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Fluorescent cell-selective ablation

using an adaptive photodynamic method

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Intravital ablation of particular cell population is necessary to decipher their role under spatiotemporal conditions. Energy transfer-based photodynamic therapy presented a conditional range for specifically inducing the death of GFP expressing cells, with little effect on normal cells. This novel system enables easy access to the functional study of cells.

The selective removal of a cell population or lineage allows us to identify their function. Numerous animal experiments with such a genetic manipulation have revealed the roles of particular cell populations. The ablations of a particular immune cell subset and specific marker-expressing stem cell were used to reveal their controversial functions.^{1–4} This may be a promising method; however, it is somewhat difficult to implement without design before the birth of new mice. Furthermore, the time and cost consumed by the process are significant. Photodynamic therapy (PDT) is a treatment modality using light and photosensitizers; the latter need to be activated by light at a specific wavelength. Activated photosensitizers convert tissue oxygen into singlet oxygen and free radicals, which can induce the death of cancer cells.⁵ herein, we used green fluorescent protein (GFP) as the linker of energy transfer between a specific wavelength light source and photosensitizers that share absorption spectrum with the emission spectrum of GFP. The applications of fluorescent

proteins such as GFP or red fluorescent protein in biological fields have been rapidly increasing with the development of fluorescent microscope and confocal microscope.^{6–9} As the conjugation of fluorescent peptide domain to a protein does not seriously interfere with cellular function and the location of original protein, fluorescent proteins are widely employed in cell dynamics studies including *in vivo* cellular tracking, cell–cell interaction, developmental studies of living animals, and cancer research including *in vivo* metastasis studies.^{10–13} In particular, by using transgenic animal models that exhibit fluorescence for cells with particular genes, researchers revealed the function of particular genes and the association with surrounding genes.^{14, 15}

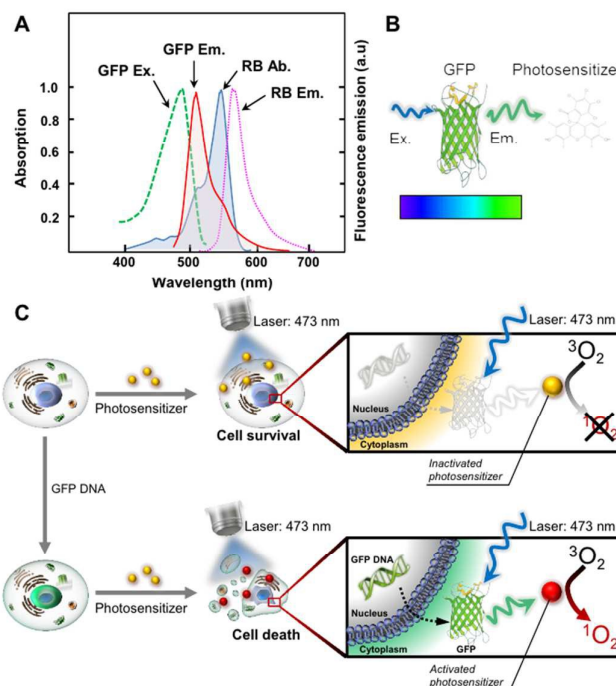


Figure 1. Concept of experiment using energy transfer between GFP and RB

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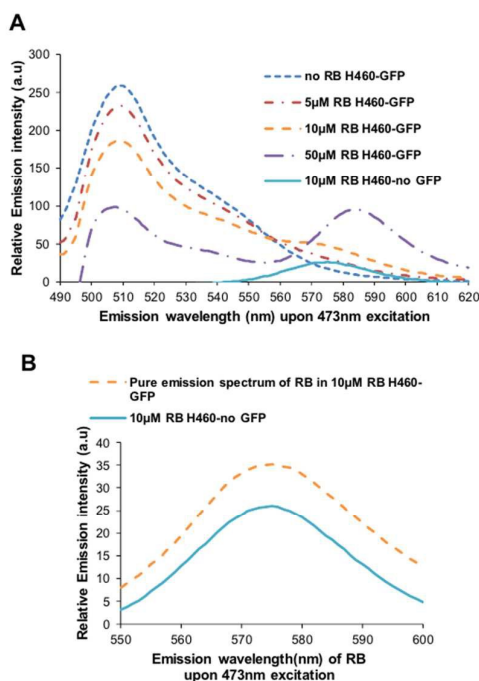


Figure 2. Emission spectrum of NCI-H460 cells according to RB concentration upon excitation at 473 nm

In this article, we verify the new function of GFP to target a cell population based on application of photodynamic method. GFP expressing cancer cells were used for proof of concept. Further, our technology may be easily applied to GFP expressing mice, which already developed to target a particular gene. To use GFP as the linker of energy transfer, wavelength of laser and absorption spectrum of photosensitizers are important. Although laser can excite GFP, laser shall have little effect on photosensitizers to minimize effects on GFP non-expressing cells. In addition, emission spectrum of GFP have to overlap absorption spectrum of photosensitizers to kill GFP expressing cells selectively. For this reason, we chose 473 nm blue laser and RB.

The maximum absorbance and shoulder absorbance spectra of RB which is photosensitizer was 549 nm and 510 nm, respectively¹⁶. Because the maximum absorbance and emission spectra of GFP are 489 and 508 nm, respectively¹⁷, the shoulder absorption range of RB overlaps with the emission spectrum of GFP when irradiated with 473 nm blue laser (Fig. 1A). Therefore, energy transfer from GFP to RB occurred upon excitation with blue laser light (Fig. 1B). RB was suggested to be activated relatively easily after irradiation with around 510 nm light, which was emitted by GFP in GFP expressing cells, and the activated RB generated radical oxygen species (ROS) from the surrounding oxygen-rich environment (Fig. 1C). However, because GFP non-expressing cells only absorbed light having 473-nm wavelength, the extent of RB activation was minimal.

To confirm energy transfer from GFP to RB, fluorescence in RB-treated NCI-H460 and NCI-H460-GFP cells after excitation at 473 nm was measured. Upon excitation at 473 nm, RB-treated NCI-H460 and NCI-H460-GFP cells showed peak emission spectra at 567 and 509 nm, which are the peak emission spectra of RB and GFP, respectively. The emission peak of GFP decreased and that of RB

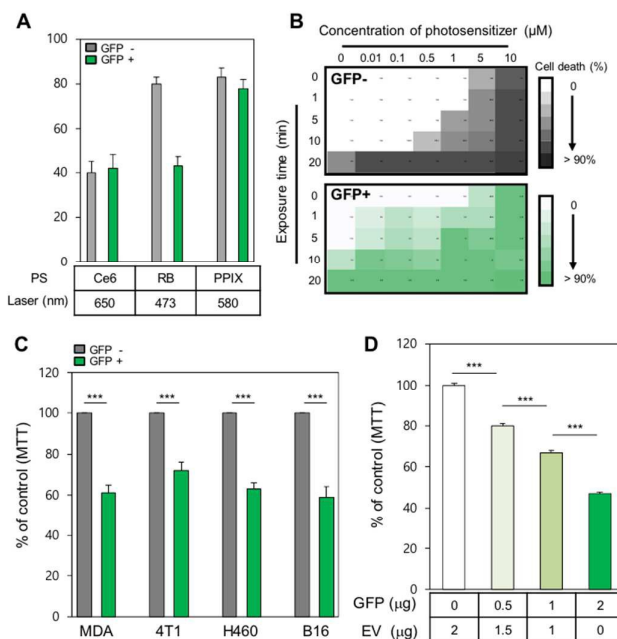


Figure 3. Measurement of cytotoxicity against GFP expressing and GFP non-expressing cells in vitro

gradually increased in NCI-H460-GFP cells with an increase in RB concentration upon excitation at 473 nm (Fig. 2A). Energy transfer efficiency in 10- μ M RB-treated NCI-H460-GFP cells was 0.279 at the peak emission spectrum of GFP (509 nm). Moreover, quenching of donor (GFP) fluorescence and linear enhancement of acceptor (RB) fluorescence were observed. This phenomenon may occur by an inner filter effect characterized by the decrease in donor (GFP) fluorescence owing to the absorption of excitation laser by acceptor (RB)¹⁸. To eliminate the interference of the inner filter effect, we performed a control experiment in which GFP⁻ cells were incubated with 10 μ M RB. The pure peak emission spectrum of RB in 10 μ M RB-treated NCI-H460-GFP cells was 35.3% higher than that in 10 μ M RB-treated NCI-H460 cells (Fig. 2B). Moreover, the pure emission energy of RB in 10 μ M RB-treated NCI-H460-GFP cells was 53.3% higher than that in 10 μ M RB-treated NCI-H460 cells. Thus, spectrum gap between the pure emission spectrum of RB in 10 μ M RB-treated NCI-H460-GFP cells and the emission spectrum of RB in 10 μ M RB-treated NCI-H460 cells can be considered as the energy transferred between GFP and RB upon excitation at 473 nm.

Energy transfer-induced selective cytotoxicity was validated in GFP expressing and GFP non-expressing cancer cells *in vitro*. Almost 60% of GFP expressing cells were selectively killed upon irradiation with 473-nm laser light after RB treatment compared with only 20% of GFP non-expressing cells (Fig. 3A). Lower death of GFP non-expressing cells than that of GFP⁺ cells can be attributed to singlet oxygen moieties produced by RB upon excitation at 473 nm. Other photosensitizers such as chlorin e6 (Ce6) and protoporphyrin IX, which are activated at 610 and 580 nm, respectively, did not specifically induce the death of GFP expressing cells. The number of dead cells varied depending on the concentration of the photosensitizer used and the duration of irradiation. Increase in RB concentration and irradiation time induced more deaths of GFP expressing cells compared with GFP non-expressing cells (Fig. 3B). However, extremely high RB concentration and extremely high exposure time exerted similar cytotoxic effects on both GFP

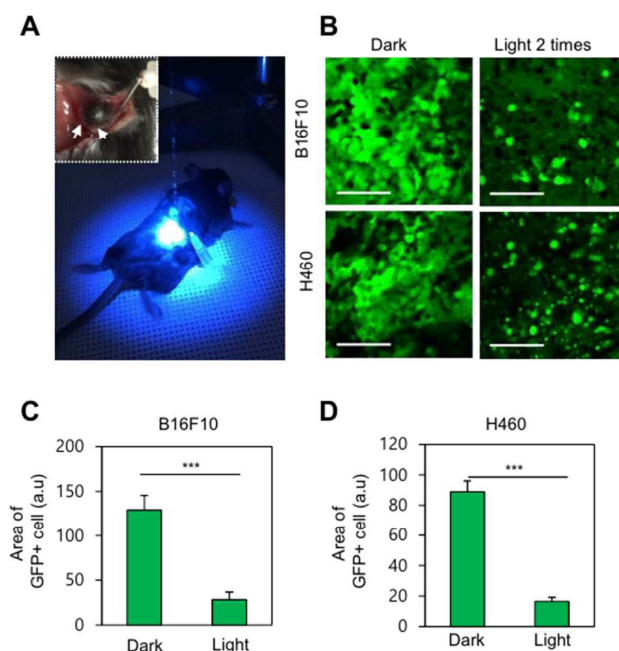


Figure 4. Selective killing of GFP expressing cells in the GFP expressing tumour cell-implanted mouse model

expressing and GFP non-expressing cells. To prevent bias according to the use of a particular cell line, we examined four cell lines from different tissues and species under the same conditions: two murine cell lines, namely, 4T1 mammary carcinoma and B16-F10 melanoma cell lines, and two human cancer cell lines, namely, MDA-MB-231 breast carcinoma and NCI-H460 non-small-cell lung carcinoma cell lines. We observed that GFP⁺ cells showed significantly higher cell death than GFP⁻ cells among these cancer cell lines examined (Fig. 3C). Although the extent of cytotoxicity appeared to be dependent on species and environmental condition, the gap of cell death tendencies of GFP expressing and GFP non-expressing cells were analogous. However, in the experiments using Ce6 and 650-nm red laser light which are unrelated to emission spectrum of GFP, no differences were spotted in the cytotoxicity against GFP expressing and GFP non-expressing cells using MTT assay (Supplementary Fig. 1). Moreover, energy transfer-induced cytotoxicity was directly proportional to the increase in the transfection efficiency of GFP (Fig. 3D, Supplementary Fig. 2).

Tumor suppression effect of energy transfer based PDT was also validated in GFP expressing cancer cells-implanted mice. A mixture of GFP expressing and GFP non-expressing NCI-H460 and B16F10 cells was implanted into the flank region of mice. Tumors in these mice were allowed to grow for 10–15 days, after which the tumors below the skin flap were repeatedly irradiated with blue laser light (473 nm) (Fig. 4A). Selective death of GFP expressing cells was observed after the second round of irradiation. GFP signals were relatively and significantly decreased in the irradiated areas compared with those before irradiation (Fig. 4B). Irradiation eliminated almost 80% of GFP expressing cells, resulting in the expansion of dark regions created by disappearance of GFP expressing tumor cells. Similar results were obtained for GFP expressing cells belonging to the different cancer cell types tested (Fig. 4C and D). The mice were also separately injected with GFP expressing or GFP non-expressing B16-F10 cells and subsequent

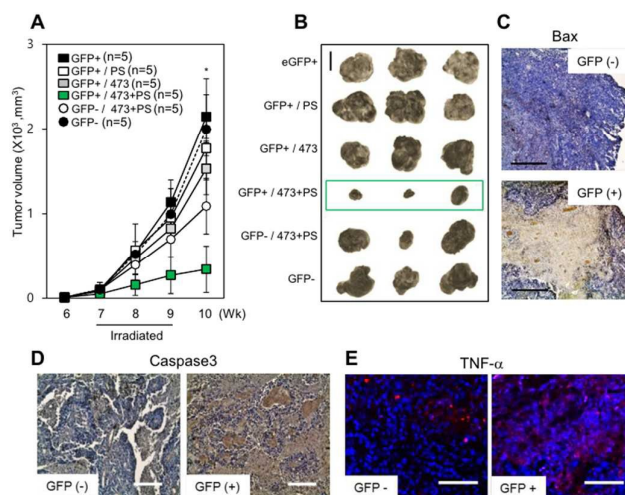


Figure 5. PDT based on energy transfer in the GFP expressing cells-implanted mouse model

tumor growth was monitored. Irradiation with 473-nm laser light slightly inhibited tumor growth from GFP expressing cells even in the absence of the photosensitizer. The combination of photosensitizers and subsequent irradiation with 473-nm laser light suppressed tumor growth more effectively in GFP expressing cells than in GFP non-expressing cells. However, statistical analysis by using Student's *t*-test showed that this inhibition was not significant. Irradiation with 473-nm laser light significantly inhibited tumor growth from RB-treated GFP expressing cells (Fig. 5A and B, Supplementary Fig. 3). Tumors in GFP expressing cell-implanted mice showed significant tissue damage and cell death. Next, we examined the expression level of cell death marker Bax to confirm the site of cell death (Fig. 5C). Extensive intra-tumoral damage was observed after treatment. Immunohistochemical analysis by using anti-caspase-3 and anti-TNF- α antibodies indicated that necrosis accounted for the death of most GFP expressing cells (Fig. 5D and E). We revealed the efficacy of *in vitro* and *in vivo* energy-transfer-based PDT and calculated the energy transfer efficiency by using changes in the emission spectrum according to RB concentration in GFP cell lines. The most important feature of this study is energy transfer, which is a fairly novel concept for explaining the harmonization of photon energy between cellular fluorescent proteins and photosensitizers. This phenomenon induced the selective death of GFP⁺ cancer cells irradiated with light of a specific wavelength. As there was no artificial manipulation of the distance between GFP and RB, the energy transfer observed in the present study is likely to be a radiative energy transfer that occurs when the distance between donor and acceptor is more than the Förster radius (~10–12 nm). However, Förster resonance energy transfer (FRET) may also account for the energy transfer observed in the present study, because the graded quenching of donor (GFP) fluorescence and linear enhancement of acceptor (RB) fluorescence, which are typical phenomena in the FRET^{19–21}, occurred. In order to rule out the interference of inner filter effect¹⁸, we observed changes in the RB fluorescence for the same concentration depending on whether the cells have GFP protein or not (Fig. 2).

In summary, our main purpose in this study was not to apply this PDT method to human patients, but to prove if fluorescence expressing cells can be responded selectively to light radiation. So,

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this novel PDT concept can be utilized for various purposes. It can be utilized for understanding the role of a gene. As there are already numerous mice types in which the *gfp* gene is genetically inserted, this method may be utilized to trace their cellular roles. In the future, we may induce the expression of GFP *in vivo* specifically in the targeted cells. This possibility was introduced by cancer-cell-specific GFP expression.²² Moreover, the development of a photosensitizer²³ or fluorescent protein²⁴ used to mutually adjust the wavelength might accelerate the process of application of this concept to human clinical trials. We conclude that this mechanism can be used in more diverse oncological and immunological fields on rare cell populations including cancer stem cells, circulating tumor cells, and several immune cell subsets.

Acknowledgements

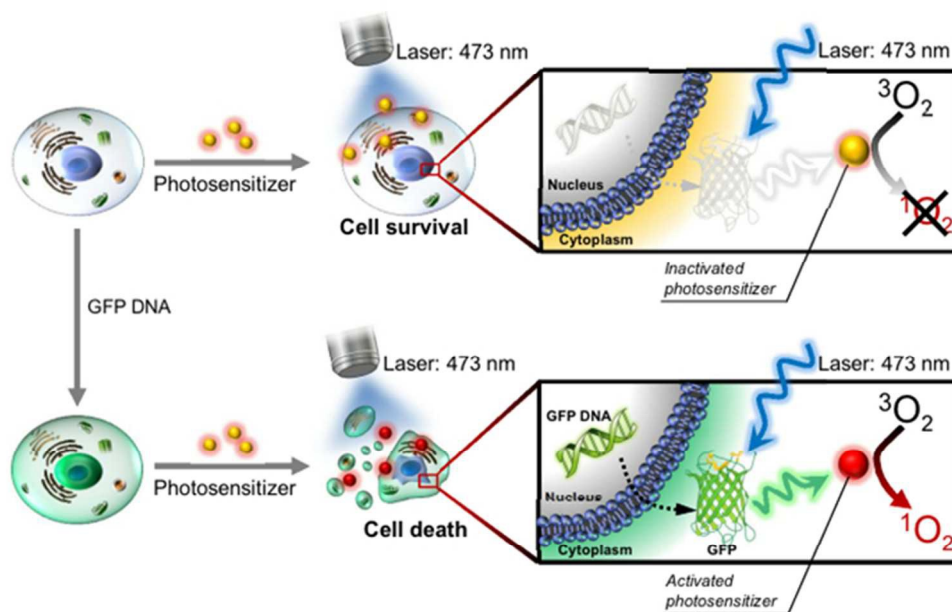
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Conflicts of interest

There are no conflicts to declare.

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