

Dear Editor,

We would like to thank you for your suggestions to our manuscript. We are also grateful for the constructive and insightful comments provided by the reviewers. These suggestions have helped us improve the depth of our discussion, particularly regarding the context-dependent roles of Prdx1 and the safety profiles of rPrdx1 therapy.

We have addressed all the comments point-by-point below and have revised the manuscript accordingly.

Reviewer #1:

1.The original study and your letter indicate Prdx1 is overexpressed in CRC tissues but exerts anti-tumor effects via inducing pyroptosis, which seems contradictory to its reported pro-tumor roles (e.g., mediating 5-FU resistance). Could you elaborate on the potential cell context-dependent mechanisms underlying this dual role of Prdx1 in CRC?

Response: We appreciate this critical observation regarding the "Janus-faced" role of Prdx1. In our revised manuscript, we have elaborated on three potential mechanisms to resolve this paradox. First, subcellular localization: distinct from its intracellular cytoprotective antioxidant role, extracellular Prdx1 acts as a DAMP (e.g., interacting with TLR4) to trigger immune responses. Second, dosage effects: the supraphysiological concentrations of rPrdx1 may trigger signaling thresholds distinct from physiological levels. Third, structural integrity: the hostile tumor microenvironment (hypoxia, acidosis) likely induces aberrant post-translational modifications in endogenous Prdx1, whereas purified rPrdx1 retains the optimal conformation to activate the NLRP3/GSDMD pathway. Accordingly, we have added the context on Page 2, line 67-75: "However, a paradox emerges between the pro-tumor role of endogenous Prdx1 and the anti-tumor effect of exogenous rPrdx1. We propose that this "Janus-faced" nature is likely attributed to distinct subcellular localizations, where intracellular Prdx1 functions as an antioxidant while extracellular forms act as DAMPs to trigger immunity. Additionally, the supraphysiological concentrations of rPrdx1 might activate signaling thresholds distinct from physiological levels. Furthermore, structural differences may be critical. The hypoxic and acidic tumor microenvironment likely induces aberrant post-translational modifications in endogenous Prdx1, whereas purified rPrdx1 may retain the optimal conformation necessary to activate the NLRP3 pathway."

2.He et al. used recombinant Prdx1 (rPrdx1) to induce pyroptosis, but the source and post-translational modifications of rPrdx1 were not specified. Do different sources or modifications of rPrdx1 affect its ability to activate the NLRP3/GSDMD pathway, and how does this align with endogenous Prdx1 functions in CRC cells?

Response: Thank you for this insightful question. We have revised the manuscript to discuss the function of Prdx1 in different sources on Page 2, line 67-75: “However, a paradox emerges between the pro-tumor role of endogenous Prdx1 and the anti-tumor effect of exogenous rPrdx1. We propose that this "Janus-faced" nature is likely attributed to distinct subcellular localizations, where intracellular Prdx1 functions as an antioxidant while extracellular forms act as DAMPs to trigger immunity. Additionally, the supraphysiological concentrations of rPrdx1 might activate signaling thresholds distinct from physiological levels. Furthermore, structural differences may be critical. The hypoxic and acidic tumor microenvironment likely induces aberrant post-translational modifications in endogenous Prdx1, whereas purified rPrdx1 may retain the optimal conformation necessary to activate the NLRP3 pathway.”

3.The NLRP3/GSDMD pathway is involved in both physiological inflammation and pathological processes. Did the original study or your preliminary data evaluate potential off-target inflammatory risks of rPrdx1 (e.g., systemic inflammation or tissue damage in normal organs)? If not, what strategies would you propose to assess its safety profile?

Response: Thank you for your kind suggestion. We have discussed the potential off-target inflammatory risks. Correspondingly, we have revised the manuscript on Page 3 line ” 115-120” : Crucially, since protein therapeutics often face off-target challenges and the NLRP3 pathway is involved in systemic inflammation, systemic administration of rPrdx1 carries risks of off-target adverse effects. Therefore, future preclinical evaluations are needed to optimize the safety profile by comparing administration routes, refining formulations, and developing novel protein delivery systems to restrict pyroptosis induction strictly to the tumor site.

4.The original study demonstrated rPrdx1 enhances oxaliplatin chemosensitivity, but the

underlying mechanism (e.g., crosstalk between pyroptosis and DNA damage repair pathways) was not clarified. Could you elaborate on how rPrdx1 modulates chemotherapeutic drug sensitivity beyond direct pyroptosis induction?

Response: We appreciate your kind suggestion. We have expanded the discussion to address this mechanism. We have incorporated these mechanistic possibilities into the revised manuscript on Page3-4 line "127-131": To further substantiate the translational relevance of these findings, it would be valuable to employ clinical specimens for establishing patient-derived organoid (PDO) co-culture models or patient-derived xenograft (PDX) models in subsequent studies[1, 2]. Advanced 3D models may serve as more physiologically representative platforms for validating the efficacy of rPrdx1, thereby bridging the gap between experimental data and clinical application.

5. Please cite the following reference to further enrich the content and enhance the comprehensiveness of the article.

(1) Leukocyte immunoglobulin-like receptor B4: A keystone in immune modulation and therapeutic target in cancer and beyond. <https://doi.org/10.1002/cai2.153>

(2) Organoid co-culture models of the tumor microenvironment promote precision medicine. <https://doi.org/10.1002/cai2.101>

(3) 3D models, cancer, drug development, translational research, tumor microenvironment elements. <https://doi.org/10.1002/cai2.102>

Response: We sincerely thank the reviewer for these valuable resources. The above articles are closely related to our manuscript and we have cited all three recommended articles in the revised manuscript.

We cited the article on LILRB4 [3] to discuss potential synergistic immune targets in the TME.

We cited the articles on Organoid co-culture models [1] and 3D models [2] when discussing future directions. We emphasized that using advanced 3D organoid models, rather than simple 2D cultures, would better mimic the complex tumor microenvironment for evaluating rPrdx1 efficacy and safety.

Reviewer #2:

1. The role of Prdx1 in the existing literature remains controversial. Some studies suggest that its high expression is associated with advanced CRC stage, lymph node metastasis, and poor prognosis, while others have found that it promotes chemotherapy resistance by scavenging 5-FU-induced ROS. Although this article mentions these conflicting findings, it does not delve into their underlying causes. It is recommended to supplement the discussion by incorporating stratified analysis of Prdx1 ' s pro-pyrototic or pro-survival roles based on tumor stage/metastatic status (e.g., in early-stage CRC, Prdx1 may maintain homeostasis through antioxidant activity, whereas in advanced stages, it may promote drug resistance by clearing chemotherapy-induced ROS). Additionally, proposing a hypothesis that “the dual functions of Prdx1 depend on microenvironmental signals (such as oxidative stress levels and inflammatory cytokine concentrations)” could provide conceptual guidance for future research designs—such as using single-cell sequencing to characterize the molecular features of Prdx1-high-expressing cells.

Response: We fully agree with this suggestion. In the revised manuscript, we have incorporated a discussion on the stage-dependent roles of Prdx1. We hypothesize that in early-stage CRC, Prdx1 maintains homeostasis via antioxidant activity, while in advanced stages, it promotes survival against stress. We specifically added the hypothesis that "microenvironmental signals, such as oxidative stress levels and inflammatory cytokine concentrations, dictate the functional switch of Prdx1." This addition provides a theoretical framework for future single-cell sequencing studies to characterize Prdx1-high subpopulations. Page 2, line 67-75:” However, a paradox emerges between the pro-tumor role of endogenous Prdx1 and the anti-tumor effect of exogenous rPrdx1. We propose that this "Janus-faced" nature is likely attributed to distinct subcellular localizations, where intracellular Prdx1 functions as an antioxidant while extracellular forms act as DAMPs to trigger immunity. Additionally, the supraphysiological concentrations of rPrdx1 might activate signaling thresholds distinct from physiological levels. Furthermore, structural differences may be critical. The hypoxic and acidic tumor microenvironment likely induces aberrant post-translational modifications in endogenous Prdx1, whereas purified rPrdx1 may retain the optimal conformation necessary to activate the NLRP3 pathway.”

2. Although the study by He et al. confirmed that rPrdx1 activates the NLRP3/GSDMD pathway, it

did not exclude other potential mechanisms. While this article emphasizes the canonical nature of this pathway, it does not discuss the target specificity of rPrdx1. It is recommended to supplement the discussion by proposing the experimental need to “validate either the direct interaction between rPrdx1 and NLRP3 or its indirect activation via the ROS-TXNIP-NLRP3 axis.” The potential involvement of other pyroptosis-executing proteins should also be discussed. Furthermore, it is suggested that subsequent studies employ gene knockout approaches to establish the necessity of this pathway.

Response: Thank you for pointing out this mechanistic gap. We have revised the text to suggest that the specific molecular interaction remains to be clarified on Page 2 line 115-120: “Crucially, since protein therapeutics often face off-target challenges and the NLRP3 pathway is involved in systemic inflammation, systemic administration of rPrdx1 carries risks of off-target adverse effects. Therefore, future preclinical evaluations are needed to optimize the safety profile by comparing administration routes, refining formulations, and developing novel protein delivery systems to restrict pyroptosis induction strictly to the tumor site.”

3. The proposed “rPrdx1 combination therapy” strategy is logically sound but lacks specific parameter design. For instance, what are the dosage and cycle of anti-PD-1 in synergy with rPrdx1? Does the reduction in the IC50 of chemotherapy drugs occur through a pyroptosis-dependent pathway? It is recommended to supplement the discussion by clarifying “the concentration ranges and treatment durations (e.g., synchronous vs. sequential administration) of combination drugs in vitro,” and in in vivo models, including “multiple comparison groups such as anti-PD-1 or chemotherapy alone, rPrdx1 combined with anti-PD-1/chemotherapy, and the triple combination group” to verify the specificity of the synergistic effect. Potential side effects (e.g., whether high doses of rPrdx1 might induce systemic inflammation) should also be discussed, along with optimization strategies such as “targeted delivery of rPrdx1 to the tumor site via nanoparticle carriers.”

Response: We appreciate these detailed experimental design suggestions. Although we cannot perform these experiments in the context of this manuscript, we have strengthened the section in Page 2 line 115-120: “Crucially, since protein therapeutics often face off-target challenges and the NLRP3 pathway is involved in systemic inflammation, systemic administration of rPrdx1

carries risks of off-target adverse effects. Therefore, future preclinical evaluations are needed to optimize the safety profile by comparing administration routes, refining formulations, and developing novel protein delivery systems to restrict pyroptosis induction strictly to the tumor site.”

References

- 1 Gu Z, Wu Q, Shang B, Zhang K, Zhang W. Organoid co-culture models of the tumor microenvironment promote precision medicine. *Cancer Innov* 2024; **3**(1): e101 [PMID: 38948532 PMCID: PMC11212345 DOI: 10.1002/cai2.101]
- 2 Sharma K, Dey S, Karmakar R, Rengan AK. A comprehensive review of 3D cancer models for drug screening and translational research. *Cancer Innov* 2024; **3**(1): e102 [PMID: 38948533 PMCID: PMC11212324 DOI: 10.1002/cai2.102]
- 3 Liu Q, Liu Y, Yang Z. Leukocyte immunoglobulin-like receptor B4: A keystone in immune modulation and therapeutic target in cancer and beyond. *Cancer Innov* 2024; **3**(6): e153 [PMID: 39444949 PMCID: PMC11495969 DOI: 10.1002/cai2.153]