OC-1 Cell Line

A 50 μM concentration of cisplatin (Sigma Diagnostics, St. Louis, MO, USA) and μM gentamycin (Sigma Diagnostics, St. Louis, MO, USA) were administered to the caveolin-1 siRNA group and non targeting siRNA control group. Cells were incubated with these drugs for 24 hours and used in subsequent experiments.

Measurement of Reactive Oxygen Species (ROS)

Differentiated cells were placed on cover glasses and apoptosis was induced by the addition of 1 M dihydrorhodamine (DHR123; Molecular Probes, Eugene, OR, U.S.A.) and 125 nM mitoTracker (Molecular Probes, Eugene, OR, U.S.A.) for 30 min at 37°C and 5% CO_2. After cells were washed with PBS, they were reactivated with 4% paraformaldehyde for 15 min. The cells were then washed with PBS containing 0.02 M glycine, and then with PBS containing 0.1% Triton X. After removing the PBS, cells were mixed with mounting medium (gelvatol), then covered and fixed on slide glasses. Prepared samples were observed using a LSM510 confocal microscope (Carl Zeiss, Tornwood, NJ, U.S.A.). A band pass filter of 505/530 nm was used for DHR123, and a 560 nm long pass filter was used for mitoTracker red. The magnification was 40 x and MetaMorph software (Universal Imaging, Westchester, PA, U.S.A.) was used for analysis.

Acknowledgements

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