

# **Photodynamic approach for teratoma-free pluripotent stem cell therapy using CDy1 and visible light\*\*.**

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## Supporting Information

### Material and Method

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## Material and Method

### Singlet-oxygen quantum yields (SQY) Assay of CDy1

To measure singlet-oxygen quantum yields, 1,3-diphenylisobenzofuran (DPBF) was used as a fluorescent probe which can carry out a highly specific reactivity against singlet oxygen ( $^1\text{O}_2$ ) forming an endoperoxide which decomposes to give 1,2 dibenzoylbenzene [1]. By monitoring fluorescence decrease of DPBF, the reaction of between DPBF and singlet oxygen were able to measure at 419 nm of wavelength. The mixture of DPBF solution was prepared. Using 96 well plates, the solution of at 100 $\mu\text{M}$  of DPBF (90  $\mu\text{L}$ ) and 15  $\mu\text{M}$  of CDy1 (10  $\mu\text{L}$ ) put together in one well as triplicated. With irradiation at 15  $\text{W}/\text{cm}^2$  of light source for 1 min, absorbance of DPBF was measured and repeated ten time point until 10 min. Rose Bengal as a reference prepared the same as CDy1. The singlet oxygen quantum yield of CDy1 were calculated with the quantum yield of RB (Rose Bengal) as a reference compound according to SQY formula (1),

$$\Phi_{CDy1} = \Phi_{RB} \frac{k_{CDy1}}{I_{CDy1}} \frac{I_{RB}}{k_{RB}} \quad (1)$$

$\Phi$  is the singlet oxygen quantum yields of CDy1 and RB,  $k$  is the first order rate constants of the substrate destruction by CDy1 and RB. I is the total absorbance of CDy1 and RB in DPBF solution.

### PSC cell culture

The mouse J1 cell line was purchased from the American Tissue Culture Collection (ATCC, VA, USA). To maintain the cells in an embryonic state, they were cultured on a feeder free in mPSC medium (DMEM (Gibco, Life technologies, NY, USA) supplemented with 15% fetal bovine serum (FBS) (Gibco), 1% nonessential amino acids (Gibco), 0.1 mM  $\beta$ -mercaptoethanol (Gibco), 0.1% gentamycin (Gibco) and 1,000 U/ml mouse leukemia inhibitory factor (mLIF) (Millipore, MA, USA)) in a humidified atmosphere at 37°C with 5%  $\text{CO}_2$ . The medium was changed daily, and the cells were passaged every 2~3 days. Human PSC (H9: Wicell Research Institute, Madison, WI, USA) were maintained in TeSR<sup>TM</sup>-E8<sup>TM</sup> medium (Stemcell Technologies, Canada) on matrigel (BD Biosciences, NJ, USA)-coated 60-mm dishes.

### CDy1 staining

mPSC and hPSC were treated with CDy1 (50 or 100 nM) for 30 min and incubated for 2 hours in a humidified atmosphere at 37°C with 5%  $\text{CO}_2$ . Red fluorescence of CDy1 was detected by fluorescence microscope (IX71 (U-MWIG2 filter), Olympus Corporation, Japan) and FACS Caliber (582/15 nm bandpass filter) (BD Biosciences). After CDy1 staining, both mPSC and hPSC were irradiated with

Millennia V laser (Spectra-Physics) at 1.2 W/cm<sup>2</sup> for 1 min.

### **Intracellular CDy1 detection**

hPSCs and mPSCs were stained with CDy1 for 30 minutes, and were cultured for 24 hours. Cells were lysed with TLB buffer, and red fluorescence in the lysates measured by microplate reader (PerkinElmer, MA, USA)

### **Annexin V staining**

hPSCs were stained with MitoTracker (Orange), MitoTracker (Red), and CDy1 respectively. Each fluorescent probe-stained hPSCs were cultured in standard medium for 2 hours. Cells were exposed by light of 532 nm and incubated for 5 hours. Cells were washed with PBS thrice, and were stained with the FITC-Annexin V and 7-AAD for 30 min at RT (25°C) in the dark. Cell death (or survival) was analysed by flow cytometry according to apoptosis detection kit I protocol (BD Biosciences). Annexin V<sup>-</sup>/7-AAD<sup>-</sup> and annexin V<sup>+</sup> cells were gated as live cell population and cell death population respectively.

### **Endothelial tube formation and ac-LDL uptake assay**

Endothelial tube formation assay was performed by sorting mEC ( $5 \times 10^4$  cells), which were subsequently seeded on 4 well matrigel (BD Biosciences)-coated plates and incubated at 37°C for 12 hours. For the ac-LDL uptake assay, mEC were incubated with 10 mg/ml DiI-labeled ac-LDL (Biomedical Technologies, MA, USA) at 37°C for 4 hours. After the cells were washed three times with PBS, images were analyzed using fluorescence microscopy (Nikon, Japan).

### **Teratoma formation**

The pluripotency of mPSC were examined in vivo through a teratoma formation assay using subcutaneous inoculation. First,  $5 \times 10^6$  CDy1-stained mPSC (with or without light-exposure) were inoculated subcutaneously under the right and left front leg of female BALB/c-nude mice. There are two groups of light-exposed mPSC (n=10) and control mPSC (n=10). Light-exposed group did not form teratomas except the control mPSC group that underwent subcutaneous injection. All mice were euthanized 21 days after the inoculation for mPSC. Tumor volumes were calculated using the formula:  $V = a \times b \times c \times \pi / 6$  ( $a$  = length,  $b$  = width, and  $c$  = depth). All animal care and experimental procedures were performed under the approval of the animal care committees in Sogang University.

### **Immunocytochemistry**

mPSC and mEC were fixed in 4% PFA (paraformaldehyde) for 20 minutes and permeabilized with 0.1%

Triton X-100 in PBS for 10 minutes. After treatment, 5% normal goat serum (blocking solution) was applied for 30 minutes and the cells were incubated with primary antibodies Oct-4 (Santa Cruz Biotech Inc.), vWF (Abcam, MA, USA), and PECAM (Millipore) in blocking solution overnight at 4°C. The cells were then washed three times with PBS and incubated with Cy2 (Jackson ImmunoResearch Laboratories), Alexa 488, and Alexa 594 (Molecular probe Inc., OR, USA) conjugated secondary antibodies for 1 hour. The images were analyzed using fluorescence microscopy (Olympus).

### **Differentiation and cell sorting**

To induce endothelial cells, mPSC were allowed to form mEB in suspension culture conditions in DMEM (Gibco) containing 5% FBS (Hyclone, GE Healthcare, UK), 20 ng/ml mouse recombinant vascular endothelial growth factor; VEGF (R&D system Inc., MN, USA) with basic medium components for 10 days. EB media was changed every 2 days for 10 days. mEBs were then plated on 0.2% gelatin coated plates and cultured in DMEM supplemented with 5% FBS for 7 days. To isolate ECs from differentiated mEBs, cell sorting was performed with a FACS Aria 3 cell sorter (BD Biosciences) using APC-conjugated anti-mouse CD31 antibody (BD Biosciences). CD31 positive cells were cultured on the 0.2% gelatin coated plates with DMEM containing 5% FBS, 20 ng/ml VEGF with basic medium components, and expanded for 2 passages.

### **Spontaneous differentiation**

To induce differentiation, mPSC were allowed to form mEB in suspension culture conditions in mPSC medium without mLIF (Millipore) for 3 days. EB media was changed every 2 days. mEBs were then plated on 1% gelatin-coated plates and cultured in mPSC medium without mLIF for 7 days.

### **Immunoblotting**

Cells were lysed with TLB buffer (20 mM Tris-HCl (pH7.4), 137 mM NaCl, 2 mM EDTA, 1% triton X-100, and 10% glycerol) supplemented with 10  $\mu$ M sodium vanadate and 1 mM protease inhibitor cocktail (Roche, Basel, Switzerland) and subjected to SDS-PAGE followed by immunoblotting using primary antibodies cleaved caspase-3 (Cell signaling, MA, USA), PARP, and  $\alpha$ -tubulin (Santa Cruz Biotech Inc., TX, USA), and horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, PA, USA).

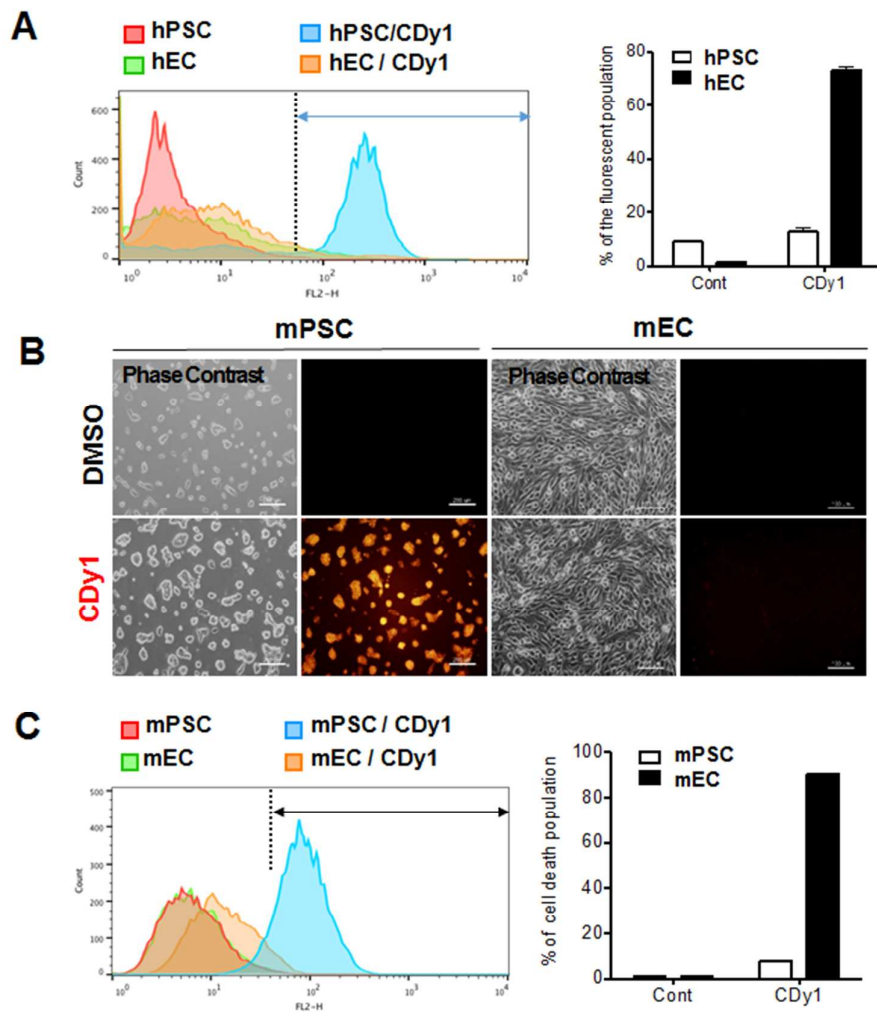
### **ROS detection**

Intracellular ROS production was determined by flow cytometry using DCF-DA as fluorescent probes.

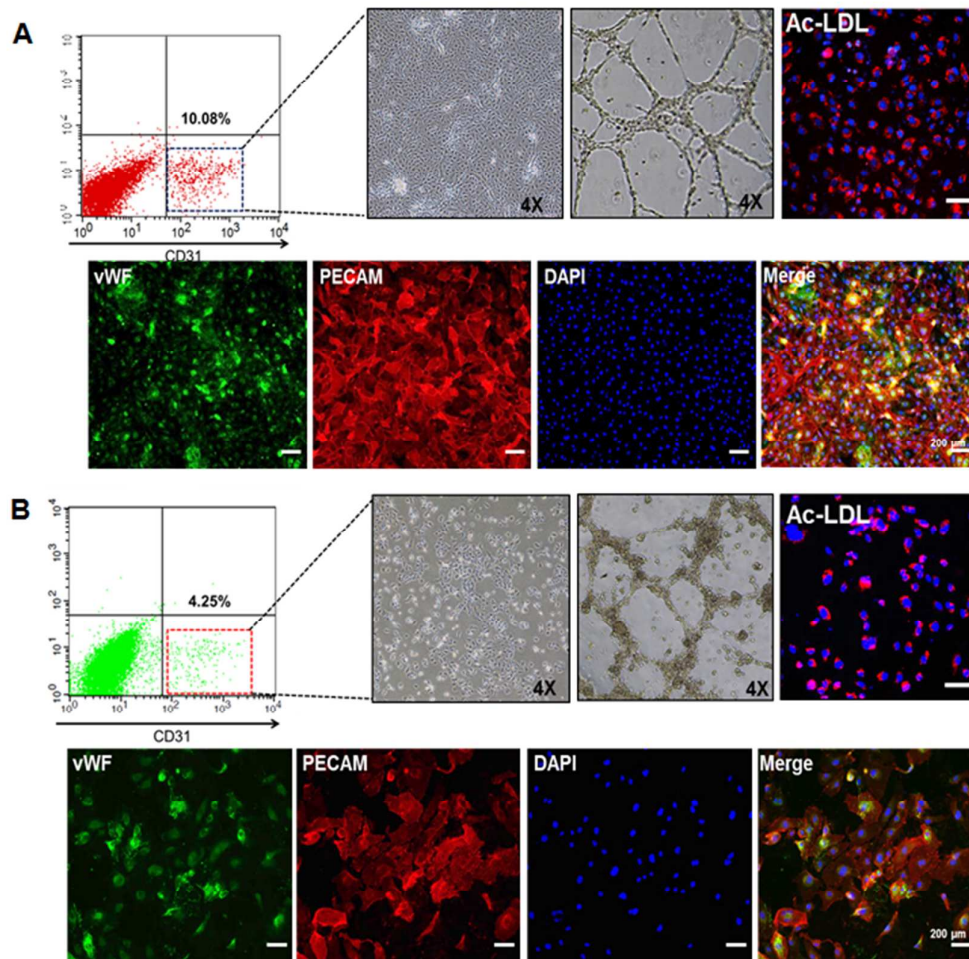
hPSCs and hECs were exposed by light of 540~580 nm for 30 seconds after CDy1 staining, and incubated for 1 hour. The cells were then also incubated with the DCF-DA (10  $\mu$ M) in HBSS (Gibco, MA, USA) for 30 min at 37°C, after which they were washed, and analyzed for fluorescence intensity using FACS Caliber (BD Biosciences, CA, USA)

### **SOD1 Immunoblotting**

hPSCs and hECs were exposed by light of 540~580 nm for about 1 minute after CDy1 staining, and incubated for 16 hours. Cells were lysed with TLB buffer (20 mM Tris-HCl (pH7.4), 137 mM NaCl, 2 mM EDTA, 1% triton X-100, and 10% glycerol) supplemented with 10  $\mu$ M sodium vanadate and 1 mM protease inhibitor cocktail (Roche, Basel, Switzerland) and subjected to SDS-PAGE followed by immunoblotting using primary antibodies SOD1 (Enzo Life Sciences, NY, USA), 4-Hydroxynonenal (Abcam, Cambridge, UK), and  $\alpha$ -tubulin (Santa Cruz Biotech Inc., TX, USA), and horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, PA, USA).

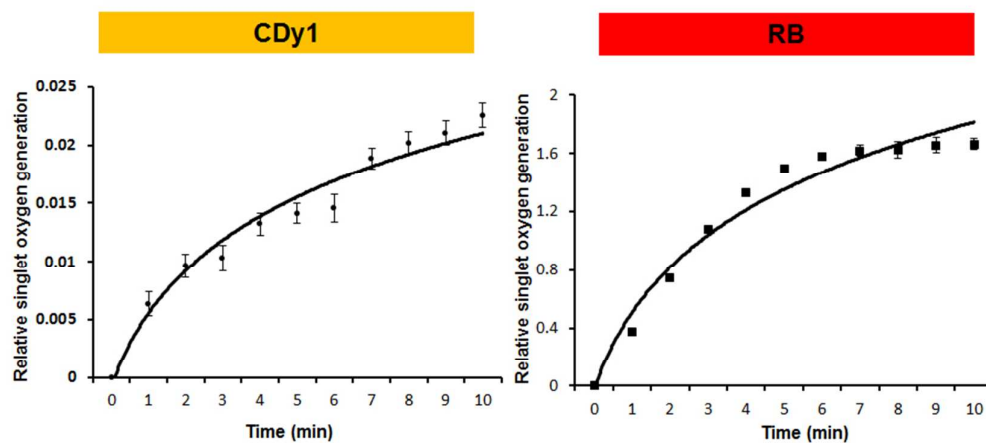


**Figure S1. A.** Intensity of red fluorescence of CDy1-stained (or non-stained) hPSCs and hECs by FACS analysis. (Scale bar, mPSC; 200  $\mu$ m, mEC; 100  $\mu$ m) Red fluorescence of mPSCs (left in B, red and blue in C) and mECs (right in B, green and yellow in C) in the absence and presence of CDy1 (50 nM) were measured by fluorescence microscopy **B.** and FACS analysis **C.** Quantitative data are presented as mean  $\pm$  SD.

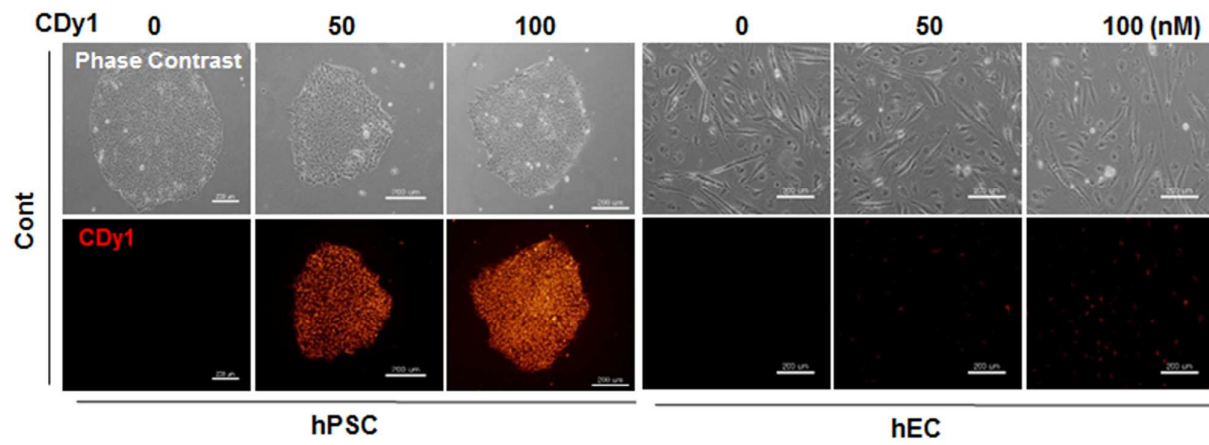


**Figure S2.** A. CD31<sup>+</sup> cell sorting from endothelial differentiation of human and B. mouse. PSCs and characterization of sorted CD31<sup>+</sup> cells by morphology, tubule formation assay, ac-LDL (red) uptake assay, and immunostaining of vWF (green) and PECAM (red) (Scale bar; 200 μm). Dapi staining (blue) for identifying the nucleus of cells.

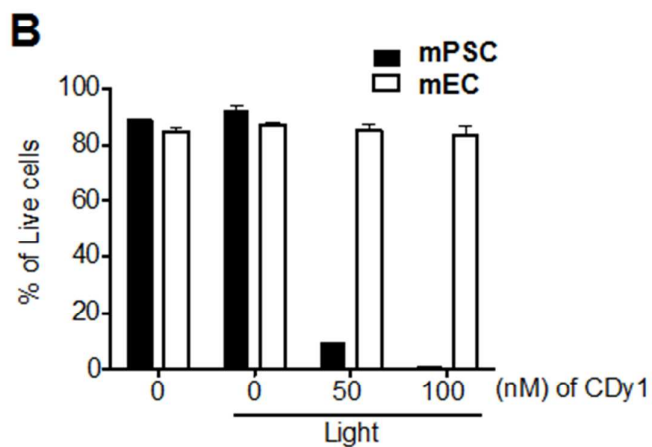
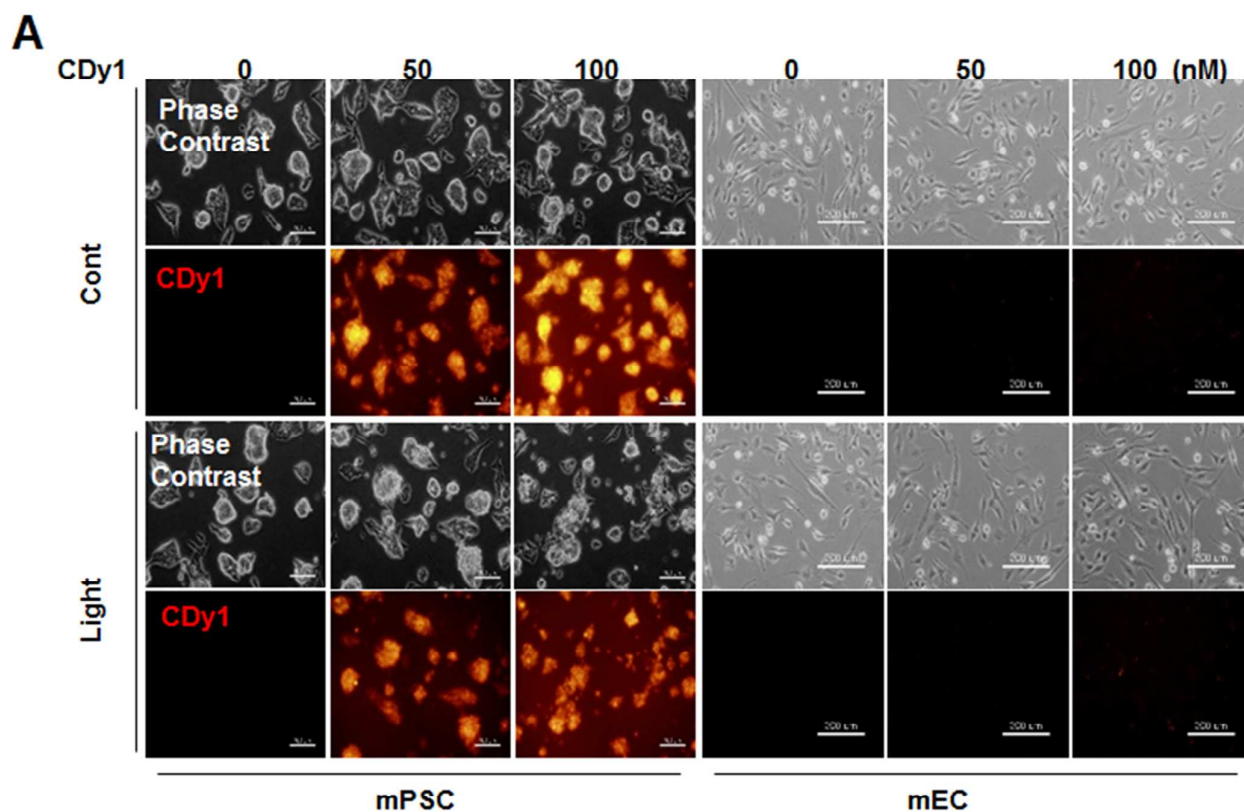




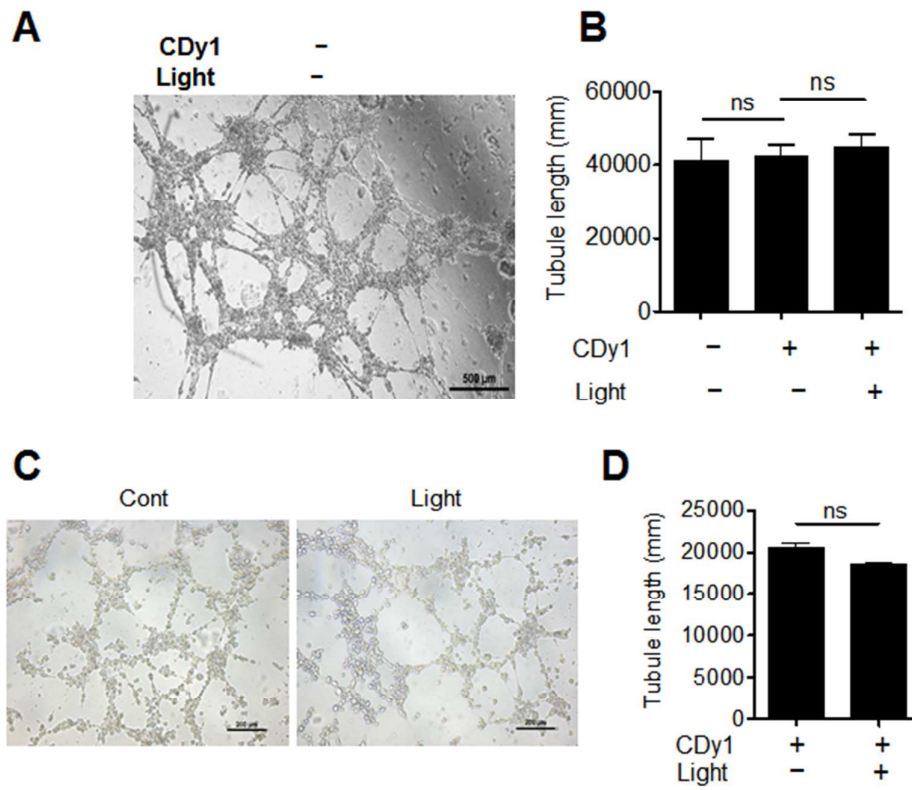
**Figure S3.** Quantification of CDy1 singlet Oxygen Generation Singlet-oxygen quantum yield (SQY) of CDy1(left) and RB(right). The quantum yield of CDy1 is 1.1% when Rose Bengal (76%) is used as a reference.



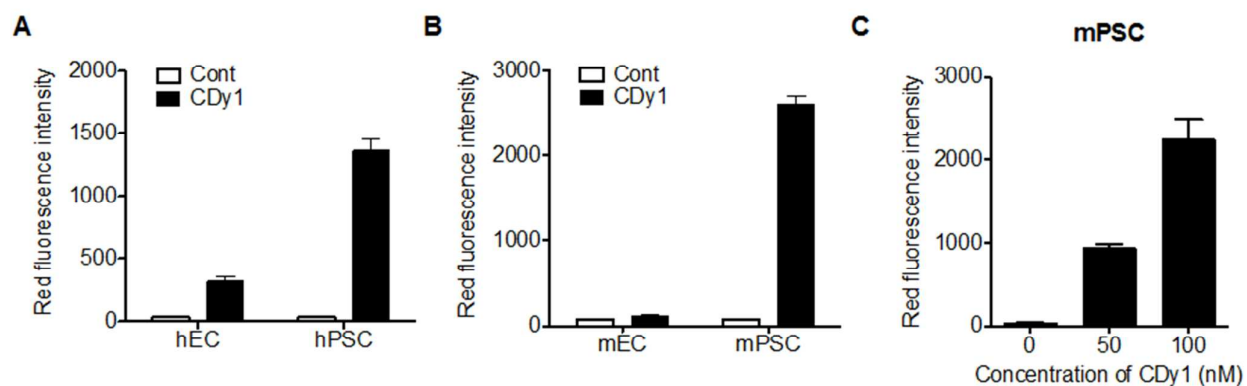
**Figure S4.** Phase contrast and fluorescence images of CDy1-treated hPSCs and hECs in a CDy1-dose dependent manner. (Scale bar, 200  $\mu$ m)



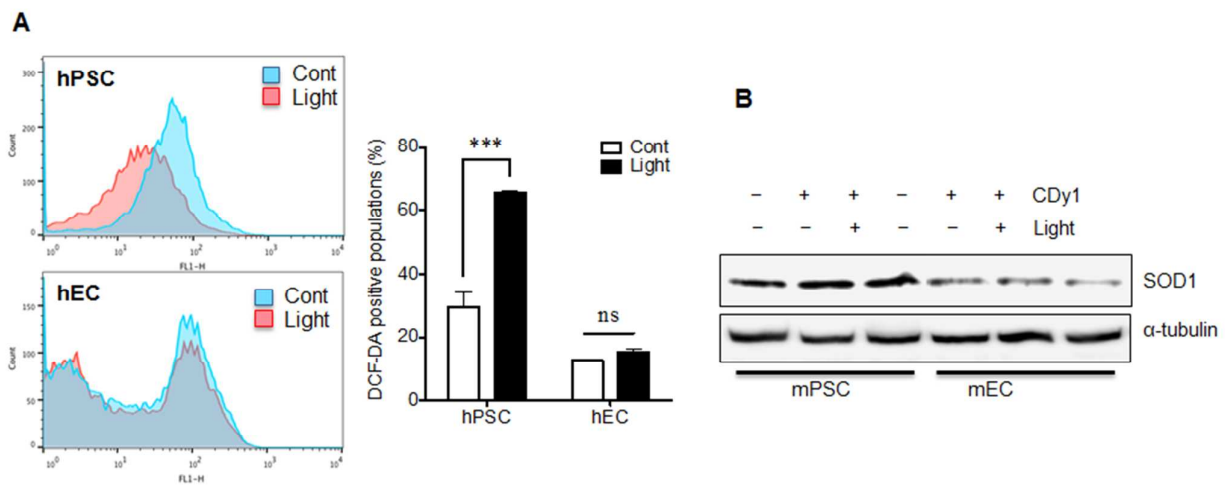
**Figure S5. A.** Phase contrast and fluorescence images of CDy1-stained or non-stained mPSC (left) and mEC (right) exposed to  $1.2 \text{ W/cm}^2$  of green light (532 nm), 1min (Scale bar, 200  $\mu\text{m}$ ). **B.** Live cell quantification by dual negative population of annexin V and 7-AAD after 5 hours of irradiation. Mock are cells without CDy1 and irradiation.



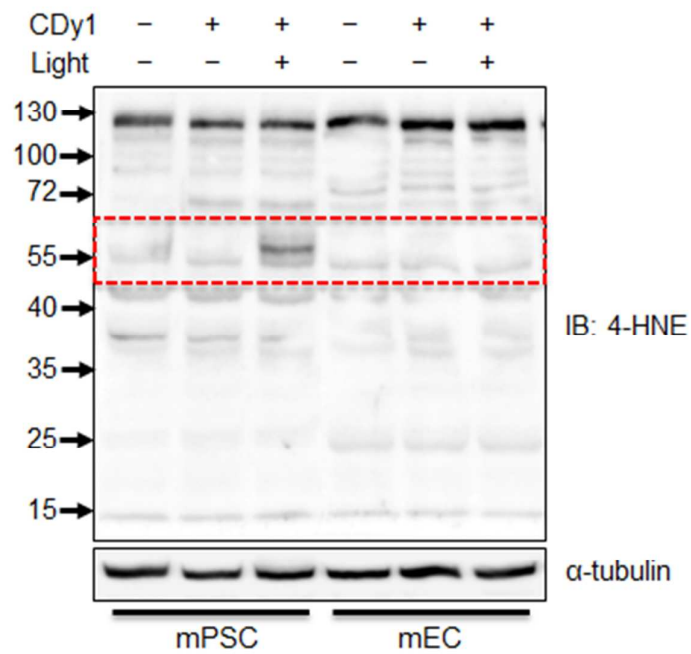
**Figure S6.** Tubule formation assay of CDy1-treated mECs (**A** and **B**) and hECs (**C** and **D**) was performed 24 hours after light exposure. (Scale bar, A; 500  $\mu$ m, C; 200  $\mu$ m) The results represent one of the experiments performed in triplicate plates. Quantitative data in B and D are presented as mean  $\pm$  SD. n/s; not significant



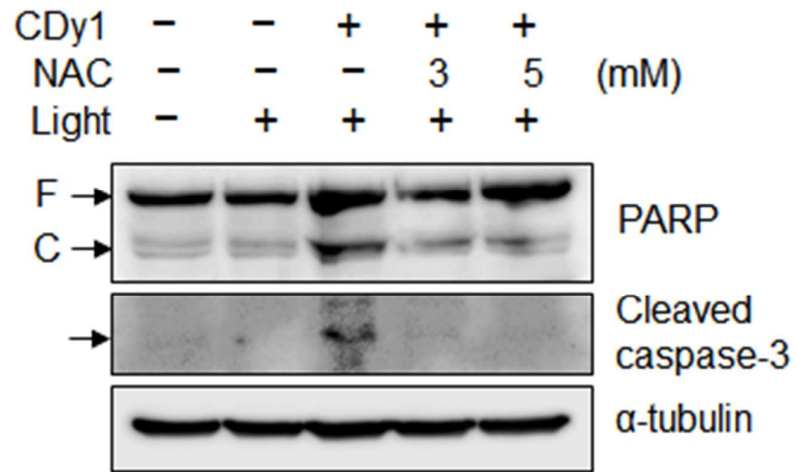
**Figure S7. A.** Detection of intracellular CDy1 after CDy1 staining CDy1-treated PSCs and ECs (human and mouse) were lysed by TLB lysis buffer. Red fluorescence intensities of lysates in hPSCs and hECs and **B.** in mPSCs and mECs were determined by fluorometer. **C.** Red fluorescence intensities of lysates in mPSCs treated with CDy1 in a dose-dependent manner were measured. Quantitative data represent mean  $\pm$  SEM of experiments performed in three independent studies.



**Figure S8.** A. ROS production and redox state in CDy1-treated hPSCs and hECs after light exposure. CDy1-treated cells were exposed to visible light, and the cells were incubated for 1 hour then stained with DCF-DA for 30 min. ROS levels were measured by flow cytometry. B. Protein level change of redox enzyme in CDy1-treated mPSCs and mECs after light exposure was monitored by immunoblotting for SOD1.  $\alpha$ -tubulin was used as an equal loading control.

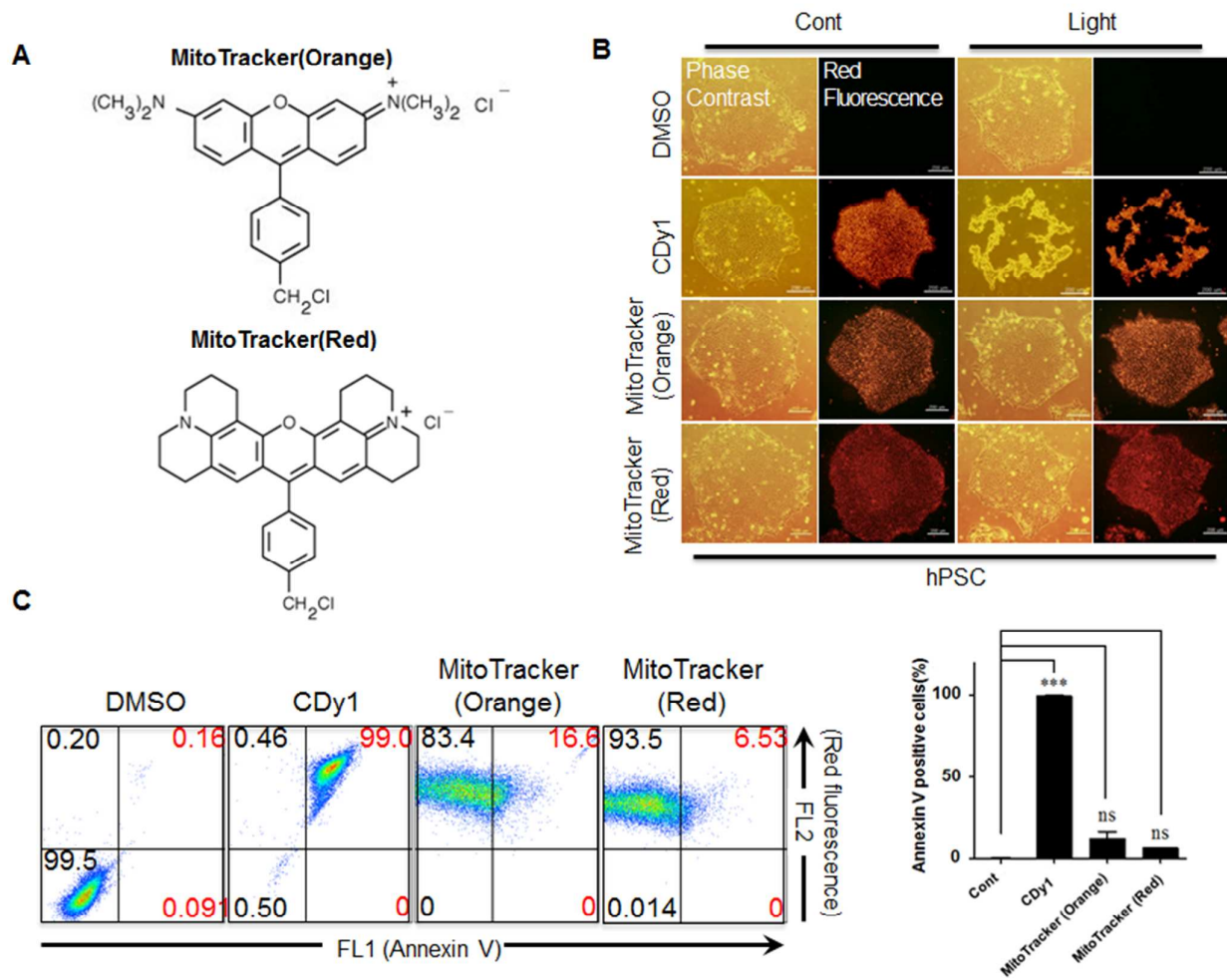


**Figure S9.** Light exposure promotes 4-HNE protein adduction in CDy1-stained mPSCs. CDy1-treated mPSCs and mECs were exposed to visible light. Immunoblotting show the level of 4-Hydroxynonenal (4-HNE) in CDy1-treated cells with or without light exposure. Red dotted line box indicates a ~55-60 KDa band sensitive to light exposure.  $\alpha$ -tubulin was used as an equal loading control.

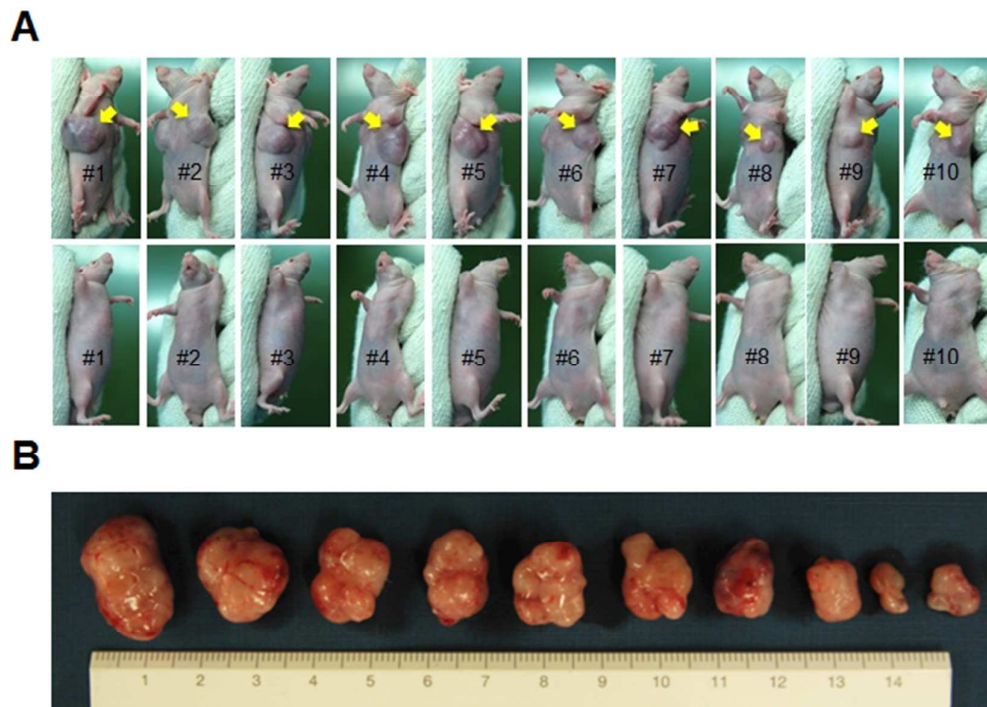


**Figure S10.** Light-induced cell death of CDy1-stained hPSCs pretreated with NAC (3 or 5 mM) for 2 hours prior to the light exposure. Apoptotic cell death of CDy1-stained hPSCs was measured by immunoblotting for cleaved PARP and caspase-3. (F; full PARP, C; cleaved PARP)  $\alpha$ -tubulin is for equal protein loading control.





**Figure S11.** Comparison of CDy1 and other fluorescent chemicals on cell death by light exposure. MitoTrackers (Orange and Red), which have similar structures with CDy1, were used for comparison of CDy1 and those fluorescent chemicals on cell death. **A.** Structures of MitoTracker (Orange and Red). **B.** Cell death of various fluorescent chemical-stained hPSCs by light exposure was measured by fluorescence microscopy and **C.** Annexin V staining and FACS analysis (Scale bar, 200  $\mu$ m). The results represent one of the experiments performed thrice. Quantitative data represent mean  $\pm$  SEM (\*\* $p$ <0.001, ns: not significant).



**Figure S12. A.** Introduction of CDy1-stained mPSCs with or without light exposure through subcutaneous injection. All images of mice and teratomas developed from CDy1-stained mPSCs with (0/10; zero out of ten mice) and without (10/10; ten out of ten mice) light exposure are shown. **B.** Teratomas extracted from animals are captured.

## Reference

(1) Amat-Guerri, F.; Lempe, E.; Lissi, E. A.; Rodriguez, F. J.; Trull, F. R. Water-soluble 1,3-diphenylisobenzofuran derivatives. Synthesis and evaluation as singlet molecular oxygen acceptors for biological systems. *J. Photochem. Photobiol., A* **1996**, *93*, 49-56.