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Original research paper

Development of a high-throughput screening system for identification of novel reagents regulating DNA damage in human dermal fibroblasts

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Ultraviolet (UV) radiation is a major inducer of skin aging and accumulated exposure to UV radiation increases DNA damage in skin cells, including dermal fibroblasts. In the present study, we developed a novel **D**NA **re**pair regul**a**ting **m**aterial discovery (DREAM) system for the high-throughput screening and identification of putative materials regulating DNA repair in skin cells. First, we established a modified lentivirus expressing the luciferase and hypoxanthine phosphoribosyl transferase (HPRT) genes. Then, human dermal fibroblast WS-1 cells were infected with the modified lentivirus and selected with puromycin to establish cells that stably expressed luciferase and HPRT (DREAM-F cells). The first step in the DREAM protocol was a 96-well-based screening procedure, involving the analysis of cell viability and luciferase activity after pretreatment of DREAM-F cells with reagents of interest and post-treatment with UVB radiation, and *vice versa*. In the second step, we validated certain effective reagents identified in the first step by analyzing the cell cycle, evaluating cell death, and performing HPRT-DNA sequencing in DREAM-F cells treated with these reagents and UVB. This DREAM system is scalable and forms a time-saving high-throughput screening system for identifying novel anti-photoaging reagents regulating DNA damage in dermal fibroblasts.

Keywords: human dermal fibroblasts, DNA damage, high-throughput screening, aging

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The skin is the largest organ and forms the outermost layer of the human body. Therefore, the skin is more easily exposed to toxic environmental agents, particularly ultraviolet (UV) radiation, than are other tissues, except for the eyes. Human skin consists of the epidermis, dermis and hypodermis. The most abundant cells in the dermis layer are human dermal fibroblasts (HDFs), which contribute to skin firmness and elasticity by upregulating

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collagen synthesis. However, chronic and continuous exposure to UV radiation leads to upregulation of matrix metalloproteinases (MMPs), collagen degradation and wrinkle formation, which are the main features of skin aging (1, 2). In addition, UV radiation generates reactive oxygen species and DNA damage that induce cellular senescence and apoptosis in HDFs (3). Furthermore, exposure to UV radiation is an important causative factor for skin diseases, such as photodermatoses, actinic keratosis and skin cancer (3). However, UV-induced skin aging is not an inevitable natural consequence, but is due to extracellular factors; in other words, the aging process is controllable by avoidance of UV irradiation (4).

Accumulating evidence indicates that the application of a chemical sunscreen, a commonly known as sun creams with an adequate sun protection factor (SPF), is able to decrease UV-induced DNA damage, such as the formation of pyrimidine dimers, in human skin (5, 6). However, the effectiveness of a sunscreen has been challenged by new research. Notably, sunscreen is only effective in preventing UV-induced DNA damage when used regularly (7, 8). Furthermore, a recent study demonstrated that sunscreens with superior UVA protection and UVB SPF 50 delayed the onset of UV radiation-induced melanoma skin cancer, but provided only partial protection, indicating that UV light can sneak past the sunscreen and cause long-term DNA damage, even in case of an SPF 50 sunscreen (9). These results indicate that sunscreen-mediated UV blockage is not 100 % protective against UV radiation, and has no lasting UV-protective effect. Sunscreen exerts only a physical protective effect within its given SPF time, not a biological protective effect against UV radiation in human skin.

Thus, identification of novel reagents exerting a biological UV protective effect in skin cells is an important future strategy for preventing UV-induced skin aging. To this end, we have developed a novel large-scale screening system, the **D**NA **re**pair regul**a**ting **m**aterial discovery (DREAM) system. This system facilitates the high-throughput identification of materials that effectively protect against and/or repair UV-induced DNA damage in HDFs.

EXPERIMENTAL

Cells and reagents

Normal human dermal fibroblasts (NHDFs) were purchased from Lonza (Switzerland) and human dermal fibroblast WS1 cells were purchased from the CLS Cell Line Service (Germany). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Life Technologies, USA) containing 10 % fetal bovine serum (FBS, Sigma-Aldrich, USA) at 37 °C and 5 % CO_2 in a humidified chamber. To analyze cytotoxicity and luciferase activity, 5×10^3 cells were seeded into a 96-well culture plate and grown. Titrated extract of *Centella asiatica* (TECA) is a reconstituted mixture comprising asiatic acid, madecassic acid, asiaticoside and madecassoside in 4:4:3 ratio and was purchased from Bayer HealthCare (Germany). Dimethylsulfoxide (DMSO), epigallocatechin gallate (EGCG), quercetin and caffeic acid were purchased from Sigma-Aldrich.

Recombinant lentivirus construction

The open reading frame (ORF) sequence of the firefly luciferase gene in the pGL3 luciferase reporter vector (Promega, USA) was amplified by polymerase chain reaction (PCR)

using the following primers adapted to the BamHI site and sensor sequence (forward primer: 5′-CGGATCCAGCCAGAGCCAGTTTTTGATGGAAGACGC3′), and the EcoRI site (reverse primer: 5′-GGAATTCCTTACACGGCGATC-3′). The PCR product was cloned into the Lenti-HT vector. The ORF sequence of hypoxanthine phosphoribosyl-transferase (HPRT) was amplified by PCR using the following primers adapted to the BamHI site: forward, 5′-CGGATCCACCATGGCGACCCGCAG-3′ and reverse, 5′-CGGATCCGGCTTTGTATTTTGC-3′. The resulting HPRT PCR product was cloned into the Lenti-HT-luciferase vector. To generate recombinant lentivirus containing the luciferase and HPRT genes, embryonic kidney 293T cells were co-transfected with the Lenti-HT-luciferase/HPRT lentiviral transfer vector, pCMV-dR8.2 (Addgene, USA), and the pCMV-VSV-G plasmid (Addgene), and then the transfected cells were incubated for 48 h. After 48 h of transfection, the medium, which contained recombinant lentivirus particles, was collected and used to infect WS1 cells. Infected WS1 cells were selected by incubation with DMEM medium containing puromycin (1 μg mL^{-1}). The selected WS1 cells were further infected with lentivirus expressing β-galactosidase (β-gal), which was previously generated by transfection with pSMPUM-MNDnLacZ vector (Cell Biolabs, Inc., San Diego, CA, USA), pCMV-dR8.2, and pCMV-VSV-G plasmid. The infected WS1 cells expressing luciferase and β-gal were inoculated into a 96-well culture plate and then subjected to puromycin selection to produce single clones. Luciferase expression in each individual clone was detected *via* a luminometer and the expression levels were analyzed by comparison with the expression level of β-gal. In the present study, the clone with the highest luciferase expression was selected and named DREAM-F cells.

UV irradiation

Before exposure to UVB radiation, NHDFs and DREAM-F cells were seeded in 96-well and 60-mm culture plates and cultured in the growth media overnight. When more than 70 % confluent, the cells were pretreated with control DMSO, TECA or EGCG for 6 h. After pretreatment, the cell media were washed out with PBS twice, and then the cells were exposed to 100 mJ cm^{-2} UVB without covering the cell culture plates, so that the UVB light was not filtered. The irradiated cells were then cultured for an additional 24 h in the growth media. In the post-treatment experiment, cells were first irradiated with UVB and then treated with the reagents for 24 h.

Cytotoxicity assay

The cytotoxic and UVB-protective effects of each reagent on dermal fibroblast cells were analyzed using a water-soluble tetrazolium salt (WST-1) assay (EZ-Cytox cell viability assay kit, Itsbio, Korea). We did not use the MTT assay, because MTT gets converted into a water-insoluble formazan and therefore requires an extra step of media suction and DMSO addition to evaluate cell viability, often resulting in higher error rates. However, WST is converted to a water-soluble formazan and there is no need of this extra step. Cells were seeded into a 96-well culture plate and pretreated with various concentrations (as indicated) of the reagents in one group and 100 mJ cm^{-2} UVB radiation in another group. After pretreatment with reagents for 24 h, cells were irradiated with UVB radiation and cultured for an additional 24 h. After pretreatment with UVB radiation, cells were treated with the indicated reagent concentrations for 24 h. After treatment, 10 μL of the WST-1 kit

solution was added to cells seeded in a 96-well culture plate and incubated at 37 °C for 0.5 h. Cell viability was determined by measuring the absorbance at 450 nm using an iMark microplate reader (Bio-Rad, USA). Three independent experiments were performed and the results are presented as the means ± standard deviation.

Flow cytometric analysis

After treatment with the reagents and UVB irradiation, the cells were harvested, twice washed with cold PBS and suspended with 1 mL of cold 70 % ethanol. Subsequently, the cells were fixed by incubation at –20 °C for 2 h. The cells were then washed with cold PBS and resuspended in 0.5 mL of propidium iodide (PI) staining solution (50 μg mL^{-1} PI, 0.5 % Triton X-100, both from Sigma-Aldrich, and 100 μg mL^{-1} RNase in PBS) and incubated at 37 °C for 1 h. The cell population in different phases of cell cycle was measured by evaluating the intensity of fluorescent PI staining of 10,000 cells using the FL2-H channel of a FACSCalibur flow cytometer (BD Biosciences, USA).

Luciferase assay

DREAM-F cells were seeded into a 96-well culture plate and cultured for 24 h. Following this, one group was pretreated with the reagents and post-treated with 100 mJ cm^{-2} UVB radiation and the other group was first exposed to UVB irradiation and then post-treated with the reagents. After treatment, the cell medium was subsequently discarded and 50 μL of passive lysis buffer (Promega) was added to each well and incubated for 5 min. Half of the resulting cell lysate from each well was then transferred to a white opaque luminometer microtiter plate and mixed with 80 μL of luciferase assay reagent (Promega). The luciferase activity of each well was analyzed using a Veritas luminometer (Turner Designs, USA). The remaining half of the cell lysate from each well was used to analyze β-gal activity using the luminescent β-galactosidase detection kit II (Clontech Laboratories, Inc., USA). The relative luciferase activity was normalized to β-gal activity. The results are the averages of three independent experiments.

Statistical analysis

All results are presented as the mean percentage ± standard deviation (SD) of three independent experiments. p-value of < 0.05, as determined by Student's t-test, was considered to indicate a statistically significant difference.

RESULTS AND DISCUSSION

Construction of lentiviruses containing the luciferase and HPRT genes

UVB radiation spontaneously induces the generation of high levels of cyclobutane pyrimidine dimers (CPDs) in genomic DNA. These CPDs inhibit mRNA transcription by stalling RNA polymerase II at the CPD sites (10). Furthermore, UVB radiation causes permanent mutation-mediated DNA damage, including C → T and CC → TT transition mutations (11). Therefore, UVB-induced CPDs and transition mutations are able to downregu-

late the expression of certain genes or induce the expression of mutant proteins with altered protein activities. Many studies have evaluated the effects and mechanisms of UV-induced DNA damage in skin. Among these, a recent study based on sequencing and comparing mutations in the housekeeping gene hypoxanthine phosphoribosyl-transferase (HPRT) concluded that not only UVB but also UVA is able to induce CPDs and the C → T transition in human skin fibroblasts (12).

Based on the above results, we aimed to develop a UV-induced DNA damage sensor system using human dermal fibroblasts. First, the UV-induced reduction in transcription and altered mutant protein level were evaluated using a luciferase system. The luciferase assay is a technique with a diverse range of applications in molecular biology and we confirmed that UV irradiation significantly reduced the activity of luciferase in luciferase vector-transfected NHDFs (data not shown). Next, to render the luciferase system more vulnerable against UV radiation, we inserted sensor sequences (SSs; 5′-AGCCAGAGC-CAGTTTTTG-3′) upstream of the luciferase ORF sequence. A study by Kreimer-Erlacher *et al*. (13) showed that in CC-containing sequences, the latter C was more commonly substituted with T than the former C (CC → CT transition). Therefore, following UV irradiation, a stop codon is likely to be created in the 'AGCCAG' sequence in the SS (AGCCAG → AGCTAG). The sequence of TTTTT in the SS has the potential to develop CPDs following UV irradiation. Furthermore, to analyze the exact number of mutations following UV irradiation, the housekeeping gene HPRT was cloned into the N-terminal region of the SS. A study of Kappes *at al*. (12) showed that irradiation with 100, 200 and 400 J m^{-2} UVB resulted in the generation of roughly 10, 23 and 59 mutations in the HPRT gene in human fibroblasts. Therefore, in summary, the HPRT-SS-luciferase fusion sequence was used to evaluate the level of UV-induced damage. To generate NHDFs expressing the HPRT-SS-luciferase protein, we used the lentiviral system, which stably infects dividing and non-

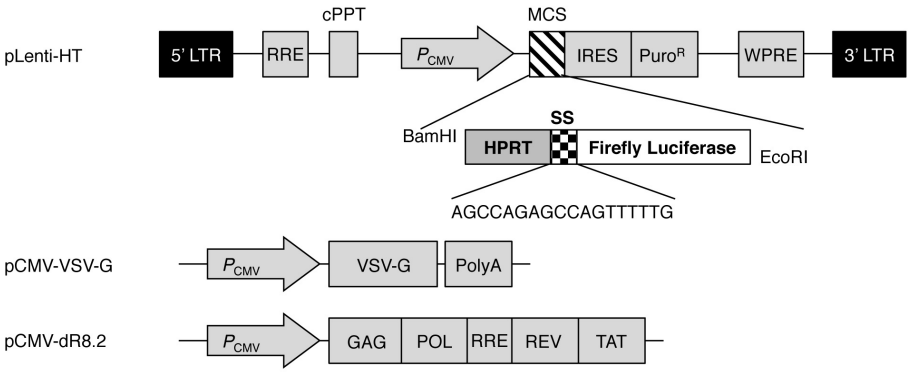

Fig. 1. Vector maps of pLenti-HT-luciferase, the packaging plasmid and the envelope plasmid. LTR-long terminal repeat sequence, RRE, rev response element; cPPT, central polypurine tract; PCMV, cmv promoter; MCS, multiple cloning sites; IRES, internal ribosome entry site; PuroR, puromycin resistant gene; WPRE, woodchuck hepatitis virus post-transcription regulatory element; VSV-G, vesicular stomatitis virus GP; PolyA, poly-A sequence.

dividing human cells. As shown in Fig. 1, the HPRT-SS-luciferase sequence was inserted between the CMV promoter and the IRES sequence in the pLenti-HT vector. The ORF region of firefly luciferase was cloned into the EcoRI site of pLenti-HT. pCMV-VSV-G is a VSV-G coding plasmid, which allowed the production of viral particles. pCMV-dR8.2 is a packaging plasmid, which encodes 4 genes, including Gag, Pol, Rev, and Tat. Subsequently, lentivirus particles were generated by transfecting 293T cells with the recombinant pLenti-HT, packaging vector pCMV-dR8.2 and pCMV-VSV-G. After 48 h of transfection, recombinant lentivirus particles were collected by filtration (Figs. 2a,b).

Generation of HPRT-SS-luciferase-expressing DREAM-F cells

After the generation of lentivirus encoding HPRT-SS-luciferase from 293T cells (Fig. 2a), human dermal fibroblast WS1 cells were infected with the recombinant lentivirus for 48 h. To select infected cells, culture medium was replaced with selection medium, containing puromycin, and the cells were further incubated for 1 week. It is commonly known that lentivirus randomly integrates within the genome; hence, the expression

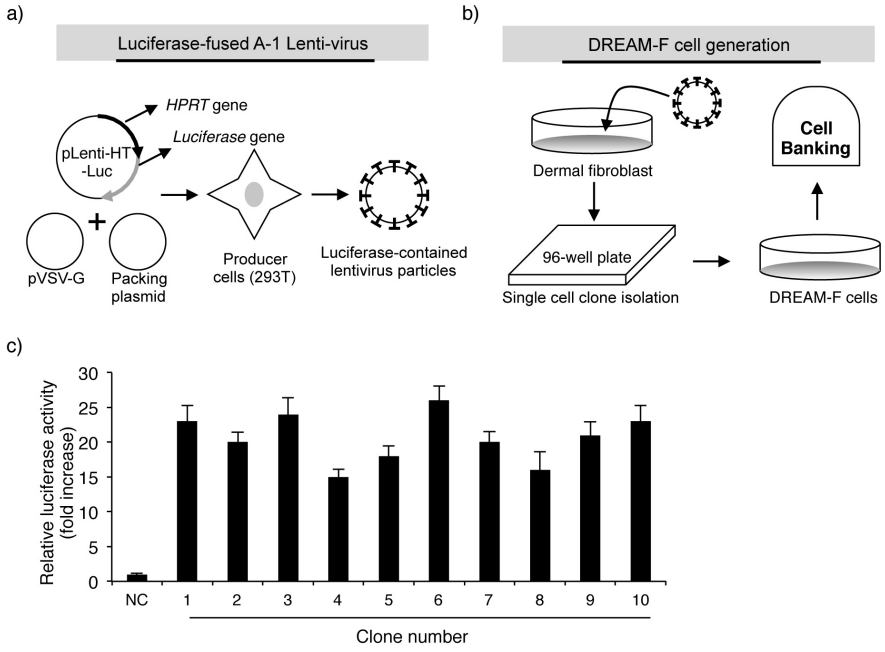

Fig. 2. Schematic diagram of luciferase-expressing lentivirus production and DREAM-F cell generation: a) Luciferase-expressing lentiviruses were produced by transfecting 293T cells with the pLenti-HT-HPRT-SS-luciferase, pCMV-VSV-G and pCMV-dR8.2 plasmids; b) Establishment of luciferase-fused WS1 cells (DREAM-F cells); c) Identification of a cell clone with the highest expression level of luciferase. The results are expressed as the means ± SD from three separate experiments; NC – negative control. Significant difference compared with control WS1 cells (*$p < 0.05$).

level of the HPRT- -SS-luciferase fusion gene varied in individual cells. Therefore, we conducted an additional selection process to obtain single clonal cells. One or two numbers of infected cells were seeded into individual wells of a 96-well plate and grown in the selection medium for 1 week. Each of the cell colonies was continuously grown to 80 % confluence in a 6-well culture plate (Fig. 2b). After single cell selection, we analyzed the level of luciferase activity, and selected the highest expressed clone, which we named DREAM-F cells (Fig. 2c).

Establishing a system to identify novel reagents regulating UVB-induced DNA damage using DREAM-F cells

The first step was to determine appropriate experimental concentrations, in terms of low cytotoxicity, of the candidate reagents. DREAM-F cells were seeded into a 96-well culture plate and treated with 4 or 5 concentrations of each reagent. After 24 h of treatment, the level of cytotoxicity was evaluated by the WST-1 assay. After evaluating the cytotoxicity of each reagent, its UV-protective or UV-induced damage-repairing effects were analyzed using DREAM-F cells. To evaluate the UV protective effect, DREAM-F cells were pretreated with low-cytotoxicity concentrations of the candidate reagents for 6 h and then exposed to 100 mJ cm^{-2} UVB light. After 24 h of further incubation, cell viability and luciferase activity were analyzed using the WST-1 and luciferase assays. To evaluate UV-induced damage repair, cells were irradiated with UVB and posttreated with the candidate reagents. We thereby identified candidate reagents showing protective or repair effects. This screening system is elucidated in Fig. 3a.

To validate this system, we used four types of phytochemicals: titrated extract of *Centella asiatica* (TECA), epigallocatechin gallate (EGCG), caffeic acid (CA), and quercetin (QC). Our group recently found that TECA exerts a UV-protective effect in human dermal fibroblasts by downregulating UV-induced cellular and DNA damage (14). Furthermore, it was demonstrated that EGCG has a UV-protective effect in human dermal fibroblasts (15). CA and QC are known to exert inhibitory effects against UV-induced oxidative stress (16, 17). As shown in Fig. 3b, relatively high concentrations of TECA and EGCG exhibited cytotoxicity in DREAM-F cells, whereas CA and QC showed no cytotoxicity. Interestingly, UV-protective and UV-induced cellular damage repair effects were only present in TECA- and EGCG-treated DREAM-F cells (Fig. 3c). Further experiments showed that TECA and EGCG inhibited UV-induced loss of luciferase activity in DREAM-F cells (Fig. 3d), indicating that these two reagents have a UV-protective and UV-induced DNA damage repair effects in human dermal fibroblasts. Our previous study demonstrated that TECA inhibits UVB-induced cellular toxicity; however, we also found that TECA directly inhibited UVB-induced DNA damage using our DREAM system. Other reports showed that EGCG reduced UVB-induced oxidative DNA damage in living skin equivalents (18). Although previous reports demonstrated CA- and QC-mediated anti-oxidative effects in hairless mice and lymphocytes, these effects might be cell-specific or may not be directly involved in the loss of cell viability mediated by UV radiation (16, 17). Overall, our results indicate that the DREAM system is useful for the identification of novel reagents regulating UV-induced DNA damage in human dermal fibroblasts.

Other potential applications of the DREAM system

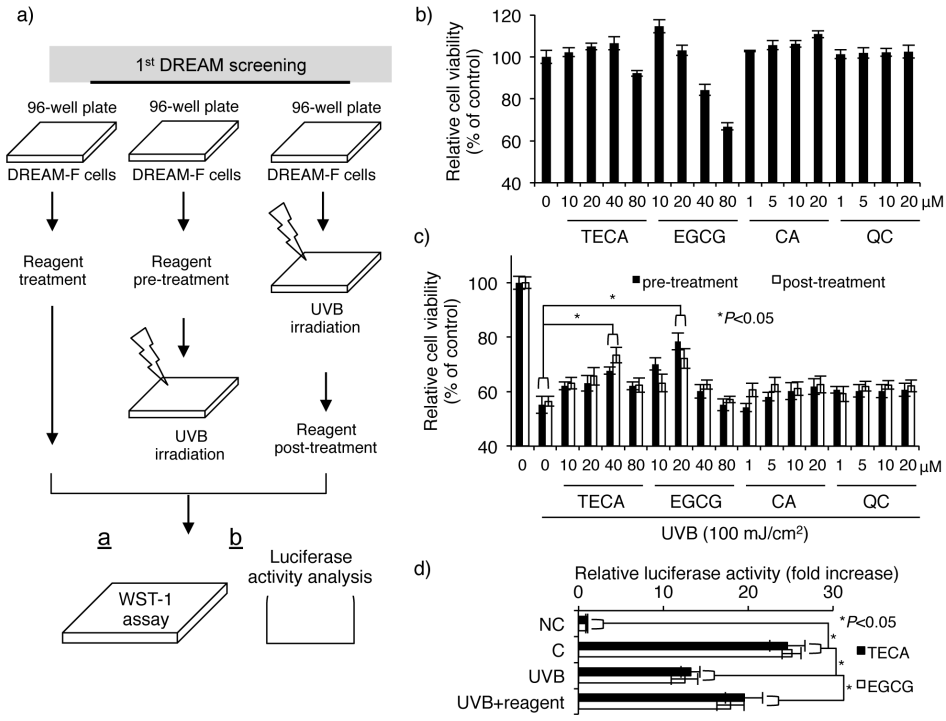

Fig. 3. Schematic diagram and confirmation of the first DREAM screening system: a) Strategy for the identification of putative reagents showing protective effect against UVB radiation and regulatory effect on UVB-induced loss of luciferase activity; b) Evaluation of cytotoxicities for TECA, EGCG, CA and QC; c) Evaluation of the protective effect against UVB-induced loss of cell viability. Significant difference compared to UVB-irradiated DREAM-F cells (*$p < 0.05$); d) Evaluation of protective effect against UVB-induced downregulation of luciferase activity (mean ± SD, $n = 3$). Significant difference compared to control WS1 cells and UVB-irradiated DREAM-F cells (*$p < 0.05$).

UV irradiation spontaneously causes DNA damage, causing accumulation of reactive oxygen species (ROS) and cell cycle arrest in skin cells (19). If such damage is not repaired or inhibited, skin cells undergo irreversible apoptosis-mediated cell death (19). Although the main purpose of the DREAM system was to identify novel reagents regulating UV-induced DNA damage in human dermal fibroblasts, the identified reagents could also be associated with protection or regeneration in cells that have undergone UV-mediated damage. Furthermore, our first prototype of the DREAM system (as shown in Fig. 3a) was able to determine the level of UV-induced DNA damage by the luciferase assay. However, the exact number of UV-induced DNA mutations could not be counted. Therefore, several reagents identified using the first DREAM screening were then used in the second DREAM system to evaluate changes in the cell cycle phase and DNA mutations after UV irradiation. As shown in Fig. 4, DREAM-F cells were seeded into 60-cm or 6-well culture plates and treated with the identified reagents before or after UV irradiation. After 24 h of incuba-

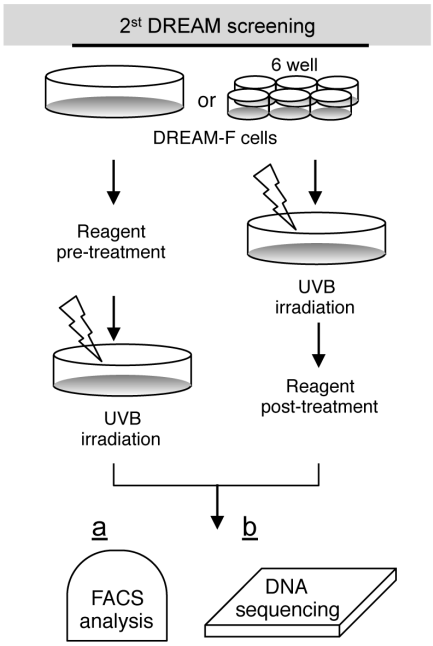

Fig. 4. Schematic diagram of the second DREAM screening system. After DREAM-F cells were exposed to UVB radiation or post-treated with a reagent, cells were gathered and subjected to PI-staining-based FACS analysis and mutation analysis through HPRT cDNA synthesis and DNA sequencing.

tion, cells were gathered and stained with fluorescent propidium iodide. The stained cells were subjected to cell cycle analysis using flow cytometry. To analyze the number of mutations, the reagents and UV-treated cells were incubated for 96 h in growth media and subjected to total RNA purification and cDNA synthesis. The fusion gene of HPRT-SS-luciferase was analyzed by cDNA sequencing. We confirmed that TECA and EGCG-treatment decreased UV-induced cell accumulation in the sub-G1 phase (apoptosis phase), indicating that these reagents block UV-induced cell death in human dermal fibroblasts (14, 15). Changes in the number of mutations after treatment of DREAM-F cells with each reagent and UV irradiation are currently under investigation.

CONCLUSIONS

Our aim was to develop a large-scale and high-throughput screening system for identifying novel reagents that protect against or repair UV-induced DNA damage in human dermal fibroblasts. For this purpose, we used a 96-well-based screening system using DREAM-F cells. In conclusion, our study demonstrates that the hybrid luciferase gene can be used for evaluating the level of DNA damage caused by UV irradiation in human dermal fibroblasts. Furthermore, we generated DREAM-F cells using lentiviruses. This hybrid cell offers a large-scale and high-throughput screening system for novel anti-aging reagents

under false-positive-free conditions. Also, we validated this DREAM system using TECA, EGCG, CA, QC reagents, and found that TECA and EGCG are novel phytochemicals showing a direct inhibitory effect on UV-induced DNA damage in dermal fibroblasts. Therefore, we believe that the DREAM system is appropriate for identification of novel pharmaceutical reagents showing anti-photoaging or anti-photocarcinogenesis in the future.

Abbreviations, acronyms and symbols. – DREAM system – DNA repair regulating material discovery system, HPRT – hypoxanthine phosphoribosyl transferase, HDFs – human dermal fibroblasts, MMPs – matrix metalloproteinase, SPF – sun protection factor, CPDs – cyclobutane pyrimidine dimers, TECA – titrated extract of *Centella asiatica*, EGCG – epigallocatechin gallate, CA – caffeic acid; QC – quercetin.

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