Supplementary material for Bol et al., Reprogramming of tumor metabolism by targeting mitochondria improves tumor response to irradiation, Acta Oncologica, 2015;54:266–74.

Supplementary Appendix

Material and methods

Cells and culture conditions

SiHa mitochondrial deficient (MD) cells were obtained by chronic exposure (8 weeks) to low concentrations of ethidium bromide (50 ng/μl) and subsequent clonal selection and were then cultured in the same media as WT parental cell line but supplemented with uridine (50 μg/ml) and pyruvate (1mM) at 5% CO₂, 21% O₂ and 37°C. The phenotype of the selected MD cells was preserved (at least) up to 20 passages and was assessed in vivo by immunostaining of the mitochondrial encoded protein subunit 2 of COXIV.

Immunofluorescence for mitochondrial encoded protein subunit 2 of COXIV

After fixation in cold methanol (5 minute), tumor cryosections (5-μm thick) were incubated with primary antibody (ab16056, 1 μg/ml, Abcam) overnight at 4°C, followed by 1 hour incubation at room temperature with Alexa Fluor 488-conjugated anti-rabbit immunoglobulin (1/1000, Life Technologies, Thermo-Fisher Scientific Inc., Rockford, IL, USA). DAPI was used to stain the cell nuclei. Images were scanned with a Zeiss Mirax fluorescence microscope (Carl Zeiss, Jena, Germany).

Pimonidazole staining and fluorescence microscopy

At the time the tumor mean diameter reached approximately 8 mm, mice were injected i.p.

with saline containing 60 mg/kg body weight of Hypoxyprobe™-1 solution. This solution distributes to all tissues and forms adducts with thiol-containing proteins in cells presenting a pO₂ lower than 10 mmHg. After 90 minutes, tumors were dissected and embedded in Tissue-Tek OCT compound and subsequently frozen in liquid nitrogen-cooled isopentane. After fixation in acetone, cryosections (5-μm thick) were incubated with diluted (1/20) Rabbit anti-pimonidazole antisera PAb2627 overnight at 4°C followed by 1 hour incubation at room temperature with Alexa Fluor 488-conjugated anti-rabbit immunoglobulin (1/100, Life Technologies). Images were scanned with a Zeiss Mirax fluorescence microscope (Carl Zeiss).

Quantification of microregional pimonidazole intensities as a function of blood vessels

After creating a binary image of the total blood vessels (threshold on CD31 staining), this binary image was next inverted and a ‘distance filter’ applied, which replaces the intensity of each pixel with intensity proportional to the distance of that pixel from the nearest vessel. This distance filtered image was then successively thresholded and binarized to select regions of the image within specific distances from vessels leading to concentric zones of 10 μm. Then, area of hypoxia (pimonidazole positive) was also converted to a binary image (by using an interactive threshold, the same used for calculating hypoxic fraction). Both images were combined (using a logical ‘AND’) and hypoxia intensities within each successive concentric zone were also obtained and median intensity levels were plotted as a function of distance from the nearest vessel.

Supplementary Figure 1. Representative picture of immunofluorescence detection for mitochondrial encoded protein anti-Oxphos Complex IV subunit II in WT and MD tumor sections. Depletion of mitochondria in MD tumors results in dramatically decrease of subunit II detection.

Supplementary Figure 2. (A, B) Quantification of ^{18}F -FDG and ^{18}F -FAZA uptake expressed as SUV Max and tumor to background ratio as a measure of tumor glucose uptake and hypoxic fraction, respectively.

Supplementary Figure 3. Representative images illustrating differences in hypoxia distribution in WT and MD tumor sections stained for blood vessels (CD31 in red), pimonidazole (hypoxyprom in green) and nucleus (DAPI in blue). Scale bar 2000 μm .

Supplementary Figure 4. Representative images illustrating microregional differences in pimonidazole (hypoxyprom in green) distribution as a function of blood vessels (CD31 in red).

Supplementary Figure 5. Tumor growth rates expressed as mean day for reaching the endpoint in WT and MD groups irradiated (IR) or not. **, $p < 0.01$ ANOVA1 ($n = 4-5$).

Supplementary Figure 6. Dose-dependent radioresistance in the five cell lines is expressed as an increase in survival fraction (SF) for cells irradiated in a closed system when compared to irradiation at an equivalent dose in open wells ($n = 3$).