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LETTER TO THE EDITOR

# p21 is necessary for the beneficial effects of fasting during chemotherapy

Dear Editor,

Short-term fasting (up to 48 hours) activates strong physiological and molecular responses [1]. We and others showed a p53-independent transcriptional activation of the cell cycle inhibitor *p21* upon fasting, most strongly in the liver, muscles, and many other tissues [2, 3]. Studies with mice [4, 5] and human patients [6] have shown the beneficial effects of short-term fasting during anti-cancer chemotherapy treatments.

The aim of this study was to clarify the role of *p21* induction in the beneficial effects of combining fasting and chemotherapy. For this, we first used the human colon cancer cell lines RKO and HCT116 and the colon non-tumoral cell line CCD-18Co. Sensitivity of colon cancer cells RKO and HCT116 to the chemotherapeutic agent oxaliplatin (50-100 $\mu$mol/L) was enhanced when cells were cultured in a short-term starvation (STS) mimicking medium, while non-tumoral CCD-18Co cells were protected from chemotherapy toxicity (Supplementary Figure S1). The detailed methods of this study can be found in the Supplementary Methods. We subjected p21-wild type (WT) and p21-knockdown (p21-KD) cells to oxaliplatin treatment with a normal or STS medium. STS and oxaliplatin single treatments induced *p21* mRNA and/or protein in p21-WT cells, while p21 expression was very low in p21-KD cells. Oxaliplatin and STS combination resulted in the highest increase in *p21* expression in both genotypes (Figure 1A and Supplementary Figure S2A-G). Combining STS with chemotherapy sensitized p21-WT tumor cells and protected p21-WT non-tumoral cells, and these STS effects were lost in p21-KD cells (Figure 1B and Supplementary Figure S2H-K). p53 protein levels, undetectable in CCD-18Co cells, followed a similar trend to p21 in HCT116 and RKO cells in p21-WT and p21-KD cells (Supplementary Figure S2B-C), suggesting a p21-mediated regulation of p53 protein levels upon chemotherapy and/or fasting.

MC38 murine colon cancer cells were sensitized to 100 $\mu$mol/L oxaliplatin toxicity by STS (Supplementary Figure S3A), and *p21* knockdown prevented this enhancing effect (Supplementary Figure S3B-F). p53 protein expression followed the same trend in MC38 cells (Supplementary Figure S3C) than in human cells, although p53 was more strongly induced with STS in MC38 cells, which might explain their enhanced sensitization to oxaliplatin treatment. We inoculated p21-WT and p21-KD MC38 cells subcutaneously into immunocompetent congenic C57BL/6 male mice. When tumors reached a certain size, the mice were divided into four treatment groups: (1) saline; (2) two cycles of 7.5 mg/kg oxaliplatin; (3) two cycles of 48 hours of fasting (24 hours before and 24 hours after saline inoculation); (4) two cycles of a combination of fasting and chemotherapy. p21-WT MC38 cells responded equally to oxaliplatin treatment than to 48 hours of fasting, as already shown for other cell types [7], and a combination of oxaliplatin and fasting induced the strongest effect (Figure 1C and Supplementary Figure S3G). In contrast, p21-KD MC38 cells did not respond to single oxaliplatin or single fasting treatments, and only the combination of both treatments prevented tumor growth (Figure 1C and Supplementary Figure S3H). These results indicated that cell-autonomous induction of *p21* with fasting above a threshold was necessary for MC38 allograft growth arrest. *p21* induction below this threshold, as attained by single treatments in p21-KD cells, was not enough to affect tumor growth. Apoptosis, measured by activated caspase 3, increased with fasting in p21-WT but not in p21-KD tumors, while fasting tended to reduce cellular proliferation, measured by Ki67, in both p21-WT and p21-KD tumors (Supplementary Figure S3I). The anti-tumor effect of fasting has been proposed to depend mostly on an enhanced immune response after chemotherapy [4, 7]. However, cancer cells cultured in an STS medium showed increased apoptosis and decreased viability, as shown in

**Abbreviations:** CDK2, Cyclin-dependent kinase 2; DNA, Deoxyribonucleic acid; KD, Knockdown; KO, Knock-out; MAPK, Mitogen-activated protein kinase; PCNA, Proliferating cell nuclear antigen; PPARa, Peroxisome proliferator-activated receptor alpha; SREBF, Sterol regulatory element-binding protein gene; STS, Short-term starvation; WT, Wild type.

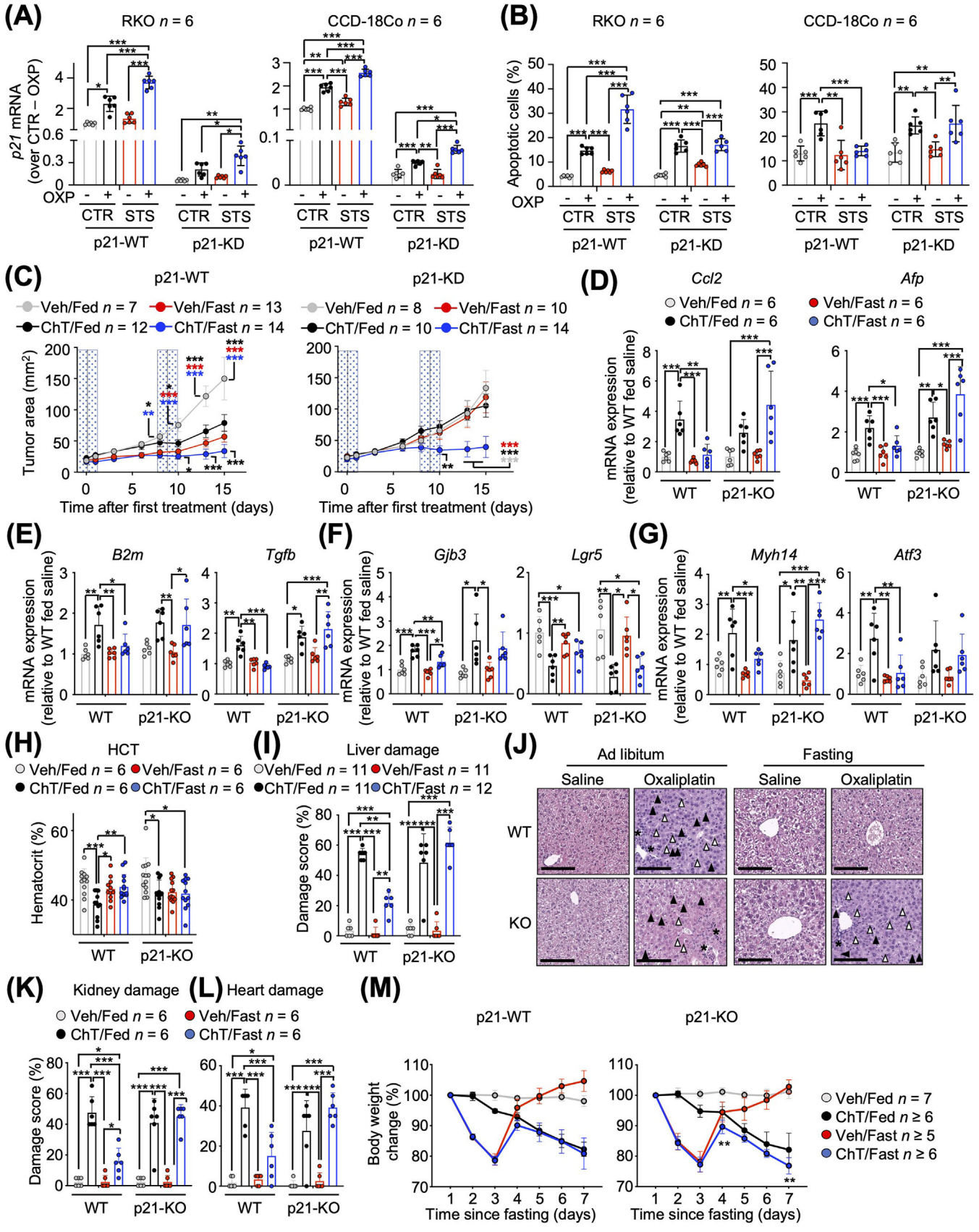


**FIGURE 1** **p21 is essential for fasting-mediated enhancement of chemotherapy. (A)** The colon carcinoma cell line RKO and CCD-18Co non-tumoral colon fibroblasts were stably transfected with an shRNA targeting *p21* mRNA. Both p21-WT and p21-KD cells were cultured in either normal (CTR) or fasting-mimicking (STS) medium for 24 hours and then treated with water or 50 $\mu$mol/L oxaliplatin (RKO)

Supplementary Figure S1 and in a previous study [8], a phenomenon termed "differential stress resistance" (DSR). Also, allograft tumors from p21-KD cells responded differently to fasting than allografts from p21-WT cells, even if the host immune response was p21-WT in both models (Figure 1C and Supplementary Figure S3G-H). Our results indicated that, apart from an effect on the immune system, there was a cell-autonomous effect of fasting in tumor cells, at least partly dependent on *p21*, that makes them more sensitive to chemotherapy.

To test if the critical role of p21 could be applied to chemotherapies different from oxaliplatin, we measured cell viability of colon cancer cell lines cultured in control or STS medium and treated with increasing concentrations of 5-fluorouracil (Supplementary Figure S4A), doxorubicin (Supplementary Figure S4B) or etoposide (Supplementary Figure S4C). Knockdown of p21 did not affect cell proliferation or viability compared with p21-WT cells in any of these cases. STS enhanced the anti-tumor efficacy of chemotherapy in p21-WT cells, while this effect was lost in p21-KD cells, indicating that the STS and p21 effects we not dependent on the type of chemotherapy.

We studied the ability of fasting to protect from chemotherapy toxicity in mice subjected to oxaliplatin treatment. Forty-eight hours of fasting were sufficient to increase *p21* mRNA in several tissues in p21-WT mice ([2] and Supplementary Figure S5A). We measured chemotherapy toxicity in mice subjected to oxaliplatin (15 mg/kg), 48 hours fasting or a combination of both treatments. We used a complete panel of biomarkers in the liver (Figure 1D and Supplementary Figure S5B), kidney (Figure 1E and Supplementary Figure S5C), small intestine (Figure 1F) and heart (Figure 1G and Supplementary Figure S5D); hematological parameters as hematocrit (Figure 1H); and histological analysis in the liver (Figure 1I, J), kidney (Figure 1K and Supplementary Figure S5E) and heart (Figure 1L and Supplementary Figure S5F). Fasting was associated with protection from chemotherapy toxicity in all these tissues in p21-WT mice. In contrast, ablation of *p21* abrogated these protective effects in all tested tissues. Fasting did not affect body weight loss after chemotherapy administration in p21-WT mice compared with mice treated with only chemotherapy. In contrast, fasting p21-KO mice suffered a stronger body weight loss after chemotherapy than mice treated only with chemotherapy, indicating increased whole-body damage with fasting in the absence of p21 (Figure 1M). We observed the same effects in female p21-WT and p21-KO mice, indicating that fasting-mediated protection from oxaliplatin toxicity was not affected by sex (Supplementary Figures S5-S7).

p21 inhibits cell cycle progression, which could explain the fasting-mediated tumor growth inhibition. However, *p21* induction occurs in many post-mitotic tissues where proliferation is rare, such as the heart, liver or kidney, where the loss of *p21* blunts fasting-mediated protection from chemotherapy toxicity. Therefore, cell cycle-independent functions of p21 are probably also responsible for this protection. p21 inhibits apoptosis by cell cycle arrest and enhancement of DNA repair, inhibition of caspase activation or inhibition of pro-apoptotic stress-activated pathways [9]. This might be one mechanism by which fasting protects from chemotherapy toxicity in non-tumoral tissues, even though p21 enhances chemotherapy-mediated apoptosis in tumoral cells, as shown in Figure 1

or 100 $\mu$mol/L oxaliplatin (CCD-18Co). Twenty-four hours later, total mRNA was extracted, and *p21* mRNA levels were measured by qPCR. **(B)** The same cells described in (A) were stained with Annexin V and propidium iodide to measure apoptosis. **(C)** C57BL/6 p21-WT male mice aged 12-16 weeks were inoculated with MC38 colon carcinoma cells. When tumors reached a certain size, mice were treated with two cycles (represented by dotted squares) of vehicle (saline, Veh) or 7.5 mg/kg oxaliplatin (ChT) while being fed ad libitum (Fed) or fasted for 24 hours before and 24 hours after chemotherapy or saline administration (Fast). Tumor size was measured periodically. Six days after the last oxaliplatin inoculation, mice were sacrificed. **(D-G)** Male mice of 12-14 weeks of age were inoculated with vehicle (saline, Veh) or 15 mg/kg oxaliplatin (ChT) while being fed ad libitum (Fed) or fasted for 24 hours before and 24 hours after chemotherapy administration (Fast). Six days after oxaliplatin inoculation, mice were sacrificed, and mRNA expression of the indicated genes was determined in the liver (D), kidney (E), small intestine (F) and heart (G). **(H)** Hematocrit (HCT) of male and female mice shown in (D-G) (males) and Supplementary Figure S5-S6 (females) was recorded at the time of sacrifice. **(I-L)** Hematoxylin & eosin stainings of the liver (I, J), kidney (K) and heart (L) from mice shown in (D-G) were analyzed to quantify histological findings related to chemotherapy toxicity-induced tissue damage. White arrowheads represent sinusoidal dilatations; black arrowheads represent vacuoles. The size bar represents 200 $\mu$m. **(M)** Body weight change each day of the protocol of the same male mice shown in D-L, normalized to initial weight before fasting. Bars represent the average of the indicated number of replicates (A-B) or the number of mice indicated in the legend (C, H-M), or n = 6 (D-G). Individual circles represent the value for each replicate/individual. Line-connected dots represent the average tumor size (C) or body weight (M) for the indicated number of mice. Error bars represent the standard deviation (A, B) or the standard error of the mean (C-M). Statistical significance was assessed using the two-way ANOVA test with Tuckey's correction for multiple comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. In C, asterisk color codes and lines indicate the groups being compared. In (M), asterisks indicate the significance between the fed oxaliplatin and the fasted oxaliplatin groups; other significances are not represented. Abbreviations: CTR, control medium; KD, knockdown; STS, short-term starvation medium; Veh, vehicle (saline); ChT, chemotherapy.

and Supplementary Figures S1-S4. Finally, p21 binds to transcriptional regulators and modulates the expression of genes related to the S phase and mitosis during DNA damage [10]; therefore, p21 induction with fasting might also regulate the transcription of genes involved in toxicity resistance.

Our mechanistic insight may help in future clinical applications of fasting during chemotherapy: patient stratification according to their *p21* induction with fasting, *p21*-inducing strategies to enhance chemotherapy treatment, or measurement of *p21* induction as a biomarker to identify interventions enhancing chemotherapy safety and efficacy.

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## AUTHOR CONTRIBUTIONS

Adrián Plaza performed most of the experiments, analyzed the data and helped writing the manuscript. Andrés Pastor Fernández helped with the cytometry and experiments. Cristina Pantoja helped in the performance and interpretation of the p21 knockdown experiments. Marta Barradas performed some p21 expression experiments. Jose L. López-Aceituno helped in the mouse experiments and management. Pablo J. Fernandez-Marcos designed the study, coordinated the work and wrote the manuscript.

## CONFLICT OF INTERESTS

The authors have declared that no conflict of interest exists.

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## ETHICS APPROVAL

All animal experiments were performed according to protocols approved by the CSIC Ethics Committee for Research and Animal Welfare in Spain and all the appropriate official entities.

## CONSENT FOR PUBLICATION

Not applicable

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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