[Cancer Biology & Therapy 6:10, 1-1, EPUB Ahead of Print: http://www.landesbioscience.com/journals/cbt/article/4948; October 2007]; ©2007 Landes Bioscience

Research Paper

Multimodality Optical Imaging and 18F-FDG Uptake in Wild-Type p53-Containing and p53-Null Human Colon Tumor Xenografts

Shulin Wang
Akiva Mintz
Kenjiro Mochizuki
Jay F. Dorsey
Joseph M. Ackermann
Abass Alavi
Wafik S. El-Deiry*

Laboratory of Molecular Oncology and Cell Cycle Regulation; Departments of Medicine (Hematology/Oncology), Genetics, Pharmacology, and Radiology; The Institute for Translational Medicine and Therapeutics, and the Abramson Comprehensive Cancer Center; University of Pennsylvania School of Medicine; Philadelphia, Pennsylvania USA

*Correspondence to: Wafik S. El-Deiry, M.D., Ph.D.; Department of Medicine (Hematology/Oncology); Abramson Comprehensive Cancer Center; University of Pennsylvania School of Medicine; Philadelphia, Pennsylvania 19104 USA; Email: wafik@mail.med.upenn.edu

Original manuscript submitted: 08/31/07
Manuscript accepted: 08/31/07

This manuscript has been published online, prior to printing for Cancer Biology & Therapy, Volume 6, Issue 10. Definitive page numbers have not been assigned. The current citation is: Cancer Biol Ther 2007; 6(10):
http://www.landesbioscience.com/journals/cbt/article/4948
Once the issue is complete and page numbers have been assigned, the citation will change accordingly.

KEY WORDS

p53, glycolytic metabolism, Warburg effect, FDG-PET imaging, cancer, bioluminescence imaging, fluorescence imaging, PET-CT

ACKNOWLEDGEMENTS

This work was supported in part by National Institutes of Health grants CA 75138, CA 75454, U54 CA105008 and by the the Littlefield-AACR award for research on metastatic colon cancer (W.S.E-D.).

ABSTRACT

During tumor development a switch to glycolytic metabolism known as the Warburg effect may provide cancer cells with a survival advantage and may also provide a therapeutic opportunity. A number of signals contribute to aerobic glycolysis including those mediated by HIF-1, c-Myc, Akt and Hexokinase. Recent studies have implicated the p53 tumor suppressor as a negative regulator of this switch. Using inducible p53 gene silencing in bioluminescent tumor xenografts we initially observed qualitatively similar levels of FDG uptake by PET small animal imaging in wild-type p53-expressing tumor xenografts and p53 gene-silenced xenografts. We further evaluated glucose uptake using FDG-PET/CT fusion imaging of green and red fluorescently-labeled wild-type and p53-null human colon tumor xenografts. Our results demonstrate that the wild-type p53-expressing tumor xenografts exhibit high levels of glucose uptake, similar to those observed in p53-null tumor xenografts, by quantitative PET imaging indicative of the glycolytic switch. Thus p53 function is not sufficient to suppress glucose uptake in cells and tumors that could theoretically support aerobic glycolysis.

INTRODUCTION

The pathogenesis of cancer involves the deregulation of various cellular pathways that orchestrate cell growth and death. It is clear that some of the changes in the pathways modulate cellular metabolism. As described by Otto Warburg about 80 years ago, virtually all cancers utilize glycolytic pathways for energy generation despite the availability of oxygen while downregulating their aerobic respiratory activity.[1] Given that glycolysis produces energy (ATP) much less efficiently than aerobic respiration, tumor cells have a much higher rate of glucose uptake than normal cells. Several mechanisms have been proposed to explain the Warburg effect. Akt was found to stimulate glucose utilization without increasing oxygen consumption leading to a shift toward aerobic glycolysis-a characteristic of many malignant cells.[2] HIF-1α has been implicated as an important regulator of glycolysis through its ability to increase the expression of genes encoding the glucose transporters and glycolytic enzymes.[3] c-Myc promotes aerobic glycolysis through directly up-regulating the glycolytic genes independent of hypoxia.[4]

As a transcription factor, the tumor suppressor p53 plays a major role in preventing cancer development. The cellular response to p53 activation is determined by differential regulation of its downstream target genes which leads to cell cycle arrest, apoptosis or senescence. The outcome of p53 activation contributes to tumor suppression by preventing or repairing the damaged DNA or elimination of potentially oncogenic cells from the proliferating population.[5] Recent results have implicated the p53 tumor suppressor protein as a regulator of mitochondrial respiration and as an inhibitor of the Warburg effect.[6] Matoba et al. identified Synthesis of Cytochrome c Oxidase 2 (SCO2) as a downstream mediator of p53 signaling and a target that promotes oxygen utilization in eukaryotic cells.[6] Data from this group indicates that the loss of p53 leads to a metabolic shift away from aerobic respiration towards the production of glycolytic ATP.[6] Bensaad et al. reported that the product of the p53 target gene, TIGAR (TP53-induced glycolysis and apoptosis regulator) is involved in the regulation of glucose metabolism; TIGAR blocks glycolysis thus promoting the activity of the pentose phosphate shunt.[7]

In the present study, we adopted ^{18}F Fluoro-2-deoxyglucose positron emission tomography (FDG-PET) to assess a fundamental property of neoplasia, the Warburg effect. FDG-PET is based on the reliance of tumor cells on glycolysis for energy even under

©2007 LANDES BIOSCIENCE. DO NOT DISTRIBUTE.

aerobic conditions.[8] Surprisingly, we found that wild-type p53-expressing tumors are still proficient at FDG uptake as compared to p53-null tumors. While p53 deficiency promoted tumor growth, evident by optical bioluminescence imaging and FDG uptake, but this did not lead to greater FDG uptake when quantified and controlled for tumor volume. Our results demonstrate that wild-type p53 is not sufficient to suppress FDG uptake in tumors which could permit aerobic glycolytic metabolism within such tumors.

MATERIALS AND METHODS

Generation of inducible p53 gene silencing and bioluminescence imaging model system. The generation of a doxycycline inducible p53 gene silencing cell line has been (described in ref. 9). Briefly, HCT116 cells, which stably co-express the PG13-luc, a p53 reporter with a Firefly luciferase gene and pRL-SV40 vector containing a cDNA encoding *Renilla* luciferase, were first transfected with a Tet repressor-expressing vector pcDNA6/TR (Invitrogen, Carlsbad, CA) alone followed by pSUPERIOR-puro-p53 plasmid along with pcDNA6/TR at a ratio of 1:2 (w:w). At 48 h after transfection, the cells were split at 1:10 dilution and grown in medium containing 0.5 μg/ml puromycin (Sigma, St. Louis, MO) and 20 μg/ml Blasticidin hydrochloride (Invitrogen).

Generation of stable GFP-expressing HCT116 and RFP-expressing p53-Null HCT116 tumor cells. To generate the fluorescent cells, pSiren-RetroQ-ZsGreen1 (Clontech, Mountain View, CA) or pSiren-RetroQ-DsRed-Express-N1 retroviral vectors were introduced into HCT116 $p53^{+/+}$ or HCT116 $p53^{-/-}$ cells, respectively. DsRed-Express-N1 was cloned from pDsRed-Express-N1 (Clontech) into pSiren-RetroQ-ZsGreen1 in place of the coding sequence for ZsGreen1 to create pSiren-Retro-DsRed-Express-N1. This vector was chosen due to the robust expression of the fluorescent protein. A non-specific control shRNA is also expressed by pSiren-RetroQ-ZsGreen1 and pSiren-Retro-DsRed-Express-N1, but should have no bearing on these studies. Either vector was transfected into Phoenix Ampho cells using Lipofectamine 2000 (Invitrogen). After 2 days, the supernatant from Phoenix Ampho cells transfected with pSiren-Retro-ZsGreen1 or pSiren-Retro-DsRed-Express-N1 was used to infect HCT116 $p53^{+/+}$ or HCT116 $p53^{-/-}$ cells. Cells expressing the respective fluorescent proteins were selected 3 times by FACS analysis to obtain stable-expressing ZsGreen or DsRed cells.

Inducible RNAi-mediated in vivo bioluminescence imaging analysis of tumor growth in mice. To analyze the effect of inducible p53 silencing on the tumorigenic capacity of HCT116 cells, 1 x 10^6 HCT116 cells stably transfected with either wild-type p53 or the p53 RNAi inducible plasmid were injected subcutaneously in each flank of female athymic *nu/nu* mice (Charles River Laboratory). To induce silencing of p53 in vivo, 5% sucrose and 2 mg/ml doxycycline was added to the drinking water. For prolonged induction, doxycycline-containing water was changed every three days. At the indicated time point, coelenterazine (0.7 mg/kg body weight) (Native Coelenterazine) (Biotium, Hayward, CA) was injected into the tail-vein. Bioluminesence images were acquired using a cooled charge coupled device (CCD) camera in the in vivo imaging system (IVIS, Xenogen, Alameda, CA) (as previously described in refs. 11 and 12). For the short term induction studies, four tumors were injected subcutaneously in each mouse, which were kept on doxycycline free diets until the tumors reached ~500 mm^3. At 72, 48 or 24 hours prior to the tracer uptake experiments, the mice were induced with doxycycline as described above.

In vitro ^{18}F-fluorodeoxyglucose (FDG)-uptake studies. In vitro uptake studies were performed as previously described. Briefly, 100,000 cells were plated in 6-well tissue culture flasks and incubated with or without doxycycline for 72 hours, during which media was changed (+/- doxycycline) every 24 hr. For the uptake studies, 1uCi of labeled tracer was added to the media and plates were incubated for 1 hr at 37°C and 5% CO2. All tracers were either purchased from American Radiolabeled Chemicals, Inc (ARC, St. Louis, MO) or obtained from the cyclotron at the University of Pennsylvania. Following incubation, cells were washed three times with cold PBS and lysed with 800 μL of 0.1 N NaOH. A sample (100 μL) of each radiotracer flask was counted by liquid scintillation. Experiments were performed in triplicates and the mean counts per minute (CPM) were calculated together with standard deviation (SD). Results were reported as relative values compared to wild-type p53 calculated as (p53KO/p53wt) or (p53-RNAi induced/non-induced).

^{18}F-fluorodeoxyglucose (FDG)-PET imaging and uptake. The above-mentioned tumor xenograft-bearing mice were injected with 0.5 mCi of ^{18}F-FDG in a volume of 0.2 ml of saline via the tail vein. PET imaging was performed at two hours post-injection on the A-PET small animal PET scanner at the University of Pennsylvania Small Animal Imaging Facility. For the non-imaging tumor uptake studies, animals were sacrificed at two hours after tracer injection and tumors were harvested. Residual radioactivity was measured in 100 mg of tissue via standard gamma counting techniques. Mean CPM of four tumors were calculated and reported with standard deviation.

Western blotting. Western blotting was carried out essentially as described previously with mouse anti-human p53 monoclonal antibody (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-human Ran antibody (BD Transduction Laboratories, San Diego, CA).[10-12]

Northern blotting. Total RNA was isolated using RNeasy total miniprep kit (Qiagen, Valencia, CA) following the manufacturer's instruction. Northern blot was carried out (as described previously in refs. 10–12).

RESULTS AND DISCUSSION

Metabolic change is one of the alterations associated with malignant progression. Cancer cells show a characteristic increase in aerobic glycolysis, which is believed to confer a survival advantage. The discovery of two p53 target genes, SCO2 and TIGAR, revealed a novel function of p53 in glucose metabolism.[6,7] However, human tumors expressing wild-type p53 avidly take up ^{18}F-FDG, a glucose analogue that is thought to correlate with tumor glucose utilization, suggesting that p53 alone may not suppress the Warburg effect. In order to test this hypothesis that wild-type p53 cannot alone suppress the Warburg effect as measured by tumor FDG uptake, we created an inducible p53 RNAi-mediated gene silencing and bioluminescence imaging model system. This system allows the precise manipulation and monitoring of p53 transcriptional activity by inducible p53 gene silencing while simultaneously visualizing the change in p53 activity and the correlation with tumor progression (Fig. 1). The levels of p53 mRNA, protein and transcriptional activity in the stable clones are regulated by doxycycline (Figs. 2 and 3B).

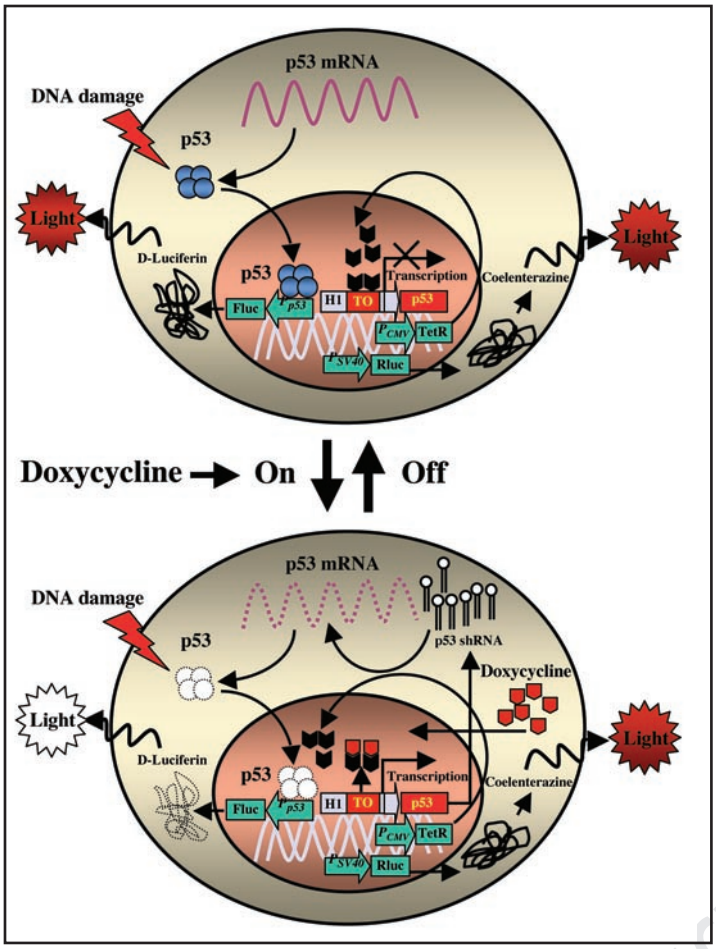


Figure 1. Schematic model of the inducible p53 RNAi-mediated gene silencing and bioluminescence imaging model system. The doxycycline-inducible p53 silencing HCT116 cells, which stably co-express the PG13-luc, a p53 reporter with a Firefly luciferase gene, and pRL-SV40 vector containing a cDNA encoding Renilla luciferase were established to monitor p53 transcriptional activity in living cells using a bioluminescence imaging system. In the presence of doxycycline, the doxycycline-induced p53 shRNA causes the degradation of p53 mRNA leading to the loss of p53-mediated transcriptional activity; thus the cells do not exhibit a bioluminescence signal upon addition of the firefly luciferase substrate D-luciferin as observed in the absence of doxycycline. Doxycycline-induced p53 shRNA has no effect on the control Renilla luciferase activity, otherwise used to image tumor volume. Therefore, this system allows use of doxycycline to control the expression of p53 and to simultaneously visualize changes in p53 transcriptional activity as well as the tumor growth.

Our data indicate that induced silencing of p53 promotes the development of larger human colonic tumor xenografts as compared to the smaller control wild-type p53 expressing xenografts (Fig. 3C). However, it is also evident from both our in vitro and in vivo data that wild-type p53-expressing tumor cells also efficiently trapped ^{18}F-FDG (Figs. 3 and 4), indicating that despite the wild-type p53 status, the Warburg effect may not be completely suppressed as measured by FDG uptake. To examine if these results were a result of incomplete p53 knockdown in our model, we measured the FDG uptake in HCT116 p53$^{+/+}$ wild type cells versus HCT116 p53$^{-/-}$ KO cells. Surprisingly, using quantitative measurements, we found that both in vitro and in mice bearing xenografted tumors, there was no significant difference in ^{18}F-FDG uptake between the HCT116 cells with wild-type p53 versus the HCT116 p53$^{-/-}$ KO cells (Fig. 5). These data strongly indicate that p53 is not solely responsible for suppression of the Warburg effect, as inferred from FDG uptake, in human tumors and that other oncogenic pathways contribute to aerobic glycolysis in human tumors despite the presence of wild-type p53. An alternative explanation may be that although the HCT116 p53$^{+/+}$ cells demonstrate characteristic wild-type p53 responses in terms of downstream target gene activation, they may not be fully wild-type with respect to all functions, specifically regarding the Warburg effect. However, our data presented here in addition to the recent results of Matoba et al. that demonstrated induction of SCO2 in the same HCT116 p53$^{+/+}$ cells, indicate that the p53 status of the tumor cells is not the only factor responsible for the Warburg effect as measured by FDG trapping.

These results have significant clinical implications due to the fact that FDG-PET imaging has become a modality of choice in diagnosis, evaluation of response to treatment and follow-up of cancer patients. FDG is thought to be trapped by tumors more avidly than it is trapped in normal tissue due to the tumor cell's propensity to utilize aerobic glycolysis for its energy needs. This inefficient utilization of glucose forces tumor cells to evolve a

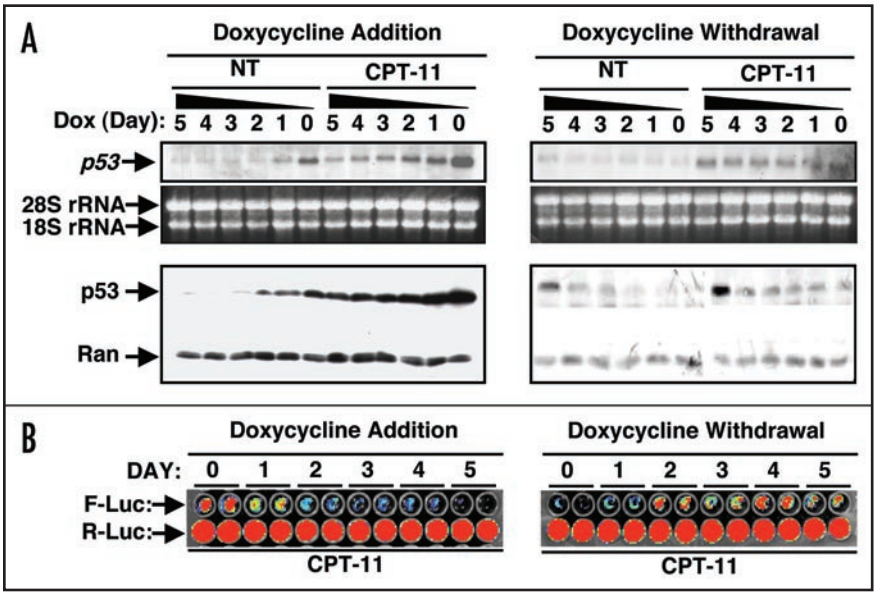


Figure 2. The expression of p53 in the inducible p53 silencing cells is tightly regulated. (A) Clones transduced with the inducible p53 shRNA were treated with 1 μg/ml doxycycline for the indicated time with (not treated, NT) or without 50 μg/ml CPT-11 for the last 16 h. (B) The cells were grown in the presence of 1 μg/ml doxycycline for 72 h and then the doxycycline was withdrawn at the indicated time. The cells were treated with or without 50 μg/ml CPT-11 for the last 16 h. The RNAs and protein extracts at each time point were analyzed by Northern and Western blots. The p53 promoter luciferase activity was determined by bioluminescence imaging. 150 μg/ml D-luciferin or 10 μg/ml coelenterazine were added to the cells. F-Luc indicates firefly luciferase activity and RLuc represents renilla luciferase control.

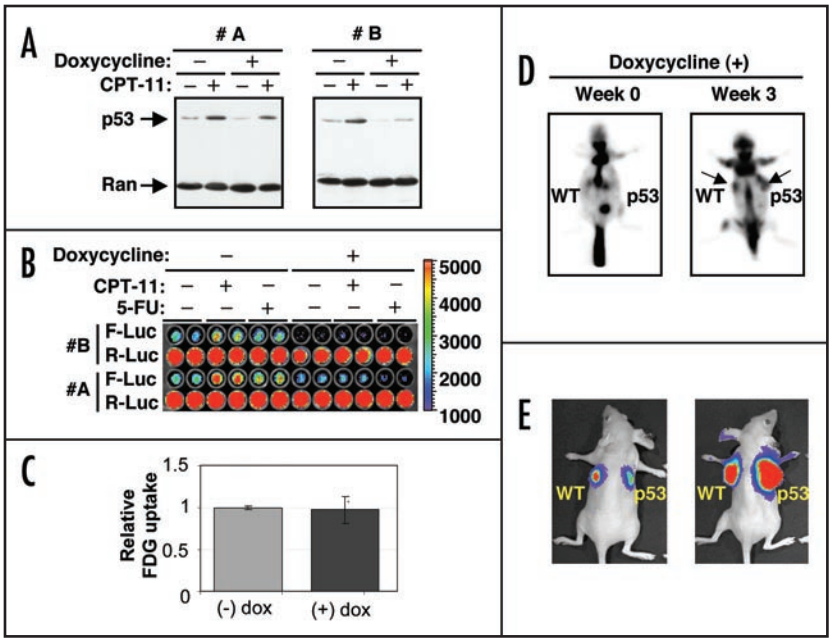


Figure 3. Inducible RNAi-mediated imaging of functional p53 activity and 18F-FDG uptake in cancer cells. (A) The stable clones (#A and #B) were grown in the absence or presence of 1 μg/ml doxycycline for 24 h and then treated with or without CPT-11 for the last 16 h. The protein extracts were analyzed by Western Blots. (B) The stable clones (#A and #B) were grown in the absence or presence of 1 μg/ml doxycycline for 24 h and then treated with or without CPT-11 or 5-FU for the last 16 h. The p53 transcriptional activities were determined by bioluminescence imaging. (C) Stable clone #B was grown in the absence or presence of 1 μg/ml doxycycline for 72 hr and then incubated with 18F-FDG for one hour. Relative uptake values were calculated as (p53-RNAi induced CPM/non-induced CPM) (D) The mice bearing wild-type and inducible p53-gene silencing in HCT116 human colonic tumor xenografts were treated with doxycycline at the indicated times. 18F-FDG-PET images were acquired at 2 h post-injection of 0.5 mCi of 18F-FDG in a volume of 0.2 ml of saline. Arrows point to the wild-type and p53 gene silenced tumors. (E) Bioluminescence imaging was performed after coelenterazine (0.7 mg/kg body weight) was injected into the tail-vein.

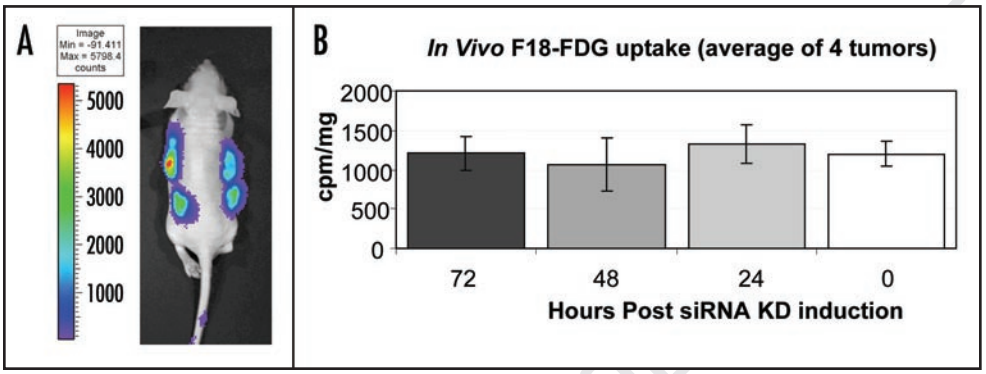


Figure 4. (A) Bioluminesence imaging of a representative mouse bearing four inducible p53-RNAi HCT116 human colonic tumor xenografts that were used in the tumor uptake studies. (B) Post-necropsy FDG uptake in HCT116 inducible p53-RNAi tumors in the non-induced state (control) or induced with doxycycline at 72, 48 or 24 prior to the administration of 0.5 mCi of 18F-FDG.

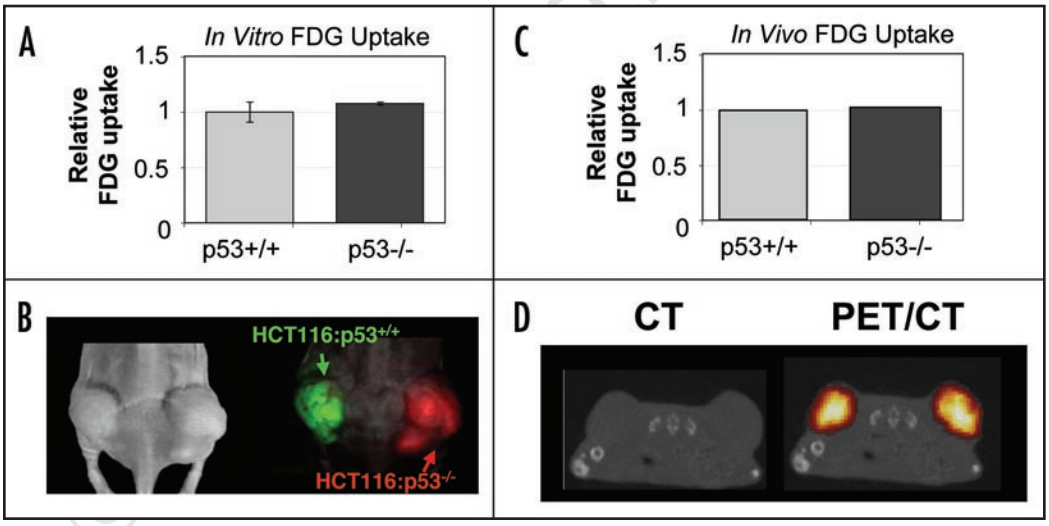


Figure 5. (A) 18F-FDG uptake in HCT116p53$^{+/+}$ cells as compared to HCT116 p53$^{-/-}$ cells. (B) Biofluorescence imaging of a mouse bearing human tumor xenografts (left) HCT116-p53$^{+/+}$ expressing EGFP and (right) HCT116-p53$^{-/-}$ expressing Ds-Red. (C) In vivo quantification of 18F-FDG uptake measured using maximum count value from the PET/CT scan shown in (D).

diversity of mechanisms to transport, trap and utilize more glucose than normal cells do. Hence, FDG is used as a clinical parameter of "tumor metabolism", which is essentially the Warburg hypothesis. If the Warburg effect and hence FDG uptake critically depended on only the p53 status of the tumor, ^{18}F-FDG would not be effectively trapped in tumors that are p53 wild-type, which is clearly not the case in the clinic. Our data experimentally demonstrates this point.

References

1. Warburg O. On respiratory impairment in cancer cells. Science 1956; 124:269-70.
2. Elstrom RL, Bauer DE, Buzzai M, Karnauskas R, Harris MH, Plas DR, Zhuang H, Cinalli RM, Alavi A, Rudin CM, Thompson CB. Akt stimulates aerobic glycolysis in cancer cells. Cancer Res 2004; 64:3892-9.
3. Semenza GL. Targeting HIF-1 for cancer therapy. Nat Rev Cancer 2003; 3:721-32.
4. Kim JW, Zeller KI, Wang Y, Jegga AG, Aronow BJ, O'Donnell KA, Dang CV. Evaluation of *myc* E-box phylogenetic footprints in glycolytic genes by chromatin immunoprecipitation assays. Mol Cell Biol 2004; 24:5923-36.

5. Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. Nature 2000; 408:307-10.
6. Matoba S, Kang JG, Patino WD, Wragg A, Boehm M, Gavrilova O, Hurley PJ, Bunz F, Hwang PM. p53 regulates mitochondrial respiration. Science 2006; 312:1650-3.
7. Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, Gottlieb E, Vousden KH. TIGAR, a p53-inducible regulator of glycolysis and apoptosis. Cell 2006; 14(126):107-20.
8. Kelloff GJ, Hoffman JM, Johnson B, Scher HI, Siegel BA, Cheng EY, Cheson BD, O'shaughnessy J, Guyton KZ, Mankoff DA, Shankar L, Larson SM, Sigman CC, Schilsky RL, Sullivan DC. Progress and promise of FDG-PET imaging for cancer patient management and oncologic drug development. Clin Cancer Res 2005; 11:2785-808.
9. Wang S, El-Deiry WS. p73 or p53 directly regulates human p53 transcription to maintain cell cycle checkpoints. Cancer Res 2006; 66:6982-9.
10. Wang S, El-Deiry WS. Inducible silencing of KILLER/DR5 in vivo promotes bioluminescent colon tumor xenograft growth and confers resistance to chemotherapeutic agent 5-fluorouracil. Cancer Res 2004; 64:6666-72.
11. Wang S, El-Deiry WS. Requirement of p53 targets in chemosensitization of colonic carcinoma to death ligand therapy. Proc Natl Acad Sci USA 2003; 100:15095-100.
12. Fei P, Wang W, Kim SH, Wang S, Burns TF, Sax JK, Buzzai M, Dicker DT, McKenna WG, Bernhard EJ, El-Deiry WS. Bnip3L is induced by p53 under hypoxia, and its knockdown promotes tumor growth. Cancer Cell 2004; 6:597-609.
13. Hammond EM, Mandell DJ, Salim A, Krieg AJ, Johnson TM, Shirazi HA, Attardi LD, Giaccia AJ. Genome-wide analysis of p53 under hypoxic conditions. Mol Cell Biol 2006; 26:3492-504.