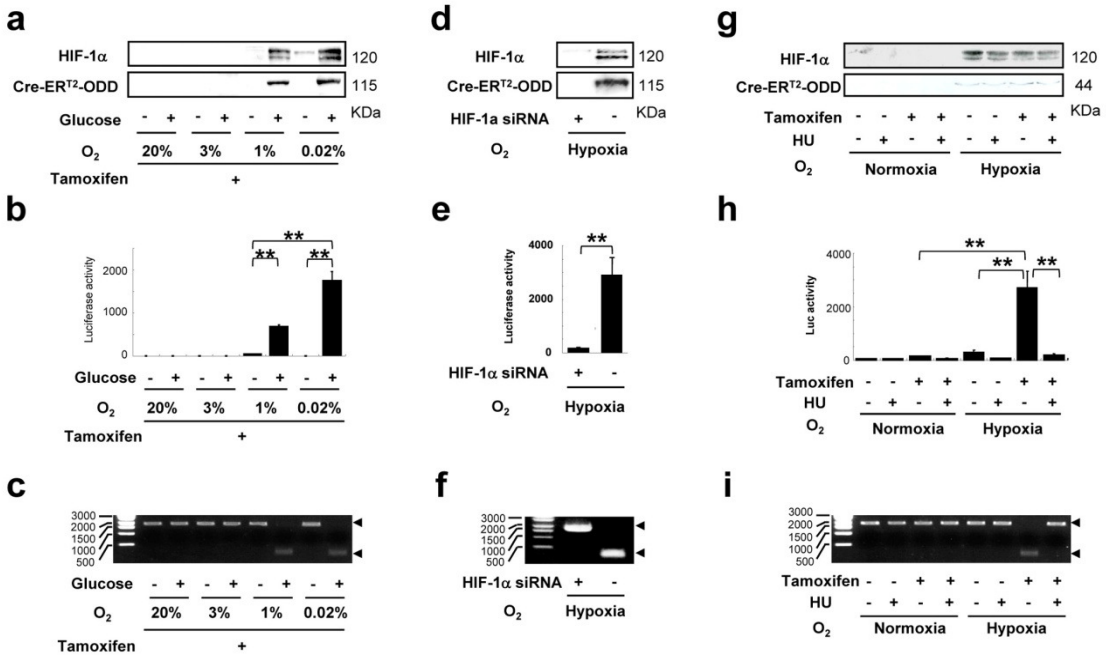
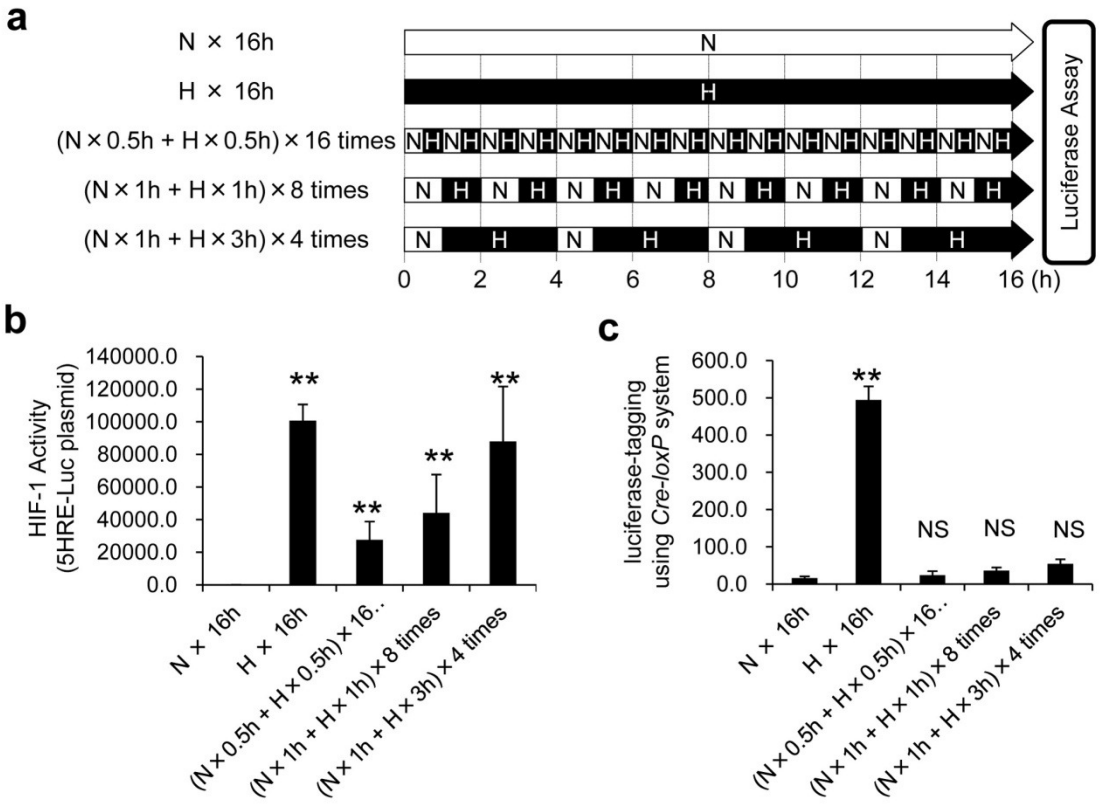
Cancer cells that survive radiation therapy acquire HIF-1 activity and translocate toward tumor blood vessels

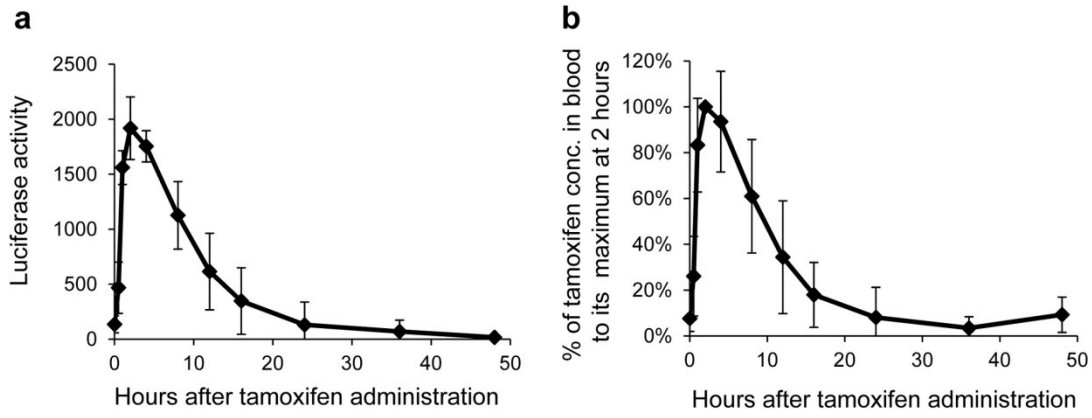
Supplementary Information



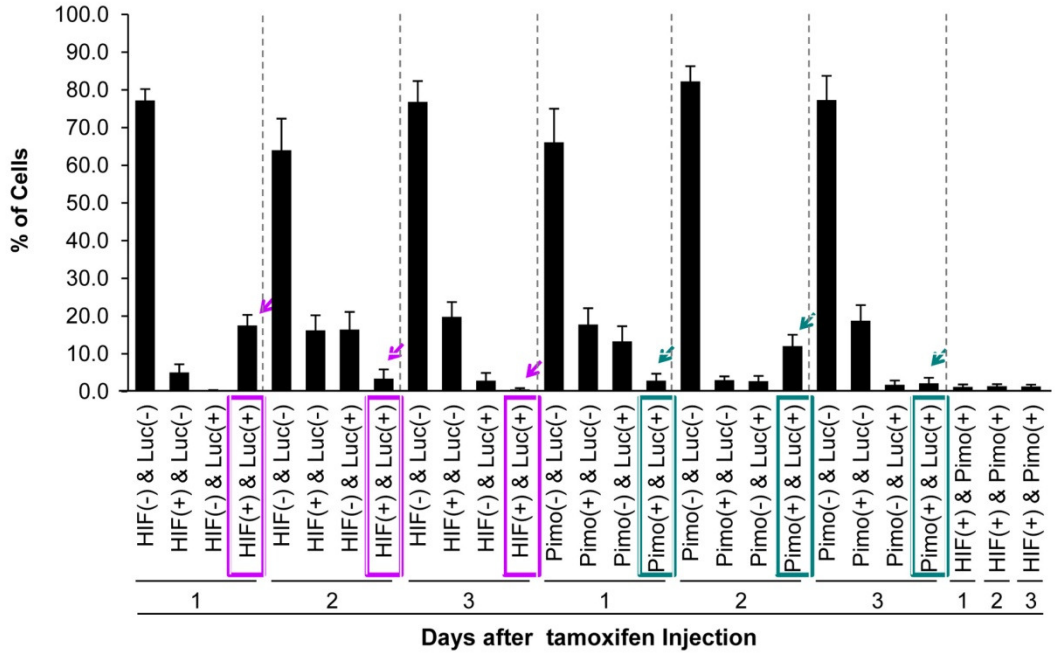
Supplementary Figure S1. Glucose-, Cell cycle progression-, HIF-1-, and tamoxifen-dependency of luciferase-tagging. (**a**) HEK293 stable transfectants with the *5HREp/Cre-ER^{T2}-ODD* gene and *CAGp/loxP-polyA-loxP-luciferase* gene were treated with tamoxifen in the presence (+) or absence (-) of glucose under the indicated O_2 tension for 24 h, and subjected to immunoblotting using anti-HIF-1α mouse monoclonal antibody (BD Biosciences; Working conc. = 0.5 μg/ml.) or anti-myc epitope tag mouse monoclonal antibody (for Cre-ER^{T2}-ODD fusion protein. Cell Signaling Technology, Inc.; × 2,000 dilution). (**b**) After the same treatment as **a**, cell lysate was subjected to luciferase assay. Mean ± *s.d.* n = 3. The statistical significance of differences was determined using Student's t-test. $^{**}P < 0.01$. (**c**) After the same treatment as **a**, genomic DNA was extracted and subjected to semi-quantitative genomic PCR assay. The primers used are shown in Fig. 1**a** as dotted arrows. (**d**) The cells were cultured under hypoxic condition (0.02% oxygen) with (+) or without (-) HIF-1α siRNA and subjected to immunoblotting using antibodies against HIF-1α, or the myc epitope tag for the Cre-ER^{T2}-ODD fusion protein. (**e**) After the same treatment as **d**, cell lysate was subjected to luciferase assay. Mean ± *s.d.* n = 3. $^{**}P < 0.01$ (Student's t-test). (**f**) After the same treatment as **d**, genomic DNA was extracted and subjected to the same semi-quantitative genomic PCR assay as **c**. (**g**) The cells were cultured with (+) or without (-) tamoxifen and hydroxyurea (HU; Working conc. = 1mM) under normoxic (20%) or hypoxic (0.02%) conditions for 24 h, and subjected to immunoblotting using anti-HIF-1α mouse monoclonal antibody or anti-myc epitope tag mouse monoclonal antibody for Cre-ER^{T2}-ODD fusion protein. (**h**) After the same treatment as **g**, cell lysate was subjected to luciferase assay. Mean ± *s.d.* n = 3. $^{**}P < 0.01$ (Student's t-test). (**h**) After the same treatment as **g**, genomic DNA was extracted and subjected to the same semi-quantitative genomic PCR assay as **c**.



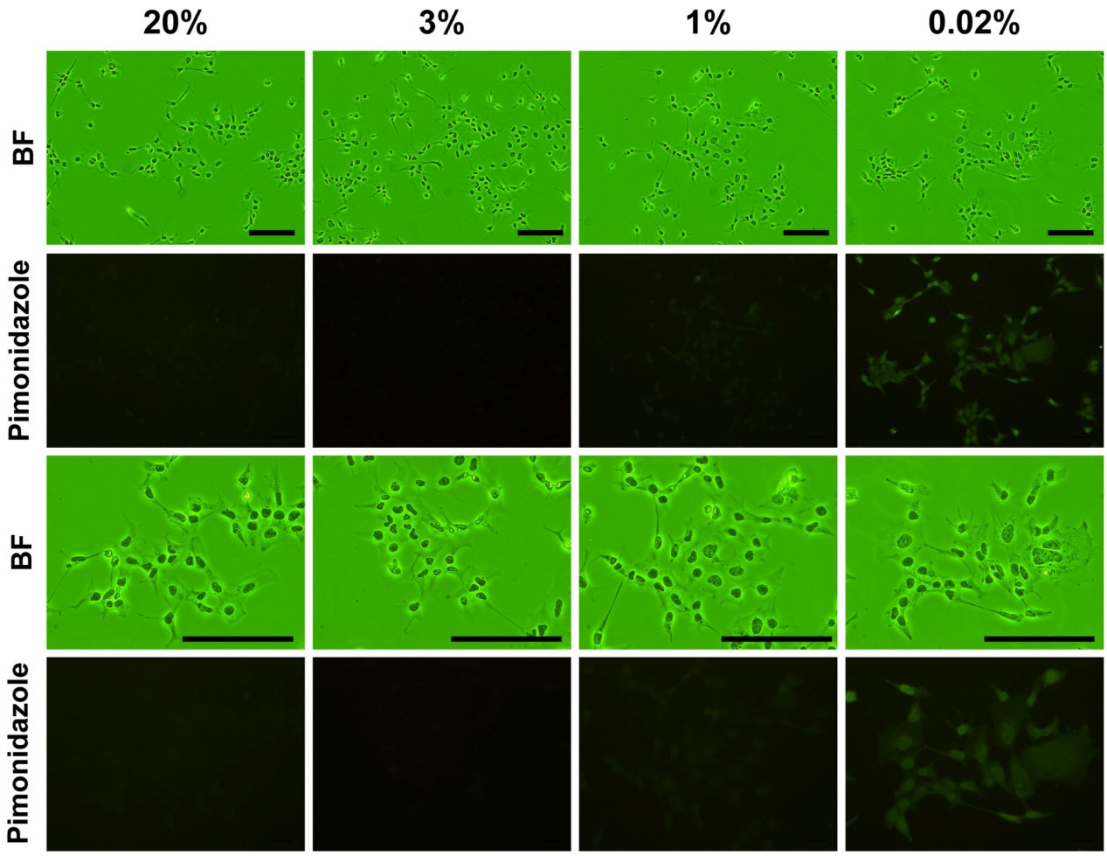
Supplementary Figure S2. Insufficient luciferase-tagging under various intermittent/cycling hypoxia *in vitro*. (**a**) Cells were treated under normoxia (N), hypoxia with 0.02% oxygen (H), or intermittent/cycling hypoxia with indicated intervals of normoxic treatments for a total of 16 h *in vitro* as schematically represented in the diagram. (**b**) HeLa/5HRE-Luc cells, which express luciferase bioluminescence under the control of a HIF-1-dependent 5HRE promoter[53,54], were treated under various oxygen conditions as represented in **a**, and subjected to a luciferase assay to quantify intracellular HIF-1 activity. Results are the mean ± *s.d.* $n = 3$. The statistical significance of differences was determined using Student's t-test. $^{**}p < 0.01$ vs N × 16 h. (**c**) Stable transfectants with both the *5HREp/Cre-ER^{T2}-ODD* gene and *CAGp/loxP-polyA-loxP-luciferase* gene were treated under various oxygen conditions as represented in **a**, and subjected to a luciferase assay to quantify the efficacy of luciferase-tagging in each condition. Results are the mean ± *s.d.* $n = 3$. $^{**}p < 0.01$ vs N × 16 h (Student's t-test).



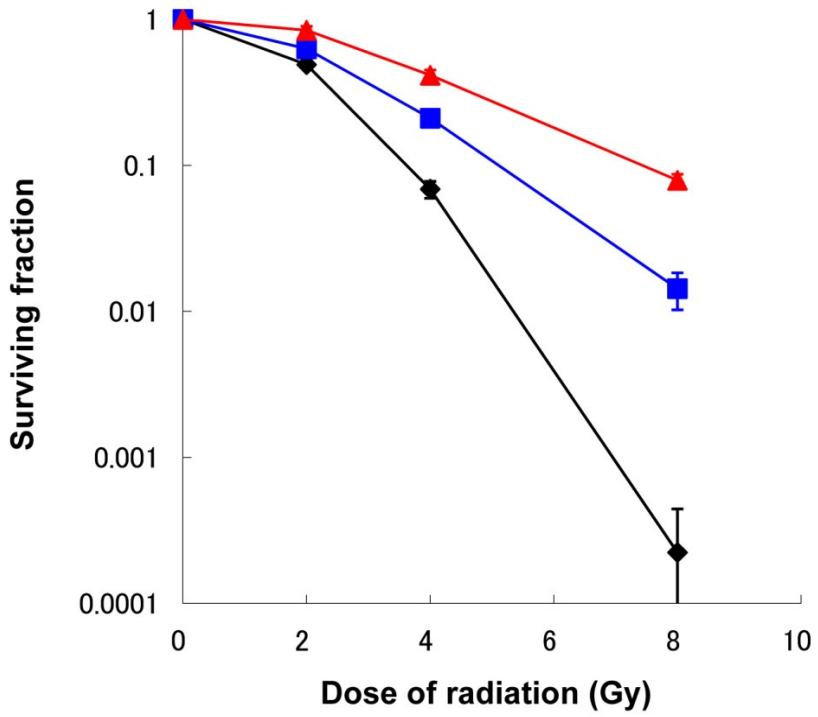
Supplementary Figure S3. Pharmacological half-life of tamoxifen in blood after administration into the footpad of tumor-bearing mice. (**a**) The tamoxifen solution was injected into the footpad of the tumor-bearing right leg, and blood was harvested at the indicated time after the tamoxifen administration. By centrifuging the blood, serum containing tamoxifen was separated from red blood cells and added to the culture medium of stable transfectants with both the *5HREp/Cre-ER^{T2}-ODD* gene and *CAGp/loxP-polyA-loxP-luciferase* gene. The cells were cultured under hypoxic conditions for 24 hours and subjected to a luciferase assay. Because luciferase activity fully depends on the concentration of tamoxifen contained in the serum, we can quantify the concentration of tamoxifen in blood at each time point indirectly but accurately. Results are the mean ± *s.d.* $n = 6$. (**b**) Luciferase activity at indicated time points was divided by that at 2 hours in order to quantify the percentage of the tamoxifen concentration in blood at each time point relative to the maximum concentration at 2 hours. Results are the mean ± *s.d.* $n = 6$.



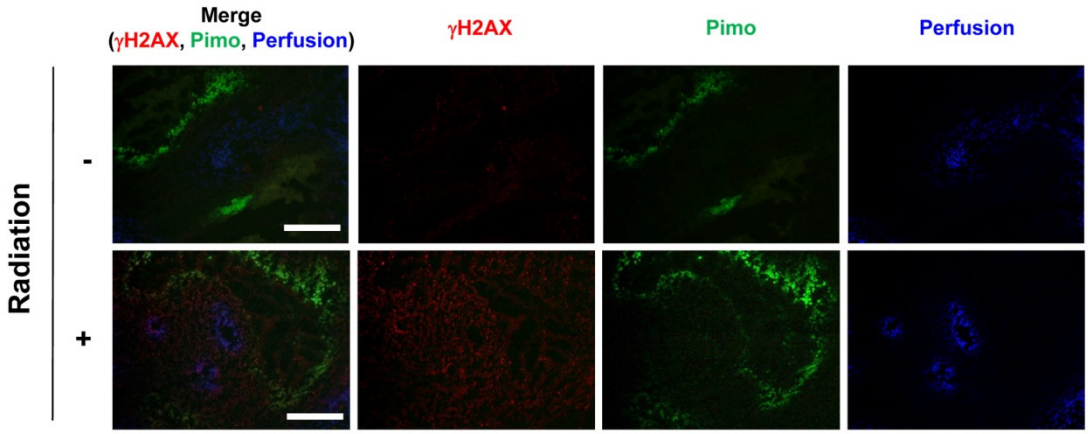
Supplementary Figure S4. Localization of luciferase-tagged cells in HIF-1-positive areas and pimonidazole-positive areas *in vivo*. Percentages of cells categorized into the indicated groups were quantified in the tumor sections in Fig. 3a with CellSens Dimension software (Olympus). Mean ± *s.d.* $n = 40$ tumor cords in 12 independent sections.



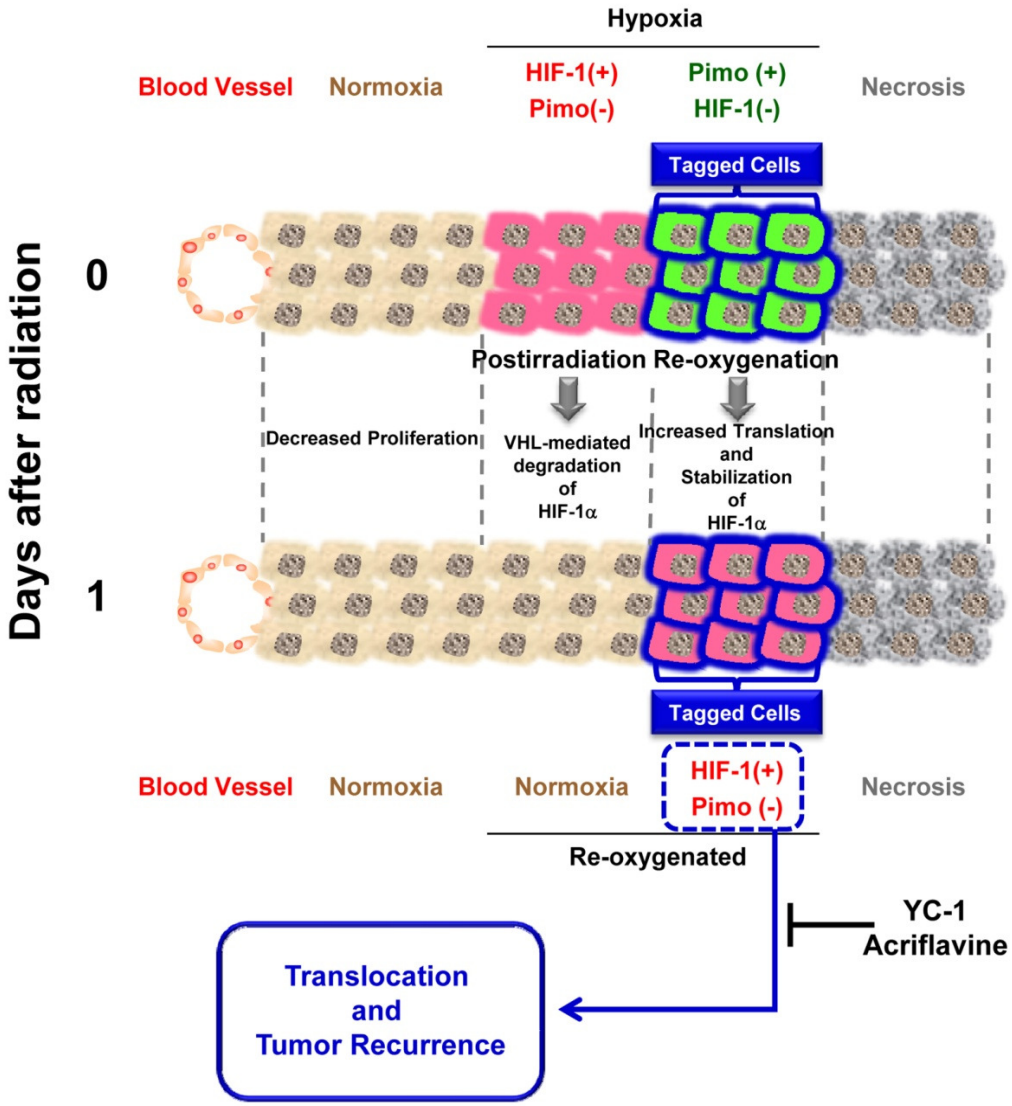
Supplementary Figure S5. Pimonidazole-staining of cells at various concentrations of oxygen. HEK293 stable transfectants were cultured with the indicated concentrations of oxygen for 24 hours and stained with a hypoxia marker pimonidazole according to the manufacturer's instructions (Natural Pharmacia International). Fluorescence was observed under an IX-71 fluorescence microscope (Olympus), and images were captured with a DP72 digital camera (Olympus). Bar = 50 μm.



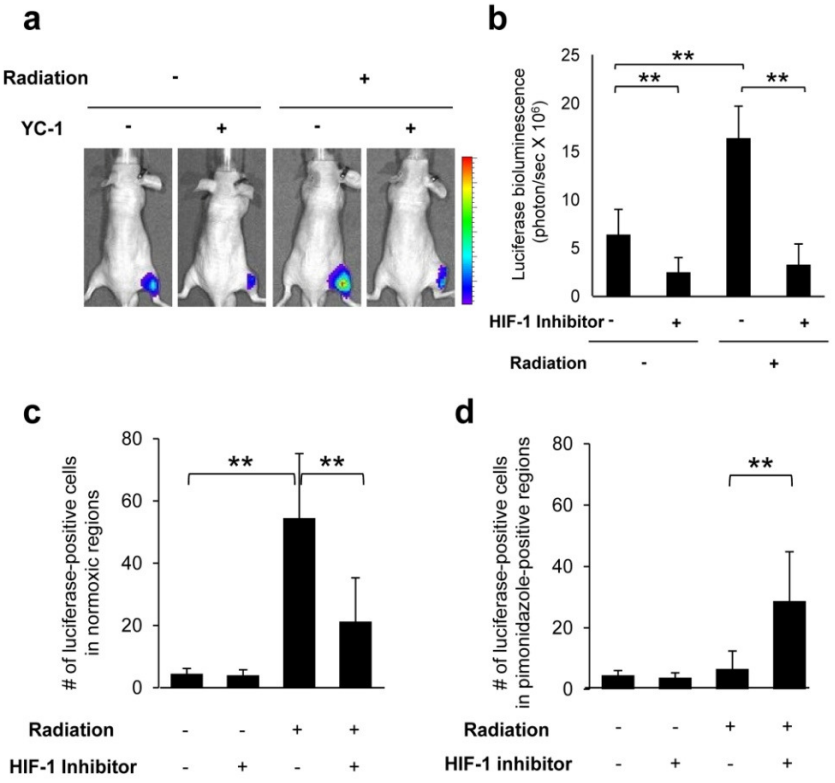
Supplementary Figure S6. Clonogenic survival assay at various oxygen concentrations. Cells were pre-cultured with the 0.02% (red triangle), 2% (blue square), and 20% (black rhombus) of oxygen for 24 h, irradiated with the indicated dose of radiation under the same oxygen conditions as the pre-culture, and then, subjected to a clonogenic cell survival assay. The plating efficiency (PE) and surviving fraction (SF) were calculated[55]. X axis: dose of X-radiation. Y axis: surviving fraction. Mean ± *s.d.* $n = 3$. Representative data of 3 independent experiments is shown.



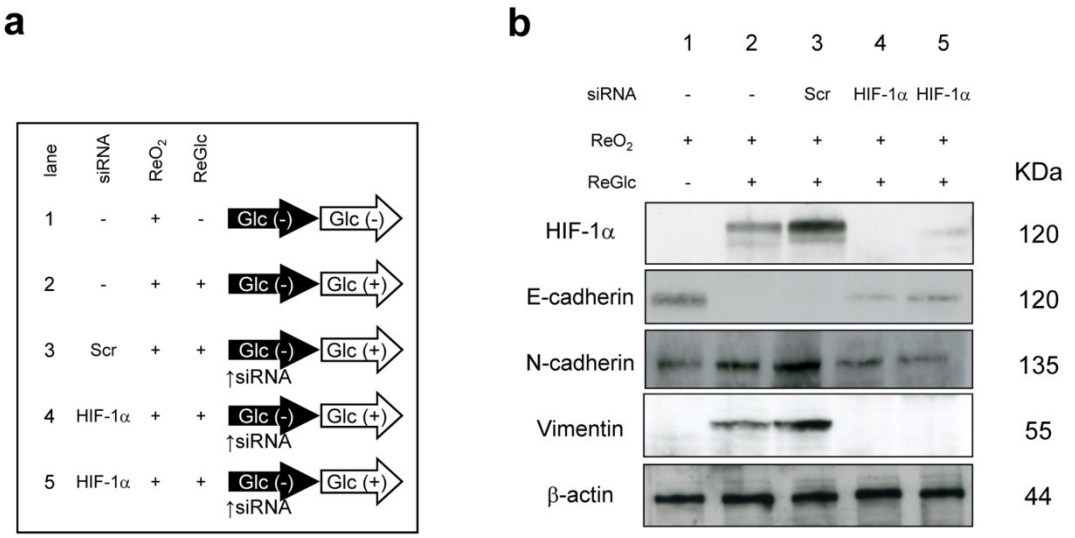
Supplementary Figure S7. Significant decrease in DNA damage in pimonidazole-positive hypoxic regions. Tumor xenografts of HEK293 stable transfectants were locally treated with ionizing radiation. The xenografts were surgically excised 30 min later, and frozen sections were stained with antibodies against pimonidazole (green) and γH2AX (red). Representative pictures are shown. Blue: perfusion marker, hoechst 33342, which was injected 1 min before sacrificing the mice. Bar = 100 μm.



Supplementary Figure S8. A model of the dynamic movement of hypoxic cells during tumor recurrence after radiation therapy. Radiation improves the availability of oxygen and nutrients in hypoxic regions, a phenomenon reported as the "re-oxygenation and re-glucose of hypoxic tumor cells"[56]. The phenomenon leads to both "VHL-dependent degradation of HIF-1α in the ex-HIF-1-positive/pimonidazole-negative area[57]" and "Akt-mTOR-dependent translation[56] and stabilization[58] of the protein in the ex-pimonidazole-positive/HIF-1-negative area. The latter is responsible for the translocation of the cells proximal to tumor blood vessels after radiation, and leads to tumor recurrence. HIF-1 inhibitors, YC-1 and acriflavine, dramatically suppress the radiation-induced activation of HIF-1, resulting in suppression of both translocation and subsequent tumor recurrence.



Supplementary Figure S9. Radiation-induced activation of HIF-1 is involved in translocation of radio-surviving cells. (**a**) Mice bearing a tumor xenograft of HeLa/5HRE-Luc cells, which express luciferase bioluminescence under the control of a HIF-1-dependent 5HRE promoter[53], were treated with or without radiation. YC-1 (+) was administered 1 min after the irradiation to suppress radiation-induced activation of HIF-1[57]. DMSO (-) was injected as a negative control. The mice were subjected to optical imaging 24 h after the radiation treatment to evaluate intratumoral HIF-1 activity. Representative imaging data are shown. (**b**) Luciferase bioluminescence in **a** was quantified. Results are the mean ± *s.d.* $n = 10$. The statistical significance of differences was determined using Student's t-test. $^{**}p < 0.01$. (**c**) The tumor-bearing mice with stable transfectants derived from HEK293 cells were irradiated (0 or 25 Gy) on the second day after the injection of tamoxifen (when the tagged cells were in the pimonidazole-positive area). A HIF-1 inhibitor, Acriflavine (+), or DMSO (-) was administered 1 min after the irradiation to suppress radiation-induced activation of HIF-1. Tumor xenografts were surgically excised 4 days after the irradiation and subjected to immunohistochemistry with both anti-luciferase and anti-pimonidazole antibodies. The numbers of luciferase-positive cells in normoxic regions were quantified. $n = 40$ tumor cords in 12 independent sections. $^{**}P < 0.01$ (Student's t-test). (**d**) The numbers of luciferase-positive cells in pimonidazole-positive regions in the sections of **c** were quantified. $n = 40$ tumor cords in 12 independent sections. $^{**}P < 0.01$ (Student's t-test).



Supplementary Figure S10. Shift in expression from an epithelial marker to mesenchymal markers in a HIF-1-dependent manner. (**a**) Cells were treated with or without siRNA for HIF-1α or a negative control (Scr) and cultured in glucose-deprived medium under hypoxic conditions for 18 h (black arrow). The cells were then cultured under normoxic conditions for reoxygenation (white arrow) in the presence (Glc+) or absence (Glc-) of glucose for an additional 6 h. (**b**) After being treated as shown in **a**, the cells were subjected to Western blotting using antibodies against HIF-1α (BD Biosciences; Clone 54; Working conc. = 0.5 μg/ml.), E-cadherin (abcam. ab15148. × 500 dilution.), N-cadherin (abcam. ab18203. Working conc. = 1 μg/ml.), vimentin (abcam. ab8069. Working conc. = 1 μg/ml.), and β-actin (BioVision. Clone B11V08. Working conc. = 2 μg/ml.).

Supplementary References

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