

Enhancement of UVB radiation-mediated apoptosis by knockdown of cytosolic NADP⁺-dependent isocitrate dehydrogenase in HaCaT cells

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Ultraviolet B (UVB) radiation induces the production of reactive oxygen species (ROS) that promote apoptotic cell death. We showed that cytosolic NADP⁺-dependent isocitrate dehydrogenase (IDPc) plays an essential role in the control of cellular redox balance and defense against oxidative damage, by supplying NADPH for antioxidant systems. In this study, we demonstrated that knockdown of IDPc expression by RNA interference enhances UVB-induced apoptosis of immortalized human HaCaT keratinocytes. This effect manifested as DNA fragmentation, changes in cellular redox status, mitochondrial dysfunction, and modulation of apoptotic marker expression. Based on our findings, we suggest that attenuation of IDPc expression may protect skin from UVB-mediated damage, by inducing the apoptosis of UV-damaged cells. [BMB Reports 2014; 47(4): 209-214]

INTRODUCTION

The ultraviolet (UV) spectrum is divided into UVC (200-280 nm), UVB (280-320 nm), and UVA (320-400 nm). UVB and UVA are of environmental significance, because UVC is filtered by the ozone layer (1). UV radiation damages skin, and results in the formation of "initiated" cells. These cells may ultimately form tumors. The initiated cells generally divide much faster than normal cells, and are transformed into cancerous cells via clonal expansion and apoptosis evasion (2). In this regard, modalities that could eliminate initiated cells may reduce the risk of cancer development. UV irradiation, especially with light in the UVB wavelength range, triggers multiple cellular targets, leading to programmed cell death (PCD) through the generation of reactive oxygen species (ROS), such as singlet

oxygen, superoxide radicals, hydroxyl radicals, and hydrogen peroxide (3, 4).

ROS rapidly react with many biological macromolecules, including nucleic acids, proteins, and lipids, and induce nucleotide damage, single and double-stranded DNA breaks, DNA-protein cross-linking, lipid peroxidation, protein degradation, protein oxidation, and mitochondria damage (5, 6). However, the sensitivity of a cell to oxidative stress depends on its antioxidant system (7). To neutralize ROS, living cells have acquired various defense systems, including ones involving enzymatic antioxidants. Superoxide dismutase (SOD) removes O₂^{•-} by catalyzing dismutation, in which one O₂^{•-} is reduced to H₂O₂ and O₂. H₂O₂ is subsequently converted into H₂O and O₂, either by glutathione peroxidase (Gpx), or catalase located in peroxisomes (8). Since the reaction catalyzed by Gpx requires reduced glutathione (GSH) as a substrate, and partially depends on the ratio of oxidized glutathione (GSSG) to GSH, the concentrations of the reactants, and their ratio that is a reflection of the cellular redox state, are important to ROS detoxification (9). NADPH is an essential reducing agent for GSH regeneration by glutathione reductase (GR) and the NADPH-dependent thioredoxin system (10, 11), which are both important for protecting cells from oxidative damage. Therefore, NADP⁺-dependent isocitrate dehydrogenases (ICDHs) as NADPH-generating enzymes may serve as antioxidants, in the presence of oxidative stress. We previously reported that cytosolic ICDH (IDPc) is involved in providing NADPH needed for GSH production, which helps prevent oxidative damage (12).

In the present study, we evaluated the effects of IDPc knockdown on UVB-mediated apoptosis, in spontaneously immortalized human HaCaT keratinocytes. We used HaCaT cells for our study, because UVB radiation is incapable of penetrating far into the skin, and only affects the epidermis, the superficial layer of the skin that is composed predominantly of keratinocytes (4). Our data suggested that attenuated expression of IDPc with small interfering RNA (siRNA) may protect skin from UVB-mediated damage, by inducing the apoptosis of UV-damaged cells.

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RESULTS AND DISCUSSION

To determine whether IDPc knockdown modulates UVB-induced apoptosis, HaCaT cells were transiently transfected with siRNA targeting IDPc mRNA. Significant attenuation of IDPc mRNA levels measured by RT-PCR (Fig. 1A) and IDPc protein expression measured by Western blotting (Fig. 1B) were observed in the IDPc siRNA-transfected cells, compared to control cells transfected with scrambled siRNA. IDPc siRNA transfection reduced IDPc activity about 80% in HaCaT cells, compared to the control. Because IDPc is susceptible to inactivation by ROS and reactive nitrogen species (RNS) (13), ROS generated by UVB irradiation may induce inactivation of IDPc. Exposure of cultured human HaCaT keratinocytes to UVB radiation resulted in a dose-dependent loss of IDPc activity (Fig. 1C), indicating that transfection with IDPc siRNA exacerbates knockdown of enzyme activity, upon exposure to UVB. When HaCaT cells were irradiated with 20 mJ/cm² of UVB, a time-dependent decrease in cell viability was observed. However, HaCaT cells transfected with IDPc siRNA were more sensitive, than the control cells transfected with

scrambled siRNA (Fig. 1D). Since cellular antioxidants act in a concerted manner, we determined whether the knockdown of IDPc expression concomitantly altered the activities of other antioxidant enzymes. Down-regulation of IDPc expression did not induce compensatory changes in the activities of other antioxidant enzymes, such as SOD, catalase, or GR (Fig. 1E).

UVB-induced apoptosis is a protective mechanism, since this process eliminates cells containing damaged DNA (2). In this regard, enhancement of UVB-induced cell death may reduce the carcinogenic risk. To determine whether reductions of HaCaT cell viability following exposure to UVB is mediated by apoptosis, we performed flow cytometry with propidium iodide staining, to monitor total cellular DNA contents. Fig. 2A shows a typical cell cycle plot of HaCaT cells that were exposed to 20 mJ/cm² of UVB. The number of apoptotic cells was estimated by calculating the number of subdiploid cells, based on the cycle histogram. We found that a significant number of HaCaT cells treated with UVB for 24 h underwent apoptosis. The number of UVB-treated apoptotic cell was markedly increased among the IDPc siRNA-transfected HaCaT cells, compared to the control cells. Apoptosis of HaCaT cells exposed to UVB

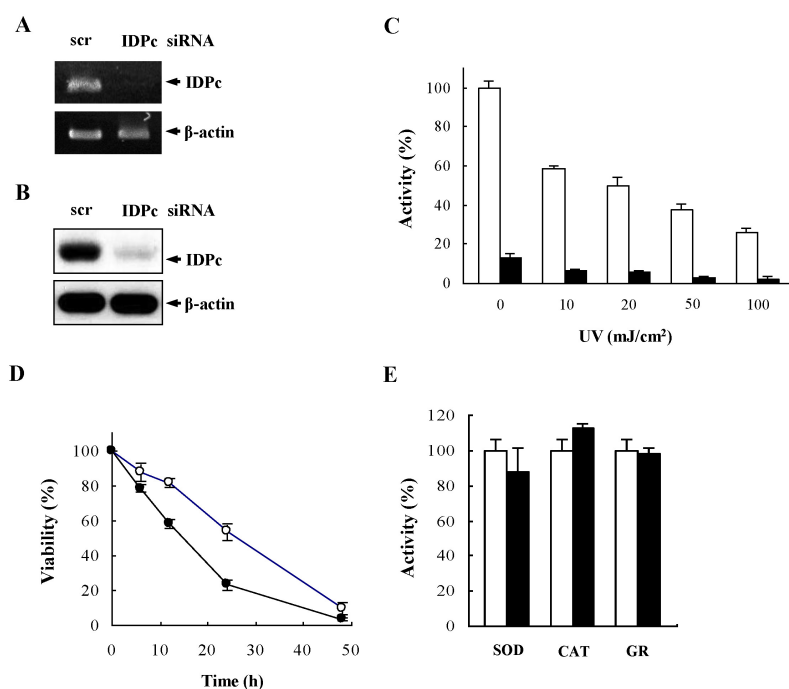


Fig. 1. Knockdown of IDPc expression by siRNA in HaCaT cells. The cells were transfected with scrambled siRNA (Scr), or IDPc-specific siRNA. After 48 h, the transfected cells were disrupted by sonication, and (A) IDPc mRNA levels, (B) protein expression, and (C) activity were evaluated. β -actin served as a control. Open and solid bars represent HaCaT cells transfected with scrambled siRNA or IDPc siRNA, respectively. Data are presented as the mean \pm standard deviation (S.D.) of three separate experiments. (D) Viability of transfected HaCaT cells exposed to UVB. After IDPc siRNA- (closed circles) or control scrambled siRNA-transfected (open circles) cells were exposed to 20 mJ/cm² of UVB for various lengths of time, cell viability was measured with an MTT assay. Data are presented as the mean \pm S.D. of three separate experiments. (E) Activity of antioxidant enzymes in the transfected HaCaT cells. Activity found in the control cells is expressed as 100%. Data are presented as the mean \pm S.D. of three separate experiments. SOD, superoxide dismutase; CAT, catalase; GR, glutathione reductase.

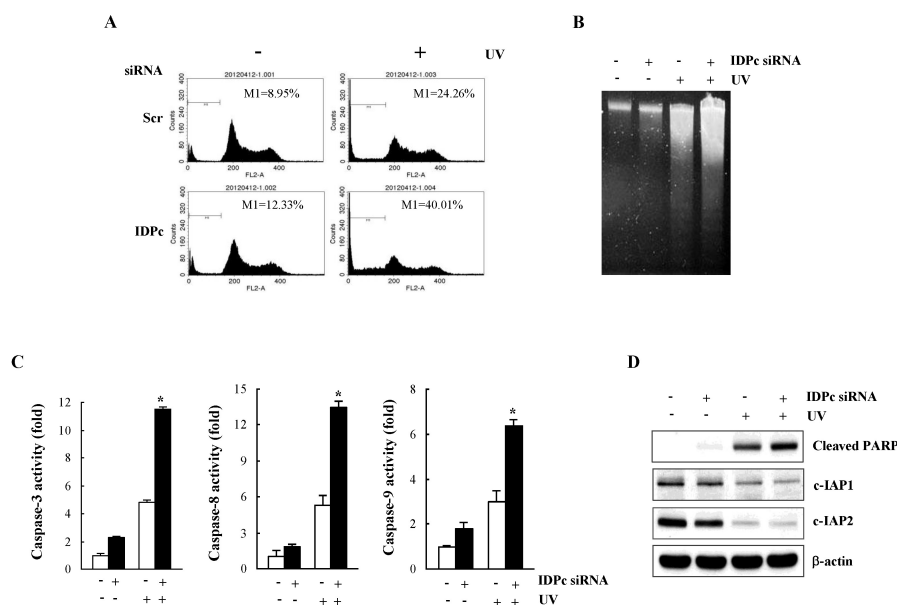


Fig. 2. UVB-induced apoptosis of HaCaT cells transfected with IDPc siRNA. (A) Cell cycle analysis and evaluation of cellular DNA contents by flow cytometry. The sub-G1 region (present as 'M1') includes cells undergoing apoptosis. The number in each panel refers to the percentage of apoptotic cells. (B) Agarose gel electrophoresis of nuclear DNA fragments from transfected HaCaT cells exposed to 20 mJ/cm² of UVB. (C) Activation of caspases in transfected HaCaT cells exposed to 20 mJ/cm² of UVB. Cells were lysed and centrifuged. The supernatant was combined with the caspase substrate, and subjected to a caspase colorimetric activity. Protease activity of the caspases was calculated, by measuring the absorbance at 405 nm. Data are presented as the mean ± S.D. of three separate experiments. *P < 0.01, versus the scrambled siRNA-transfected cells exposed to UVB. (D) Immunoblot analysis of apoptosis-related proteins, in transfected HaCaT cells exposed to 20 mJ/cm² of UVB. Cell extracts were subjected to 10-12.5% SDS-PAGE and immunoblotting with antibodies, against cleaved PARP, c-IAP1, and c-IAP2. β-actin was used as an internal control.

was also monitored, by measuring DNA fragmentation with agarose gel electrophoresis. As shown in Fig. 2B, DNA fragmentation was more significant in IDPc siRNA-transfected cells, compared to the control cells, after exposure to UVB. We next evaluated changes in the expression of apoptotic marker proteins, as a result of UVB exposure. Caspase-3, -8, and -9 are pivotal factors in the apoptotic pathways. Both caspase-8, and -9 can activate caspase-3, through proteolytic cleavage. Caspase-3 may then cleave vital cellular proteins, or activate additional caspases via proteolytic cleavage (14, 15). Caspase-3, -8, and -9 activation in HaCaT cells was assessed with a caspase colorimetric assay. Activity of all these enzymes was markedly increased in IDPc siRNA-transfected HaCaT cells, compared to the control cells, after exposure to UVB (Fig. 2C). The formation of fragments that represents proteolytic cleavage of PARP indicates initiation of the apoptotic process. The level of cleaved PARP was increased in IDPc siRNA-transfected cells, compared to the control cells, after UVB exposure (Fig. 2D). Many members of the inhibitors of apoptosis (IAP) family block proteolytic activation of caspase-3 and -7 (16). Our results showed that c-IAP-1 and c-IAP-2 expression was decreased in IDPc siRNA-transfected cells treated with UVB (Fig. 2D). Taken together, these data indicate that IDPc knockdown may help promote UV radiation-induced apoptosis.

Recent studies on UV-induced apoptosis have shown that ROS formation increases the number of apoptotic cells (17). It has been shown that UVB induces the production of superoxide anion radicals (O₂⁻) and hydrogen peroxide (H₂O₂) that can be subsequently converted into highly reactive hydroxyl radicals (·OH), via the Fenton and Haber-Weiss reactions (8). To determine whether the difference in apoptosis levels observed between the control and IDPc siRNA-transfected cells after exposure to UVB was associated with ROS formation, the concentrations of intracellular peroxide in the HaCaT cells were measured by flow cytometry, using the oxidant-sensitive probe dihydrorhodamine 123, 2',7'-dichlorofluorescein diacetate (DCFH-DA). As shown in Fig. 3A, increased DCF fluorescence was observed in HaCaT cells exposed to UVB. In addition, the level of O₂⁻ production in the cells transfected with IDPc siRNA, and exposed to UVB radiation, was evaluated by fluorescence microscopy, using dihydroethidium (DHE) as a fluorescent probe (Fig. 3B). GSH is one of the most abundant intracellular antioxidants, and measuring changes in the concentration of this factor is an alternative method for monitoring cellular oxidative stress (18). Cellular GSH levels in HaCaT cells exposed to UVB were evaluated by fluorescence microscopy with the GSH-sensitive fluorescent dye 5-chloromethyl-fluorescein diacetate (CMF-DA). As shown in Fig. 3C, CMF-DA

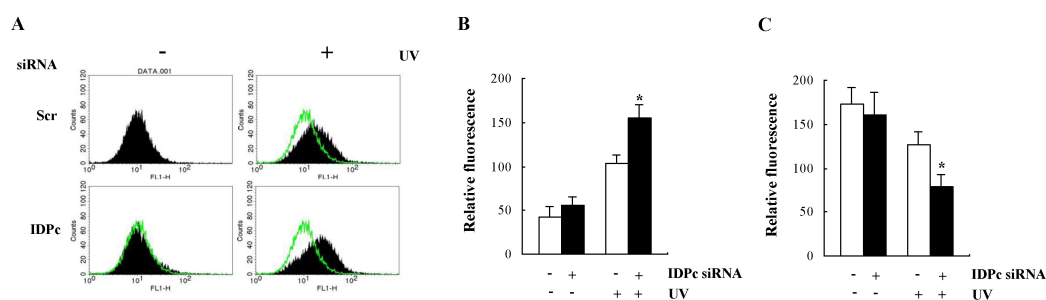


Fig. 3. Cellular redox status of HaCaT cells transfected with IDPc siRNA. (A) DCF fluorescence in transfected HaCaT cells exposed to 20 mJ/cm² of UVB was measured by flow cytometry. (B) DHE fluorescence was detected by microscopy. (C) Effect of IDPc siRNA transfection on GSH levels. Fluorescence image of CMF-DA-loaded cells was obtained with microscopy. (B-C) The relative fluorescence intensity was calculated, as previously described (22). Data are presented as the mean \pm S.D. of three separate experiments. *P < 0.01, versus the scrambled siRNA-transfected cells exposed to UVB.

fluorescence was significantly decreased in IDPc siRNA-transfected cells, compared to the control cells.

Mitochondrial dysfunction may play a critical role in the apoptotic pathway. Mitochondrial permeability transition (MPT) is a very important pro-apoptotic event, and ROS is a major stimulus that alters the MPT (19). To determine whether IDPc knockdown modulates MPT upon exposure to UVB radiation, we evaluated changes in the MPT, using the fluorescent probe 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazole carbocyanine iodide (JC-1). As indicated by JC-1 fluorescence ratios, knockdown of IDPc expression caused substantial disruption of the mitochondrial membrane potential (Fig. 4A). This loss of mitochondrial membrane potential was presumably due to impairment of mitochondrial electron transport, which is also responsible for incomplete O₂ reduction, leading to an increment in ROS production (20). To determine whether the alteration of mitochondrial integrity was associated with changes in mitochondrial ROS production, the levels of intracellular peroxides in the mitochondria of HaCaT cells were measured by fluorescence microscopy, with the oxidant-sensitive probe dihydrorhodamine (DHR) 123. As shown in Fig. 4B, the level of DHR fluorescence was significantly higher in IDPc siRNA-transfected cells, compared to the control, following exposure to 20 mJ/cm² of UVB. These results indicate that UVB radiation most likely leads to increased mitochondrial injury, and IDPc knockdown exacerbates this damage.

In summary, we found that the suppression of IDPc expression enhances the ability of HaCaT cells to undergo apoptosis, as a result of damage from UVB exposure. Based on our findings, we suggest that IDPc knockdown may protect skin from UVB-mediated injury. This is accomplished through the induction of apoptosis in damaged cells.

MATERIALS AND METHODS

Materials

β -NADP⁺, isocitrate, NADPH, and 3-(4,5-dimethylthiazol-2-

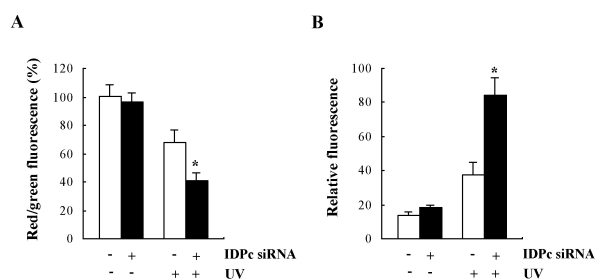


Fig. 4. Mitochondrial dysfunction in HaCaT cells transfected with IDPc siRNA. (A) HaCaT cells were incubated with a JC-1 probe for 30 min. Mean (red/green) fluorescence intensity expressed as a percentage of the control indicated the ratio of (high/low) mitochondrial membrane potential. Data are presented as the mean \pm S.D. of three separate experiments. *P < 0.01, versus the scrambled siRNA-transfected cells exposed to UVB. (B) DHR 123-specific fluorescence was measured with a fluorescence microscope. The relative fluorescence intensity was calculated, as previously described (22). Data are presented as the mean \pm S.D. of three separate experiments. *P < 0.01, versus the scrambled siRNA-transfected cells exposed to UVB.

yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO). DHR 123, DCFH-DA, DHE, CMF-DA, and JC-1 were purchased from Molecular Probes (Eugene, OR). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), or Cell Signaling (Beverly, MA). Antibodies against IDPc were prepared in IDPc-immunized rabbit, and purified by Protein A affinity chromatography.

Cell culture and cytotoxicity

Immortalized, nontumorigenic human HaCaT keratinocytes were maintained as a monolayer in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 50 units/ml penicillin, and 50 μ g/ml streptomycin. The cells were incubated at 37°C, in a humidified atmosphere of 95% air and 5% CO₂. UVB irradiation

was delivered with a UVB lamp (Spectronics Corporation, Westbury, USA), equipped with a dosimeter (20-200 sec irradiation for 10-100 mJ/cm²). The irradiated cells were then maintained for 12 h at 37°C, to perform the experimental observation. Cytotoxicity was assessed with an MTT assay, as previously described (12).

Knockdown of IDPc expression by siRNA

IDPc-specific and control (scrambled) siRNA was purchased from Samchully Pham (Seoul, South Korea). Sequences of the IDPc-specific and control dsRNA used for the current experiments are as follows. The IDPc siRNA sequences were 5'-GGACUUGGCUUGCAUdTdT-3' (sense), and 5'-AAUGCAAGCAGCCAAGUCCdTdT-3' (antisense); while the scrambled control siRNA sequences were 5'-CUGAUGACCU-GAGUGAAUGdTdT-3' (sense), and 5'-CAUUCACUCAGGU-CAUCAGdTdT-3' (antisense). HaCaT cells were transfected with 2.5 nM of oligonucleotide, using RNAiMAX (Invitrogen, Carlsbad, CA) under serum-free conditions, according to the manufacturer's protocol. After incubating at 37°C for 24 h, the cells were washed, and maintained in fresh normal medium, containing 1% FBS, 50 units/ml penicillin, and 50 µg/ml streptomycin.

Enzyme assay

IDPc activity was measured, by monitoring the production of NADPH at 340 nm at 25°C, as previously described (12). To measure caspase activity, the cells were washed three times with chilled PBS, and then incubated with 75 µl of lysis buffer [50 mM Tris-HCl, pH 7.4; 1 mM EDTA, 10 mM EGTA, 10 µM digitonin, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)], for 30 min at 37°C. The lysates were subsequently centrifuged at 16,000 g, for 20 min at 4°C. The supernatant was mixed (1 : 1) with reaction buffer (100 mM HEPES, 1 mM EDTA, 10 mM DTT, 0.5 mM PMSF, and 10% glycerol). The reaction was initiated by adding 5 µL (5 mg/ml) of each caspase substrate. After 1 h of incubation at 37°C, caspase activity was measured as the absorbance at 405 nm.

Immunoblot analysis

Proteins were resolved in a 10-12.5% SDS-polyacrylamide gel, transferred onto nitrocellulose membranes, and subjected to immunoblot analysis. Primary antibody binding was detected with horseradish peroxidase-labeled anti-rabbit IgG, and an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

RT-PCR analysis of IDPc expression

Total RNA was isolated using an RNeasy kit (Qiagen, Hilden, Germany), according to the manufacturer's instruction. cDNA was produced by quantitative RT-PCR, using the following primers: IDPc, 5'-GCT TCA TCT GGG CCA GTA AAA ACT ATG-3' (forward), and 5'-GTA AAC CTT TAA TGC TAG CAG CCA AGT CC-3' (reverse); and β-actin, 5'-TCT ACA ATG AGC TGC GTG

TG-3' (forward), and 5'-ATC TCC TTC TGC ATC CTG TC-3' (reverse). The amplified DNA products were resolved in a 1% nondenaturing agarose gel, and stained with ethidium bromide.

Assessment of cellular redox status

Intracellular ROS generation was measured, using the oxidant-sensitive fluorescent probe DCFH-DA. Cells were treated with 10 µM DCFH-DA, for 30 min at 37°C, and ROS levels were then analyzed by fluorescence-Activated Cell Sorting (FACS). Superoxide anion production was monitored, using the fluorescent probe DHE with a fluorescence microscope, as previously described (20). Intracellular GSH levels were determined, using the GSH-sensitive fluorescence dye CMF-DA. HaCaT cells were incubated with 5 µM CMF-DA, for 30 min. The images of CMF-DA cell tracker fluorescence by GSH were analyzed by fluorescence microscopy.

Measurement of mitochondrial redox status and damage

The fluorescent probe JC-1 was used to estimate mitochondrial membrane potential. JC-1 (10 µM) was added to the cells, and incubated at 37°C for 30 min. The ratio of the intensity of green fluorescent monomers to that of the JC-1 aggregates is directly proportional to the mitochondrial membrane potential (21). The levels of mitochondrial ROS were measured, using the oxidant-sensitive fluorescent probe DHR 123 with a fluorescence microscope, as previously described (20).

Assessment of DNA fragmentation

To evaluate the degradation of chromosomal DNA into nucleosome-sized fragments, a 500-µl aliquot of lysis buffer (100 mM Tris-HCl, pH 8.5; EDTA, 0.2 mM NaCl, 0.2% SDS, and 0.2 mg/ml proteinase K) was added to the cell pellet (2 × 10⁵ cells), and incubated overnight at 37°C. DNA was obtained by ethanol precipitation, separated in a 0.8% agarose gel, and visualized under UV light.

Quantitation of relative fluorescence

The average fluorescence intensity observed in fluorescence images was calculated, as previously described (22).

Statistical analysis

Differences between two mean values were analyzed with Student's t-test. P values < 0.05 were considered statistically significant.

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