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Silencing peroxiredoxin-2 sensitizes human colorectal cancer cells to ionizing radiation and oxaliplatin

María Belén Cerda, Rodrigo Lloyd, Milena Batalla, Florencia Giannoni, Mariana Casal and Lucia Policastro

b. Consejo Nacional Investigación Científicas y Técnicas (CONICET), Argentina. Av. Rivadavia 1917, Ciudad Autónoma de Buenos Aires, Argentina
c. Instituto de Oncología Angel H. Roffo, Universidad de Buenos Aires. Av. San Martín 5481, Ciudad Autónoma de Buenos Aires, Argentina.

Corresponding autor

Lucia Policastro, e-mail: policast@cnea.gov.ar

María Belén Cerda, e-mail: belencerda@cnea.gov.ar
Abstract

Colorectal cancer (CRC) remains one of the leading causes of cancer-related death worldwide. Antioxidant enzymes decrease the generation of ionizing radiation (IR)-induced free radicals and therefore are associated with radioresistance. The main goal of this work is to study the involvement of peroxiredoxin-2 (Prx2) in the radio and chemoradiotherapy response in CRC cells in vitro and in vivo. We found that Prx2 oxidation state is associated with differential response to ionizing radiation in CRC cell lines. HCT116 radioresistant CRC cell line have lower ROS levels and a higher monomer/dimer Prx2 ratio, compared to halfway resistant Caco-2 and T84, and radiosensitive LoVo cell line. Constitutive and transient Prx2 silencing in CRC cells increase ROS levels, and most importantly, enhance in vitro radiation sensitivity. In addition, we showed that administration of IR plus oxaliplatin in down regulated Prx2 HCT116 cells has higher cytotoxic effect than in control cells. Finally, radiosensitizing effect of Prx2 depletion was confirmed in vivo. These results suggest that Prx2 is an important component in tumoral radiation response, and their inhibition could improve radio and chemoradiotherapy protocols in patients with CRC.

1. Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in males and the second in females. Nowadays, despite significant advances in CRC treatment, it remains as one of the leading causes of cancer-related death worldwide.\(^1\) Pre-surgical radiochemotherapy is commonly used to improve CRC treatments outcome; however, only a few percentage of patients have shown a complete response to this treatment.\(^2,3\) Intrinsic cellular resistance is one of the most important constraints that yet remain to be solved and new therapeutic strategies should be explored to overcome this barrier. Oxidative stress generated by IR damages cells and induces the activation of antioxidant enzymes.
signal transduction pathways associated with radioresistant phenotypes.\textsuperscript{4} Thus, antioxidant enzymes decrease the generation of IR-induced free radicals and therefore reduce radiation damage. This phenomenon has been established as one of the main mechanism of cancer radioresistance.\textsuperscript{5}

Peroxiredoxins (Prxs) enzymes constitute a family of antioxidants that consist of six isoforms (Prx1–Prx6) encoded by distinct genes in mammals, which are classified into three mechanistic subgroups (typical 2-Cys, atypical 2-Cys, and 1-Cys).\textsuperscript{6} All Prxs contain a conserved NH2-terminal cysteine residue that serves as the active site for catalysis and typical 2-Cys Prxs, (Prx1–Prx4) have an additional conserved C-terminal cysteine residue responsible for resolving the oxidized active site cysteine.\textsuperscript{7} Prxs protect cells by removing constitutive levels of H\textsubscript{2}O\textsubscript{2}, hydroperoxides and peroxynitrite.\textsuperscript{8,9} In this process, the conserved catalytic cysteine of typical 2-Cys, named peroxidatic cysteine (Cys–SPH) is oxidized to a cysteine sulfenic acid (Cys–SOH), which is attacked by the resolving cysteine (Cys–SRH) located in the C terminus of the other subunit.\textsuperscript{10} This condensation reaction results in the formation of a stable inter-subunit disulfide bond.\textsuperscript{11} This modification is completely reversible by the subsequent reduction that regenerates the active cysteine.\textsuperscript{12}

Prxs are up-regulated in many cancers, including cancers of the lung,\textsuperscript{13} thyroid,\textsuperscript{14} breast,\textsuperscript{15} and mesothelioma,\textsuperscript{16} suggesting a possible role of these enzymes in cancer cell maintenance. Particularly in CRC has been reported Prx2 overexpression in biopsies tumor tissue versus normal tissue.\textsuperscript{17} So, here we studied the role of Prx2 in the intracellular ROS levels regulation and in the tumor response to IR and chemotherapeutic drugs as oxaliplatin (OXLP). We show that Prx2 knocking down through RNAi sensitizes CRC cells to IR and IR plus OXLP, in addition to the strong decrease of cell proliferation and tumorigenicity of this type of cells.
2. Materials and methods

2.1 Cell culture

Human CRC cell lines HCT116, Caco-2, T84 and LoVo were kindly given by Dr Podhajcer (Fundación Instituto Leloir, Argentina). Cell lines were cultivated in complete Dulbecco's modified Eagle's medium/F12 (DMEM/F12, Invitrogen Argentina SA) supplemented with 10% (vol/vol) fetal bovine serum (FBS, NatoCor, Argentina), 50 units/mL of penicillin G and 50 μg/mL of streptomycin sulfate, at 37 °C and 5% (vol/vol) of CO₂ in humidified atmosphere. Subcultures were performed following standard procedures.

2.2 Prx2 small hairpin RNA (shRNA) transfection and isolation of clones expressing Prx2 shRNA

Prx2 specific shRNA (plasmid pRFP-shPrx2, TR310194) and the negative scrambled control (psh-control plasmid TR30015) were purchased from OriGene Technologies, Inc (USA). The targeted sequence of pRFP-shPrx2 was 5’-CTGACTTCAAGGCCACAGCGGTGGTTGAT-3’ and the control sequence does not share homology with any known sequences in the human genome.

HCT116 cells were seeded on 60-mm-culture plates and transfected either with 0.5 µg of pRFP-shPrx2 or psh-control plasmid at 24h (70-80% confluence). Transfection was performed using DreamFectGold (Quiagen, USA), according to the manufacturer’s guidelines. Cells were incubated for 48 h, and then were reseeded at low density (1/16 dilution) in complete medium, puromycin (1 μg/mL) was added as selection antibiotic. Prx2 knock-down was evaluated by Western Blot. The clone with the most decreased expression of Prx2 was selected and named as HCT116shPrx2. Control clone was named HCT116shc.

2.3 Prx2 small interfering RNA transfection
Prx2 small interfering RNA (Prx2 siRNA) was purchased from Ambion (USA). The sense strand nucleotide sequence for Prx2 siRNA was 5’-AGAUCAUCGCGUUCAGCAAAtt-3’, and the anti-sense sequence was 5’-UUGCUAGACGUGAUACUCg-3’. The negative control siRNA (Neg siRNA) sense sequence was 5’-CUUACGCUGACGUACUUGAtt-3’, and the antisense sequence was 5’-UCGAAGUGACUCAGGUAAAGtt-3’. The Neg siRNA does not share homology with any known sequences in the human genome. siRNA duplexes were transfected into HCT116 cells using DreamFectGold (Quiagen, USA) according to the manufacturer’s guidelines and using magnetofection technique in Caco-2 and T84 cell lines as described elsewhere. Prx2 knock-down was evaluated by Western blotting.

2.4 Clonogenic survival assay

Clonogenic survival assays were used to determine the radiosensitivity of cells transfected with Prx2 siRNA, Neg siRNA or without transfection. Exponentially growing cells were seeded at 800 cells per T25 culture flask, incubated for 12 h until were irradiated with γ-rays from a $^{137}$Cs source (IBL-437C Irradiator; CIS BioInternational, CEBIRSA, Argentina) at a dose rate of 6 Gy/min. After 14 days, colonies were fixed with methanol/acetic acid solution 3:1 and stained with 1% of crystal violet in 25% methanol. The fraction of clonogenic cells was determined by scoring colonies containing $\geq$50 cells. Surviving fraction (SF) corresponding to each dose was determined as the number of colonies formed at the different doses divided the number of colonies formed at 0 Gy. Survival curves were fitted to the linear quadratic model $S = \exp - (\alpha D + \beta D^2)$. Clonogenic survival assays were used to determine the response of cells transfected with Prx2 or Neg siRNA to subsequent treatment with OXLP and IR. Before irradiation cells were incubated with the IC30 of OXLP (5 µM).
for 2 h. Then the medium was changed and the irradiation was performed as described above.

Clonogenic survival assays were also used to determine the sensitivity of the clones HCT116shPrx2 and HCT116shc to H$_2$O$_2$. In this case, 12 h after cells seeded in 60-mm tissue culture plates cells were treated with 0–100 µM H$_2$O$_2$. Twenty-four hours following treatment, medium was change by fresh medium and the cells were maintained for 14 days. Colonies staining and surviving fraction determination was performed as described above.

2.5 Western blot analysis

Prx2 protein expression in CRC cells and their induction post irradiation with 2Gy was examined by Western blotting. Cells were grown to 70-80% confluence in 60-mm tissue culture plates, washed twice in phosphate buffered saline (PBS), lysed in RIPA buffer, stored 30 min on ice and then centrifuged for 10 min at 4 °C. Supernatants were collected and protein concentrations were determined using the Bio-Rad kit (Bio-Rad Laboratories). Cell lysates containing 20 µg proteins were mixed with sample buffer (250 mM Tris-HCl pH6.8, 8% SDS, 0.04% bromophenol blue, 40% glycerol and 20% β-mercaptoethanol) and heated for 5 min at 95 °C. They were then resolved by SDS-PAGE (10% polyacrylamide gels), and transferred to nitrocellulose membranes (Hybond ECL Membrane, Amersham Biosciences, GE Health Care) by electroblotting. The membranes were blotted with 5% nonfat milk, washed in Tris-Buffered saline Tween (TBS-T) and incubated with primary rabbit polyclonal antibodies anti-Prx-2 (1:5000) or mousse polyclonal anti-β-actin (1:2000) (Sigma-Aldrich, Argentina) overnight at 4 °C. The membranes were washed with TBS-T and then were incubated with anti-rabbit or anti-mousse horseradish peroxidase conjugated IgG (1:2000) for 60 min at RT. Fluorescence detection was conducted using the enhanced
chemiluminescence detection system (ECL Western Blotting Substrate, Invitrogen) and the detector Bioespectrum® (UVP). Quantification was performed by densitometric analysis with NIH Image J software. To analyze the Prx2 thiol oxidative status in CRC cells, immunoblot assays were performed in the absence of β-mercaptoethanol reducing reagent.

2.6 Measurement of intracellular ROS
Intracellular ROS were detected using the cell-permeable probe DCFH-DA (Invitrogen, Argentina). Cells were incubated in 10 µM DCFH-DA for 20 min at 37°C and were collected by trypsinization, washed three times with PBS and suspended in 2 ml of this buffer. Fluorescence was detected with a FACSCalibur (Becton Dickinson, San Diego, CA, USA).

2.7 Measurement of sulfhydryl groups
The sulfhydryl group reacts with 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB; Ellman’s reagent) and produces a yellow-pigmented 5-thio-2-nitrobenzoic acid (TNB). Measurement of the absorbance of TNB at 405 nm provides an accurate estimation of sulfhydryl groups in the sample. 50 µg protein sample of HCT116, HCT116shc and HCT116shPrx2 cells were placed in 96-well plates and brought to a final volume of 180 µl with reaction buffer (0.1M sodium phosphate, 1 mM EDTA) and 20 µl of 10 mM DTNB (Thermo Scientific, Argentina). Plates were incubated for 15 min at RT and the absorbance was determined at 412 nm.

2.8 Measurement of cell growth and doubling time
Cell growth and proliferation were determined using a doubling time assay. Cells were seeded at 2x10^3 cells/well of a 96-wells plate, collected and counted every 24 h during 72 h. Doubling time was calculated as \( dt = \frac{\log 2 \times t}{(\log N - \log N_0)} \), where \( dt \) = doubling
time, t=time point of counting (h), N=number of cells counted, and N₀=initial number of cells seeded.

2.9 Tumor xenograft experiments

Fifteen 5-week-old female athymic *nu/nu* BALB/c mice (obtained from the animal facility of the National Atomic Energy Commission; Buenos Aires, Argentina) were divided into 3 groups as follows: control group (implanted with HCT116 cells), control shRNA group (implanted with HCT116shc cells), Prx2 shRNA group (implanted with HCT116shPrx2 cells). Tumor cells (1x10⁷) were injected subcutaneously into each lateral flanks. Four days after implantation tumors reached approximately 4–5 mm in diameter and only right side tumors were irradiated. Animals were anesthetized with ketamine/xylazine (80-10 mg/kg and then 2Gy from a γ-rays source of 192Ir were applied (GammaMedplus, Varian Medical System, Instituto de Oncología Ángel H. Roffo). Tumor sizes were measured by an electronic caliper every three days and volumes were estimated as following equation tumor volume (mm³) = 0.5×DM×(Dm)², where DM and Dm are the major and minor tumor diameters respectively. Forty days later, all mice were sacrificed. Animal care and experimental procedures were followed institutional guidelines approved by the National Institutes of Health.

2.10 Statistical analysis

GraphPad Prism 4 software (GraphPad Software Inc., San Diego, CA, USA) was used for statistical analyses. Data are represented as means ± standard deviation (SD) for three independent experiments. Student’s unpaired t test or two-way analysis of variance (ANOVA) was used to determine differences between groups, differences were considered significant at p<0.05.
3. Results

3.1 Doses response curves of CRC cells to $\gamma$-radiation.
The doses response curves of CRC cells to $\gamma$-radiation were obtained from clonogenic assays and the survival curves were fitted to the linear quadratic model ($S = \exp(- (\alpha D + \beta D^2))$) (Figure 1). The parameters $\alpha$, $\beta$ and FS2 are detailed in Table 1. According to statistical difference between $\alpha$ parameters, CRC cell lines were arranged in descending order of radioresistance as following: HCT116> T84≈ Caco-2> LoVo; being HCT116 the most radioresistant cell line in this model ($p<0.05$).

3.2 Differential intracellular ROS level and Prx2 redox status is associated to radioresistance in CRC cells
DCF fluorescence, proportionate to the intracellular ROS level, was monitored by flow cytometry in CRC cells (Figure 2A). The radioresistant HCT116 cells showed significantly less intracellular ROS levels compared to halfway resistant Caco-2 ($p<0.05$) and T84 ($p<0.05$) cells, and also compared to the radiosensitive LoVo cells ($p<0.05$).

We evaluated Prx2 level and its oxidation state in CRC cell lines by immunoblot analyze in presence and absence of $\beta$-mercaptoethanol reducing agent, respectively. No significant difference in total Prx2 expression among cell lines was observed, however higher Prx2 monomers/dimers ratio was detected in radioresistant HCT116 cells compared to Caco-2, T84 and LoVo cells ($p<0.05$ Figure 2B). The Prx2 expression was not increased after irradiation (2 Gy) in any cell lines (data not shown).

3.3 Knocking down Prx2 expression increase radiosensitivity to IR
Stably expressing Prx2 or control shRNA HCT116 clones were successfully established and Prx2 was also efficiently silenced by siRNA in HCT116, Caco-2 and T84 (Figure 3A). To determine whether the inhibition of Prx2 would sensitize CRC cells to IR,
clonogenic survival assay was performed following exposure cells to different doses of γ-radiation. Transient Prx2 knockdown decreased the survival of irradiated HCT116, Caco-2 and T84 cells. In addition, HCT116shPrx2 clone was also more sensitive than HCT116shc cells to IR (Figure 3B). In order to quantify the radiosensitizer effect of Prx2 silencing, the dose enhancement ratio (DER) was calculated as the ratio of doses needed to obtain a surviving fraction of 0.1 (DER0.1) or 0.37 (DER0.37) without and with Prx2 inhibition. DER=1 stands for the absence of any effect, DER<1 stands for radioprotection and DER>1 stands for radiosensitization. DER0.1 and DER0.37 of cells transfected with Prx2 siRNA were 1.1 and 1.4 for Caco-2, 1.4 and 1.7 for T84, 1.4 and 1.8 for HCT116, respectively. In HCT116shPrx2 clone DER0.1 and DER0.37 was 1.6 in both cases. These results show that Prx2 down regulation has a strong radiosensitizer effect in CRC cells.

3.4 Knocking down Prx2 expression increase sensitivity to consecutive treatment with OXLP and IR

In order to evaluate the effect of IR administered consecutively to OXLP treatment, clonogenic assays were performed in HCT116, HCT116shc and HCT116shPrx2 cells. First, SF analysis revealed a significant decrease of SF2 in HCT116 cells pre-incubated with OXLP, confirming the radiosensitizer effect of OXLP reported in other cell lines19 (Figure 3C). Also, down regulated Prx2 cells were even more sensitive to IR in combination with OXLP respect to control cells. SF2 in HCT116shPrx2 cells pre-incubated with OXLP was 0.07±0.01 (p<0.01) while SF2 was 0.25±0.01 in HCT116shc control cells (Figure 3C). These results suggest that Prx2 down-regulation could increase the cytotoxic effect of radiochemotherapy protocols.

3.5 Effect of Prx2 down-regulation on ROS production, sulfhydryl groups status and cell proliferation
To verify the antioxidant function of Prx2, the intracellular ROS level was measured in stably or transiently down-regulated Prx2 cells by DCF assay. ROS level was significantly increased in HCT116 cells with stably (HCT116shPrx2 vs HCT116shc p<0.05) or transiently decreased expression of Prx2, (Prx2 vs Neg siRNA transfected HCT116 p<0.05) (Figure 4A). These results suggest that Prx2 play an important role in the regulation of intracellular ROS, and the increase of ROS levels could contribute to radiosensitization of CRC cells.

Oxidation of reduced sulfhydryl groups (SH) is one of the modifications induced by oxidative stress, so total levels of SH groups were determined by Ellman's assay. A significant decrease in the amount of SH groups in clone HCT116shPrx2 respect to HCT116shc was observed (p <0.001). There were no significant difference between HCT116 and HCT116shc control cells (Figure 4B). The decrease of reduced sulfhydryl groups in HCT116shPrx2 would be a result of increased ROS levels due to Prx2 depletion.

Due to the regulation of the cell cycle is intricately linked to cellular redox status; we also determine whether decreasing Prx2 levels alters cell proliferation. The mean doubling times for HCT116shPrx2 and control HCT116shc cells were 31.2 ± 2.2 h and 21.7 ± 1.6, respectively, indicating that Prx2 down-regulation alters cell proliferation.

3.6 Knocking down Prx2 expression sensitizes HCT116 cells to oxidative stress

To determine whether inhibition of Prx2 would sensitize CRC cells to oxidative stress, clonogenic survival assay was performed in HCT116shPrx2 cells following exposure to H₂O₂. Exogenous treatment with H₂O₂ (0–100 µM) revealed a dose-dependent reduction in cell survival. This effect was more pronounced in HCT116shPrx2 cells compared to control HCT116shc cells (For 10 µM and 20 µM H₂O₂ HCT116shPrx2 vs
HCT116shc p<0.05, Figure 4C). Thus, these data suggest that decreasing Prx2 expression does indeed sensitize CRC cells to oxidative stress.

### 3.7 Inhibition of tumor growth and enhancement of tumor radiosensitivity by knocking down Prx2 expression

A tumor xenograft model was used to examine the radiosensitizing effect of Prx2 depletion in vivo, using three experimental groups: HCT116, HCT116shc and HCT116shPrx2 inoculated animals. Tumors in HCT116shPrx2 group disappeared completely at day 22±3 post inoculation, while tumors in HCT116 and HCT116shc grew up to 1530±223 mm$^3$ and 1797±323 mm$^3$ respectively at day 40, indicating that Prx2 down regulation decreased significantly tumorigenicity of HCT116 cells (Figure 5A). All animals were healthy at day 40 post inoculation when the experiments were finished, no difference in animal weights between different groups was found at this time.

In order to evaluated irradiation effects over HCT116shPrx2 tumors, right flank tumors of different experimental groups were irradiated with 2Gy at day 4 post inoculations. No significant differences in average tumor size between different experimental groups were observed at this time (p>0.05). As shown in Figure 5B, irradiated HCT116shPrx2 tumor size decreased significantly at day 6 post irradiation compared to non-irradiated HCT116shPrx2 tumor group (p <0.05), while no difference was found between irradiated and non-irradiated control HCT116 and HCT116shc tumors. These results indicate that Prx2 down regulation had also a radiosensitizer effect in vivo.

### 4. Discussion

In this work, we showed novels findings about intracellular ROS levels, Prx2 state and its association with cellular radioresistance in CRC: 1- HCT116 radioresistant CRC cell
line has lower ROS levels and a higher monomer/dimer Prx2 ratio, compared to halfway resistant Caco-2 and T84 and radiosensitive LoVo cell line, indicating a possible association between radioresistance and Prx2 redox status in CRC; 2- the inhibition of Prx2 expression sensitizes CRC cells to IR and to IR combined with OXLP in \textit{in vitro} assays; and 3- the inhibition of Prx2 expression sensitizes CRC cells to irradiation \textit{in vivo}. The results shown in this work supports the hypothesis that modulation of ROS levels by inhibition of antioxidant enzymes could affect the cellular response to IR in CRC tumors.

Basal ROS levels are higher in cancer cells compared to their normal counterparts.\textsuperscript{20,21} ROS levels in malignant cells have been related to cell growth, angiogenesis, and metastasis.\textsuperscript{22-24} Indeed, CRC cells have shown higher levels of ROS generation than normal cells\textsuperscript{21} and some evidences have suggested that the generation of ROS may play important role in colorectal carcinogenesis.\textsuperscript{25,26}

We found that Prx2 oxidation state was remarkably different among CRC cell lines. In HCT116 radioresistant cells which have low level of intracellular ROS, a higher proportion of reduced enzyme was detected. This is the monomeric Prx2 form which is the active conformation of the enzyme, with a catalytic cysteine, which becomes oxidized during catalysis forming a disulfide bridge with other monomer. This dimer is then reduced, in a reducing environment, to regenerate the active Prx2, contributing to an important cellular redox regulatory mechanism. Taking account our results, it is possible to suggest that the higher level of monomeric Prx2 respect to dimer in HCT116 radioresistant cells could act as a reservoir of active enzyme, which allows a fast response against oxidative damage. So, higher levels of monomers respect of dimers could be consider as useful marker of radioresistance, while high dimer levels could act as useful markers of altered redox homeostasis.\textsuperscript{27} However, Gomez Diaz et al have
reported high Prx2 dimer formation in a model of acquired resistance in MCF-7 breast cancer cells (MCF+FIR3). It was suggested that the formation of Prx2 dimers was an adaptive response of these cells to the oxidative environment created by chronic irradiation. In the other hand, some authors have reported the induction of Prx2 post irradiation, but we did not observe induction of Prx2 in response to IR en CRC cells. The same result has been observed in radioresistant leukemia cells. In order to study the involvement of Prx2 on oxidative stress modulation and the response to radiation in CRC cells, we silenced the Prx2 expression through RNAi in our CRC model. In both cases, transient or constitutive Prx2 silencing, we observed an increase in ROS levels in HCT116 cells, and most importantly, Prx2 down regulation cause sensitization to IR in all CRC cells in vitro. This in vitro effect has been also reported in head and neck cancer cells, breast cancer cells and glioma cells to radiation. Furthermore in our study, the radiosensitizing effect of Prx2 depletion was confirmed in in vivo experiment. These results suggest that despite the enzymatic redundancy of antioxidant system, Prx2 is an important component in the regulation of intracellular ROS levels and in the response to IR in CRC cells. The importance of Prx2 as a non-replaceable component of antioxidant system by other compensatory antioxidant molecules is also emphasized by Pearson et al. that have reported that Prx2 inhibition in glioma cells does not alter the expression of other antioxidant enzymes such as catalase and superoxide dismutase.

In addition, we also evaluated the effect of Prx2 down regulation combined to the administration of IR and OXLP. The results have shown that the administration of IR plus OXLP in Prx2 down regulated cells has higher citotoxic effect than in cells transfected with a control siRNA. This suggests that Prx2 inhibition could improve radio and chemoradiotherapy protocols in patients with CRC.
Prxs, as thiol peroxidases that remove hydroperoxides, are emerging as important players in cellular redox signaling.\textsuperscript{32} In this regard, Prx2 had shown a main role in the control of overall balance of thiol groups oxidized/reduced in cellular microenvironment. HCT116shPrx2 clone showed a significant decrease in total reduced thiol groups compared to HCT116 and HCT116shc controls. Sato et al have shown that thiol redox status is one of the key factors of the apoptotic pathway in Jurkat T cells.\textsuperscript{33} We hypothesize that a similar mechanism could be present in CRC cells contributing to citotoxic effect of Prx2 depletion, however future studies need to be done to deep into the specific mechanism of this signaling.

On the other hands, enzymes containing thiol groups are able to regulate other proteins,\textsuperscript{34} and also have been shown to affect Ca\textsuperscript{2+} signaling pathways,\textsuperscript{35} therefore this unbalance of thiol oxidized/reduced groups produced by Prx2 silencing could alter signaling pathways involved in cell survival and proliferation.\textsuperscript{32,36} Furthermore, Prx2 inhibition was shown to inhibit the growth of SW480 and SW620 CRC cell lines.\textsuperscript{37} We also observed a decreased proliferation rate in HCT116shPrx2 clone \textit{in vitro} and also our study demonstrated that Prx2 shRNA-treated cells had a reduced ability to form tumors in a nude mouse xenograft model. These data are consistent with the findings of Lu et al. that suggest that cell growth inhibition as consequent of Prx2 inhibition is in part due to the suppressed WNT/b-catenin signaling.\textsuperscript{37}

In brief, we demonstrated that Prx2 inhibition had radio- and chemoradiosensitizing effects and has the potential to inhibit cell growth in vivo. All these results further emphasize the potential of Prx2 inhibition as therapeutic intervention in patients with CRC. However, further study is needed to clarify the specific molecular mechanisms for the role of Prx2 in the response to radio-chemotherapy and tumor growth of CRC cells.
5. Conflict of interest

The authors declare no financial or other conflict of interest with regard to this work.

6. Acknowledgement

This work was supported by the National Agency for Promotion of Science (ANPCyT) grants PICT-2013/1751 and 2014/2436 and grants from Roemmers Foundation. Cerda MB was the recipient of the Ph.D. fellowship from CONICET and National Institute of Cancer, Argentina.

7. References


Figure legends

Fig. 1. Survival curves of CRC cells post-irradiation. Cells received 0–5 Gy of IR and colonies were detected 14 days after irradiation. Survival curves were fitted to the linear quadratic equation: $S = \exp (- (\alpha D + \beta D^2))$. Data represent means ± SD of three independent experiments with triplicates for each dose.

Fig. 2. Intracellular ROS levels and Prx2 redox status of CRC cells. (A) Intracellular ROS levels of CRCs cells were detected by DCFH-DA probe staining. ROS levels were expressed as mean fluorescence intensity measure by FACS analysis (HCT116 vs Caco-2, T84 or LoVo, *p<0.05). (B) Prx2 expression was evaluated by western blotting (up) and the quantification was performed by densitometric analysis (down). Left: Prx2 total expression. Right: Prx2 monomers/dimers ratios evaluated in non-reducing condition and expressed as % of monomers respect to total Prx2. HCT116 vs Caco-2, T84 or LoVo, * p<0.05.

Fig. 3. Knocking down Prx2 expression increases radiosensitivity to IR and OXLP plus IR. (A) Prx2 protein expression in transiently transfected Caco-2, T84 and HCT116 cells with Prx2 or Neg siRNA at 72 h post-transfection, and Prx2 levels in stably expressing Prx2 (HCT116shPrx2) or control shRNA (HCT116shc) HCT116 clones. (B) Survival curves fitted to the linear quadratic equation of gamma irradiated Prx2 silenced cell lines. Cells received 0–5 Gy of IR at 72 h post-transfection and colonies were detected 14 days after irradiation. For all cell lines, statistical significance of the difference between control and Prx2 down-regulated cells: 1 Gy, $P <0.05$; 2 Gy, $P <0.05$. (C) HCT116, HCT116shc and HCT116shPrx2 surviving fractions obtained from clonogenic assays after cells exposure to OXLP (5 µM) for 2 h and irradiation with 2Gy. HCT116shPrx2 OXLP vs HCT116shPrx2 *p<0.05, HCT116shPrx2 OXLP vs HCT116shc OXLP **p<0.01. Data represent means ± SD, n=3.

Fig. 4. Effect of Prx2 down regulation on ROS production, sulfhydryl group status and cellular response to oxidative stress. (A) DCFH-DA probe staining in constitutively (left) or transiently (right) Prx2 down regulated HCT116 cells. ROS levels were expressed as mean fluorescence intensity measured by FACS analysis, *p<0.05.(B) Sulfhydryl groups (SH) in HCT116shPrx2, HCT116shc or HCT116 cells determined by Ellman's assay expressed as SH mmol/mg total protein, *p<0.05. (C) Survival curves obtained from clonogenic assays of HCT116shPrx2, HCT116shc and HCT116 cells exposed to different $H_2O_2$ doses (0-100 µM), statistical significance of the difference between control and Prx2 down-regulated cells: 10 µM and 20 µM *p<0.05.

Fig. 5. Tumor growth inhibition and enhancement of tumor radiosensitivity by knocking down Prx2 expression. (A) Growth of xenograft tumors in nude mice derived from HCT116, HCT116shPrx2 or HCT116sh cells was monitored over time. Four days after cells implantation in both mice side, a dose of 2Gy were applied only in right flank of irradiated groups (grays curves). (B) Quantification of radiosensitizer effect. Tumors sizes at day 6 post-irradiation compared to non-irradiates groups, HCT116shPrx2 vs irradiated HCT116shPrx2 *p <0.05.
Table 1. Radiobiological parameters of CRC cell lines obtained from survival curves fitted to the linear quadratic model.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>$\alpha$ (Gy$^{-1}$)</th>
<th>$\beta$ (Gy$^{-2}$)</th>
<th>FS2</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCT116</td>
<td>0.41±0.03$^a$</td>
<td>0.06±0.06</td>
<td>0.34±0.03</td>
</tr>
<tr>
<td>T84</td>
<td>0.59±0.05$^b$</td>
<td>0.02±0.02</td>
<td>0.30±0.01</td>
</tr>
<tr>
<td>Caco-2</td>
<td>0.62±0.05$^b$</td>
<td>0.03±0.03</td>
<td>0.29±0.02</td>
</tr>
<tr>
<td>LoVo</td>
<td>0.88±0.09</td>
<td>~ 0</td>
<td>0.20±0.03</td>
</tr>
</tbody>
</table>

FS2: surviving fraction at 2Gy. $^a$ HCT116 vs T84, Caco-2 or LoVo p<0.05; $^b$ T84 vs LoVo p<0.05; $^c$ Caco-2 vs LoVo p<0.05
Survival fraction vs. Irradiation dose (Gy)

- Caco-2
- T84
- Lovo
- HCT116
A

Caco-2

- Neg siRNA
- Prx2 siRNA*

T84

- Neg siRNA
- Prx2 siRNA*

HCT116

- Neg siRNA
- Prx2 siRNA*

HCT116shc

- HCT116shc
- HCT116shPrx2*

B

Surviving fraction vs Dose (Gy)

C

Surviving fraction (2 Gy)

- HCT116shc
- HCT116shPrx

without OXLP
OXLP

**

*
A

![Graph showing mean tumor volume over time for different cell lines and conditions.]

- HCT116shc
- HCT116shc + IR
- HCT116shPn2
- HCT116shPn2 + IR

B

![Bar graph comparing tumor volume on irradiation day and Day 6 post irradiation.]

- HCT116
- HCT116shc
- HCT116shPn2

Legend:
- Black bars: Irradiation day
- Gray bars: Day 6 post irradiation
Highlights

1. Prx2 oxidation was associated with radiation response in colorectal cancer (CRC) cells.
2. Radioresistant cells have higher monomer/dimer Prx2 ratio than radiosensitive cells.
4. Prx2 inhibition sensitizes CRC cells to irradiation in vitro and in vivo.