ORIGINAL RESEARCH COMMUNICATION

Binding to WGR Domain by Salidroside Activates PARP1 and Protects Hematopoietic Stem Cells from Oxidative Stress

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Abstract

Aims: A component of the base excision repair pathway, poly(ADP-ribose) polymerase-1 (PARP1) functions in multiple cellular processes, including DNA repair and programmed cell death. We previously showed that Salidroside, a phenylpropanoid glycoside isolated from medicinal plants, prevented the loss of hematopoietic stem cells (HSCs) in native mice and rescued HSCs repopulating in transplanted recipients under oxidative stress. The aim of this study was to investigate the mechanism by which PARP1 activation by Salidroside maintains HSCs under oxidative stress. **Results:** We found that although there were no spontaneous defects in hematopoiesis in *Parp1*^{-/-} mice, oxidative stress compromised the repopulating capacity of *Parp1*^{-/-} HSCs in transplanted recipient mice. A biochemical study using truncated proteins lacking the defined functional domains of PARP1 showed that the tryptophan-glycine-arginine-rich (WGR) domain of PARP1 was critical for Salidroside binding and subsequent PARP1 activation under oxidative stress. Functionally, complementation of *Parp1*^{-/-} HSCs with full-length PARP1_{WT}, but not the PARP1_{R591K} mutant in WGR domain restored Salidroside-stimulated PARP1 activation *in vitro*. Mechanistically, activated PARP1 by Salidroside enhanced the repopulating capacity of the stressed HSCs by accelerating oxidative DNA damage repair. **Innovations and Conclusion:** Our findings reveal the action of mechanism for Salidroside in PARP1 stimulation and a novel role of PARP1 activation in maintaining HSC function under oxidative stress. *Antioxid. Redox Signal.* 20, 1853–1865.

Introduction

REACTIVE OXYGEN SPECIES (ROS) are the chemical reactive molecules containing oxygen, which are routinely generated during a metabolic or inflammatory process (28). An imbalance between ROS production and antioxidant defense, defined as oxidative stress, can cause or amplify genotoxic stress and stimulate inflammatory responses (9). It has been considered as an important pathogenic factor in leukemia-prone bone marrow (BM) diseases by inducing a variety of responses in hematopoietic stem cells (HSCs), such as stem cell differentiation and apoptosis (1, 14, 15, 32). ROS can drive HSCs into cell division, which appears to be essential for DNA repair processes (46). It has been shown that oxidative DNA damage repair (ODDR) is less efficient in quiescent stem cells than in progenitor cells, and that ROS-

induced DNA damage impairs the self-renewal capacity of human HSCs (43). Mice deficient in genes functioning in

Innovation

The present studies demonstrate the intrinsic repopulating defect of *Parp1*^{-/-} hematopoietic stem cells (HSCs) under oxidative stress and identifies the tryptophan-glycine-arginine-rich (WGR) domain of poly(ADP-ribose) polymerase-1 (PARP1) required for Salidroside binding and PARP1 activation thereby preventing oxidative stress-induced premature HSC exhaustion. These findings not only provide a molecular explanation for Salidroside-stimulated PARP1 activation in HSC maintenance under oxidative stress, but also suggest new targets for therapeutically exploring the pathogenic role of oxidative stress in hematologic diseases.

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oxidative stress responses, including *Atm*, *Fancc*, *Fancd2*, *FoxO*, and in DNA damage repair, including *Lig4*, *Dna-pk*, *Ku80*, *Xpd*, *mTR*, show elevated ROS levels and sustained DNA damage accumulation leading to premature HSC exhaustion (2, 4, 5, 18, 19, 24, 26, 28, 31, 33, 34, 41, 47).

Poly(ADP-ribosyl)ation (PARylation) is a post-translational protein modification by creating poly(ADP-ribose) (PAR) covalently attached onto target proteins that mediate gene transcription, DNA damage repair, and cell death signaling (4, 17, 23). Poly(ADP-ribose) polymerase-1 (PARP1) is the founding member of the PARP family with a highly conserved structure and six domains: three Zinc finger DNA-binding domain (DBD: Zn1, Zn2, and Zn3), the auto-modification domain (AD), the tryptophan-glycine-arginine-rich (WGR) domain, and the catalytic domain (CAT) that is composed of two subdomains—the helical subdomain (HD) and the ART subdomain (17, 23). It has emerged as a promising drug target for cancer therapy due to its role in maintaining genome stability (25). Although increasing evidence indicates that PARP1 is involved in oxidative DNA damage response (3, 7, 26, 45), how PARP1 functions in HSC maintenance under oxidative stress remains to be elucidated.

Salidroside, a phenylpropanoid glycoside, is the major active substance of *Rhodiola rosea*, which grows in dry and sandy ground and has been used traditionally and pharmacologically for a long period of time in many European countries in the 19th century (8, 38). The main use was as a brain tonic, as a roborant, and to alleviate headache (8, 38). Recently, it has been documented in experimental animals for protective effects of oxygen, cold, radiation, and heavy physical exercise (8), suggesting that Salidroside has various pharmacological properties, including antiaging, anticancer, anti-inflammation, hepatoprotective, and antioxidative effects (13, 16, 20).

We previously showed that Salidroside stimulated the PARP1 activity and protected HSCs from oxidative stress (26). To further understand the action of mechanism for Salidroside in PARP1 stimulation in the context of ODDR in HSCs, we constructed several truncated proteins lacking the defined functional domains of PARP1 and assessed the effect of these mutants in HSCs under oxidative stress and their binding to Salidroside. The results showed that Salidroside activated PARP1 through its binding to the WGR domain, while truncated proteins lacking the WGR domain or carrying R591K mutation in the WGR domain failed to bind and respond to Salidroside under oxidative stress. Functionally, Salidroside stimulated PARP1 activation in *Parp1*^{-/-} HSCs expressing the wild-type (WT) PARP1 and prevented HSCs from H₂O₂-induced cycling and repopulating exhaustion. Taken together, we identified a specific binding domain of PARP1 required for Salidroside-stimulated PARP1 activation and a crucial role for PARP1 in maintaining HSC function under oxidative stress.

Results

Genetic dissection of Salidroside action on HSC function under oxidative stress

We previously showed that activation of PARP1 by Salidroside, a phenylpropanoid glycoside isolated from the medicinal plant *R. rosea* prevents the loss of HSCs in native mice and rescues HSCs repopulating in transplanted recipi-

ents under oxidative stress (26). To provide genetic evidence that Salidroside protects HSCs from oxidative stress through stimulating the PARP1 activity, we employed *Parp1*-deficient mice (*Parp1*^{-/-}). We first determined the optimal dose of hydrogen peroxide (H₂O₂); consistently with the previous finding (26), we found that 0.25 μ mol/g body weight was the optimal dose that effectively induced oxidative stress, as evidenced by a dose-dependent increase in ROS (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/ars) and lipid peroxide (LPO) (Supplementary Fig. S1B). We then treated WT and *Parp1*^{-/-} mice with or without Salidroside followed by H₂O₂ injection (0.25 μ mol/g body weight). We found that H₂O₂ treatment led to significant expansion of Lin⁻c-kit⁺Scal⁺ (LSK) cells in WT mice, which was partially limited by Salidroside (Fig. 1A). However, in *Parp1*^{-/-} mice, Salidroside failed to prevent H₂O₂-induced LSK expansion. Specifically, there was a significant increase in LSK frequency in H₂O₂-treated *Parp1*^{-/-} mice and Salidroside treatment did not reverse the effect (Fig. 1A). It is noteworthy that neither Salidroside nor H₂O₂ treatment altered the morphology of these primitive LSK cells (Fig. 1B). Further analysis of the LSK compartment indicated that H₂O₂ treatment caused a significant reduction of LT-HSCs (LSK-CD150⁺CD48⁻) in WT mice and a further decrease of LT-HSCs in *Parp1*^{-/-} mice (Fig. 1C). Salidroside almost completely abrogated H₂O₂-induced loss of LT-HSCs in WT mice, while this effect was absent in *Parp1*^{-/-} mice. Specifically, the LT-HSC number in H₂O₂-injected *Parp1*^{-/-} mice in the presence of Salidroside remained significantly lower compared with WT mice (Fig. 1C). These results suggest that PARP1 plays an important role and is required for the action of Salidroside in maintaining a HSC pool under oxidative stress.

We then determined whether Salidroside could improve the repopulating ability of oxidative stressed *Parp1*^{-/-} HSCs by transplanting LSK cells from H₂O₂-treated WT C57BL/6 mice or *Parp1*^{-/-} mice (CD45.2⁺) pretreated with or without Salidroside into lethally irradiated congenic recipients (CD45.1⁺). We observed a significant decrease of donor-derived chimera (CD45.2⁺) in the recipients injected with LSK cells from H₂O₂-treated WT mice, which was partially reversed by Salidroside at 4 months after transplantation (Fig. 1D). Although HSCs from vehicle-treated *Parp1*^{-/-} mice gave rise to comparable donor-derived chimera (72.37% $\pm$ 5.95% in the WT group and 69.93% $\pm$ 5.09% in the *Parp1*^{-/-} group) in the recipients, oxidative stress compromised the repopulating capacity of *Parp1*^{-/-} HSCs (10.83% $\pm$ 5.58%) more when compared with WT HSCs (31% $\pm$ 4.11%). Salidroside failed to restore the ability of stressed *Parp1*^{-/-} HSCs in reconstituting mouse hematopoiesis in the recipient mice (11.5% $\pm$ 3.84%). Mechanistically, Salidroside appeared to prevent HSCs from oxidative stress-induced cycling (Fig. 1E) rather than apoptosis (Fig. 1F), as detected by bromodeoxyuridine (BrdU)/7-aminoactinomycin D (7AAD) and Annexin V/7AAD staining, respectively. These results collectively provide genetic evidence that PARP1 is required for the action of Salidroside in influencing HSC function under oxidative stress.

Salidroside binds to the WGR domain of PARP1

PARP1 has a highly conserved structural and functional organization, including an N-terminal zinc finger DBD, a

nuclear localization signal, a central AD, and a C-terminal catalytic domain (4, 17, 23). We first determined whether Salidroside bound directly to PARP1. To this end, we constructed several truncated proteins lacking the defined functional domains of PARP1 (Fig. 2), and assessed their ability

to bind to Salidroside. We employed microscale thermophoresis to determine the binding between Salidroside and the PARP1 derivatives. This technology probes for fluorescent changes in the hydration shell of molecules to measure protein–protein or protein–small molecule interactions with

FIG. 1. Oxidative stress compromises self-renewal capacity of *Parp1*^{-/-} HSCs. (A) Salidroside does not limit H₂O₂-induced LSK expansion in *Parp1*^{-/-} mice. BMCs from WT C57BL/6 mice or *Parp1*^{-/-} mice pretreated with or without SD (75 μg/g body weight) followed by H₂O₂ (0.25 μmol/g body weight) injection were harvested for LSK (Lin⁻Sca1⁺c-kit⁺) cell frequency assessment using flow cytometry. Representative flow graph (left) and quantification (right) are shown. Results are mean ± SD of three independent experiments (*n* = 3 per group per experiment). (B) H₂O₂ and Salidroside treatment does not alter the morphology of the primitive cells. LDBMCs isolated from *Parp1*^{-/-} mice pretreated with H₂O₂ in the presence or absence of Salidroside were subjected to ImageStream analysis. Cells were gated on LSK population. Representative images are shown. (C) Salidroside does not prevent H₂O₂-induced LSKCD150⁺CD48⁻ cell loss in *Parp1*^{-/-} mice. BM cells described in (A) were subjected to flow cytometry analysis for LSKCD150⁺CD48⁻ frequency. Representative flow graph (left) and quantification (right) are shown. Results are mean ± SD of three independent experiments (*n* = 3 per group per experiment). (D) Salidroside does not enhance *Parp1*^{-/-} HSC repopulation in recipient mice. LSK (Lin⁻Sca1⁺c-kit⁺) cells from WT C57BL/6 mice or *Parp1*^{-/-} mice (CD45.2⁺) pretreated with or without Salidroside (75 μg/g body weight) followed by H₂O₂ (0.25 μmol/g body weight) treatment were isolated by cell sorting using FlowAria II. One thousand sorted LSK cells plus 1 million c-kit-depleted competitors (CD45.1⁺) were injected to lethally irradiated recipients. Donor chimerism was examined at 4 months after transplantation using peripheral blood from recipients. Representative images (left) and quantifications (right) are shown. Results are mean ± SD of three independent experiments (*n* = 5 per group per experiment). (E) Salidroside fails to prevent *Parp1*^{-/-} HSC cycling *in vivo*. BMCs were harvested from mice described in (D) followed by 7AAD and BrdU staining to determine cycling cells in CD45.2⁺ LSK CD34⁻ population. Representative images (left) and quantifications (right) are shown. Results are mean ± SD of three independent experiments (*n* = 3 per group per experiment). (F) Salidroside and H₂O₂ have no effect on apoptosis of LSK CD34⁻ cells. BMCs were harvested from mice described in (D) followed by 7AAD and Annexin V staining to determine apoptotic cells in the CD45.2⁺ LSK CD34⁻ population. Representative images (left) and quantifications (right) are shown. Results are mean ± SD of three independent experiments (*n* = 3 per group per experiment). **p* < 0.05; ***p* < 0.01; ****p* < 0.001. BMCs, bone marrow cells; LDBMCs, low-density bone marrow cells; BrdU, bromodeoxyuridine; 7AAD, 7-aminoactinomycin D; HSC, hematopoietic stem cell; PARP1, poly(ADP-ribose) polymerase-1; SD, Salidroside; WT, wild type. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 1. (Continued)

high sensitivity in near-native conditions (1). Thermophoresis of the protein in the presence of varying concentrations of chemicals was analyzed for 30 s. Measurements were performed at room temperature and standard deviation was calculated from three independent experiments. The results show that full-length PARP1 specifically bound Salidroside, but not 1-(2-(glucopyranosyl)-phenyl)-ethanone, a chemical with a similar structure to Salidroside (Supplementary Fig. S2), in titration assays and yielded a K_d value of ~ 84 nM (Fig. 3A), while constructs without the WGR domain (PARP1 mutants deleted for C-terminus), including constructs 1 (PARP1₁₋₂₁₅, Zn1, and Zn2), 2 (PARP1_{Indash;375}, Zn1, Zn2, and Zn3), 3 (PARP1₁₋₄₇₉), and 4 (PARP1₁₋₅₂₅, Zn1, Zn2, Zn3, and BRCT) failed to bind Salidroside (Supplementary Fig. S3 and Table 1). We also found that PARP1 mutants deleted for N-terminus, including constructs 5 (PARP1₂₁₆₋₁₀₁₄, N-terminal deletion of Zn1), 6 (PARP1₃₇₆₋₁₀₁₄, N-terminal deletion of Zn1 and Zn2), 7 (PARP1₅₂₆₋₁₀₁₄, WGR-CAT domain), and 8

(PARP1₄₈₀₋₁₀₁₄, DsDB-WGR-CAT domain, 37) had relatively comparable binding affinity to full-length PARP1 (Supplementary Fig. S3 and Table 1), suggesting that the C-terminal of PARP1 is required for Salidroside binding. To further reveal the binding between PARP1 and Salidroside, we mutated residue Arg⁵⁹¹ in the WGR domain (Fig. 3B), which has been shown critical for the Zn1 DNA damage interface and the CAT (24). The result shows that mutagenesis of the WGR domain (R591K) disrupted the binding of PARP1 to Salidroside (Fig. 3C). Taken together, these results suggested that the WGR domain is required for Salidroside-PARP1 binding.

Salidroside activates PARP1 through binding to the WGR domain in vitro

To elucidate the action of mechanism for Salidroside in PARP1 stimulation, we isolated LSK cells from *Parp1*^{-/-} mice and transduced them with the lentiviral vector expressing

FIG. 2. Expression and purification of PARP1 proteins. (A) Schematic presentation of functional domains of PARP1 protein. (B) Generation of PARP1 derivatives. Sequences corresponding to the indicated PARP1 domains were cloned into the pET32a vector. (C) Expression of truncated PARP1 proteins. *E. coli* HMS174 (DE3) pLysS harboring different domains of PARP1 were grown in the presence (+) of IPTG to induce expression of the truncated proteins. Fifty micrograms of cell lysates was analyzed by coomassie blue staining. Arrows donate the corresponding truncated proteins. (D) Western blot analysis of the truncated PARP1 proteins using the anti-histidine antibody. Truncated proteins were purified by affinity chromatography on a nickel-sepharose column, followed by gel filtration on a PD-10 column. IPTG, isopropyl- β -D-1-thiogalactopyranoside.

the Venus-only, Venus-PARP1_{WT}, or Venus-PARP1_{R591K} mutant. Transduction efficiency was comparable between groups (around 90%; Supplementary Fig. S4). We found that complementation of *Parp1*^{-/-} cells with PARP1_{WT} restored its activation under oxidative stress in response to Salidroside, as determined by *in vitro* PARP1 enzyme activity assay (Fig. 4A), which was correlated with accelerated DNA damage clearance as detected by immune blotting for γ -H2AX, a robust indicator of DNA strand breaks (Fig. 4B). Specifically, Salidroside stimulated PARP1 activity in LSK cells from *Parp1*^{-/-} mice complemented with PARP1_{WT}. Furthermore, the level of γ -H2AX decreased as early as 4 h after Salidroside treatment in *Parp1*^{-/-} cells complemented with PARP1_{WT}. In contrast, *Parp1*^{-/-} cells expressing the WGR mutant (PARP1_{R591K}) failed to respond to Salidroside and showed sustained accumulation of DNA damage up to 12 h post H₂O₂ treatment (Fig. 4B). Correspondingly,

PARP1_{WT} but not the PARP1_{R591K} mutant restored *in vitro* the self-renewal capacity of *Parp1*^{-/-} hematopoietic stem progenitor cells as determined by the colony-forming unit (CFU) assay (Fig. 4C). Together, these results indicate that Salidroside stimulates PARP1 activation through its binding to the WGR domain of PARP1.

The WGR domain is critical for PARP1 activation by Salidroside *in vivo*

To substantiate the *in vitro* studies, we next sought to test whether the WGR domain of PARP1 was required for Salidroside-stimulated PARP1 activation *in vivo*. To this end, we transduced LSK cells isolated from *Parp1*^{-/-} mice with lentiviral vectors expressing Venus-only, Venus-PARP1_{WT}, or Venus-PARP1_{R591K} mutant, followed by H₂O₂ treatment in the presence or absence of Salidroside. One thousand

FIG. 3. Solidoside binds to WGR domain of PARP1. (A) Solidoside specifically binds to WGR domain of PARP1. Purified full-length or truncated PARP1 proteins were first labeled with Alexa-647 fluorescence dye followed by microscale thermophoresis analysis. Solidoside was titrated between 3 and 50,000 nM to a constant amount of labeled PARP1 proteins (100 nM). The reaction was performed in 50 mM HEPES, 50 mM NaCl, 0.01% Tween20, and 2 mM MgCl₂. The samples were incubated at room temperature for 30 min before the measurements. Data were normalized to either $\Delta F_{\text{norm}} [\%]$ ($10 \times (F_{\text{norm}}(\text{bound}) - F_{\text{norm}}(\text{unbound}))$) or Fraction bound ($\Delta F_{\text{norm}} [\%] / \text{amplitude}$). The average K_d values $\pm$ SD were calculated from three replicates. Results are mean $\pm$ SD of three independent experiments. (B) Mutagenesis of WGR domain. (C) WGR mutant (PARP1_{R591K}) fails to respond to Solidoside stimulation. Purified full-length PARP1 proteins carrying the R591K mutant were used for the experiment described in (A). WGR, tryptophan-glycine-arginine-rich.

treated Venus⁺ LSK cells were then transplanted to lethally irradiated BoyJ recipients. Four months after bone marrow transplantation (BMT), donor-derived chimera was detected by flow cytometry. The results showed that cells expressing PARP1_{WT}, but not the empty vector or the PARP1_{R591K} mutant responded to Solidoside stimulation and succeeded in reconstituting recipient hematopoiesis, as determined by flow cytometry for CD45.2⁺ cell frequency in the BM of the recipient mice (Fig. 5A). We also examined the morphology of the donor-derived LSK cells after hematopoietic reconstitution by ImageStream analysis, and found no significant change in the morphological appearance of these LSK cells after reconstitution in the irradiated recipient mice (Fig. 5B).

To provide functional evidence that binding to the WGR domain of PARP1 contributes to the antioxidant effects of Solidoside in HSC maintenance, we next determined the effect

of WGR-Solidoside interaction on HSC quiescence in recipients transplanted with Venus-only-, Venus-PARP1_{WT}-, or Venus-PARP1_{R591K}-transduced LSK cells. We performed pyronin Y staining, an established dye to stain RNA and correlated to actively cycling cells (22). Upon H₂O₂ injection, we observed a significantly increased cell cycle progression (pyronin⁺) in empty vector- or the WGR mutant (PARP1_{R591K})-transduced *Parp1*^{-/-} HSCs, which was not attenuated by Solidoside (Fig. 5C). Consistently, the level of DNA strand break marker, γ -H2AX, remained high in the presence of Solidoside in Venus-only or Venus-PARP1_{R591K} mutant-transduced *Parp1*^{-/-} HSCs (Fig. 5D). In contrast, PARP1_{WT}-complemented *Parp1*^{-/-} HSCs were prevented from H₂O₂-induced cycling by Solidoside (Fig. 5C) and showed reduced DNA damage (Fig. 5D). We also performed secondary BMT to confirm the functional relevance of binding to the WGR domain of PARP1 by Solidoside in LT-HSC maintenance. To this end, we transplanted 10 million whole bone marrow (WBM) cells from the Solidoside- and H₂O₂-treated primary recipients of *Parp1*^{-/-} cells expressing Venus-only, PARP1_{WT}, or the WGR mutant (PARP1_{R591K}), into lethally irradiated secondary WT recipients. Four months post-transplantation, we analyzed hematopoietic reconstitution derived from donor (CD45.2⁺) cells. The results showed that Solidoside failed to enhance donor-derived hematopoiesis in recipients transplanted with *Parp1*^{-/-} HSCs expressing the WGR mutant protein (Fig. 5E). Thus, the effect of Solidoside on HSC maintenance under oxidative stress requires the WGR domain of PARP1.

To explore the clinical significance of Solidoside in HSC maintenance in a disease model, we employed the Fanconi anemia (FA) mouse model, in which oxidative stress has been identified as a physiologic mediator of HSC loss (10, 29, 48). To this end, we treated *Fancc*^{-/-} or *Fancc*^{-/-} mice and their

TABLE 1. SUMMARY OF BINDING AFFINITY OF TRUNCATED PARP1 TO SALIDOSIDE

Truncated protein	K_d	Binding
PARP1 ₁₋₂₁₅	—	—
PARP1 ₁₋₃₇₅	—	—
PARP1 ₁₋₄₇₉	—	—
PARP1 ₁₋₅₂₅	—	—
PARP1 ₂₁₆₋₁₀₁₄	92.62 nM	+
PARP1 ₃₇₆₋₁₀₁₄	114.7 nM	+
PARP1 ₅₂₆₋₁₀₁₄	256.1 nM	+
PARP1 ₄₈₀₋₁₀₄₀	73.13 nM	+
PARP1 _{WT}	84.01 nM	+
PARP1 _{R591K}	—	—

PARP1, poly(ADP-ribose) polymerase-1.

FIG. 4. Salidroside stimulates PARP1 activation through WGR domain. (A) WGR domain is required for Salidroside-stimulated PARP1 activation. LSK cells isolated from *Parp1*^{-/-} mice were transduced with the lentiviral vector expressing Venus-only, Venus-PARP1_{WT}, or Venus-PARP1_{R591K} mutant. Transduced cells were then treated with or without H₂O₂ in the presence/absence of Salidroside followed by flow cytometry analysis for PARP1 activity. Representative flow graph gated on Venus⁺ cells are shown. (B) Repair kinetics. Lin⁻ cells isolated from *Parp1*^{-/-} mice were transduced with lentiviral vector expressing Venus-only, Venus-PARP1_{WT}, or Venus-PARP1_{R591K} mutant followed by cells sorting for the Venus⁺ population. Venus⁺ cells were then treated with Salidroside (250 μ M) for 2 h, then H₂O₂ for an additional 2 h, and released for indicated time intervals. Whole cell lysates (30,000 cells/lane) were subjected to SDS-PAGE and immunoblotted with antibodies against γ -H2AX, FLAG-M2, and β -actin. (C) Salidroside restores progenitor activity in PARP1_{WT}-complemented *Parp1*^{-/-} LSK under oxidative stress. Cells described in (A) were sorted for the Venus⁺ population. One hundred Venus⁺ LSK cells were then plated in a cytokine-supplemented methylcellulose medium for colony formation assay. Colonies were counted on day 7. Results are mean $\pm$ SD of three independent experiments ($n=3$ per group per experiment). * $p<0.05$; ** $p<0.01$; *** $p<0.001$. SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

littermates (CD45.2⁺) with or without Salidroside followed by H₂O₂ injection. One thousand sorted LSK cells from the treated mice plus 1 million c-kit-depleted competitors (CD45.1⁺) were then transplanted into lethally irradiated recipients (CD45.1⁺). Consistent with previous reports that FA murine HSCs have defective reconstitution after replicative stress-generating BMT (48, 49), we found that *Fancc*^{-/-} and *Fancc*^{-/-} HSCs showed poor engraftment in the recipient mice and oxidative stress further compromised their reconstituting ability (Supplementary Fig. S5). Significantly, Salidroside enhanced the ability of FA HSCs in repopulating recipient hematopoiesis (Supplementary Fig. S5). These results suggest that Salidroside may have a therapeutic value for hematologic diseases like FA, where oxidative stress plays a pathogenic role.

Discussion

The present study investigated the role of PARP1 in oxidative DNA damage responses and identified the WGR do-

main as the structural element for Salidroside binding to PARP1. We propose that WGR-Salidroside binding is critical for PARP1 activation and protects quiescent HSCs from oxidative stress-induced cycling and subsequent exhaustion. We have provided several lines of evidence to support the following notions: (i) Salidroside binds to the WGR domain of PARP1 and stimulates the PARP1 activity *in vitro*; (ii) R591K mutation in the WGR domain of PARP1 disrupts the binding of Salidroside to PARP1; (iii) complementation of *Parp1*^{-/-} cells with WT PARP1, but not the WGR mutant restores the Salidroside-stimulated PARP1 activation and ODDR; (iv) *Parp1*^{-/-} HSCs expressing WT PARP1, but not the WGR mutant collaborate with Salidroside in long-term hematopoietic repopulation under oxidative stress.

We found that HSCs deficient in the *Parp1* gene are more sensitive to H₂O₂ treatment (Fig. 1), which leads to significant long-term HSC loss and HSC exhaustion. Recently, premature stem cell exhaustion due to accumulation of ROS or DNA damage in mice deficient in several oxidative stress

FIG. 5. WGR domain is required for PARP1 function *in vivo*. (A) WGR domain is essential for Salidroside-enhanced repopulating ability of stressed HSCs. LSK cells isolated from *Parp1*^{-/-} mice were transduced with the lentiviral vector expressing Venus-only, Venus-PARP1^{WT}, or Venus-PARP1^{R591K} mutant followed by cell sorting for Venus. Venus⁺ cells were then treated with or without H₂O₂ in the presence/absence of Salidroside followed by BMT by transplanting 1000 Venus⁺ cells to lethally irradiated BoyJ recipients. Four months after BMT, donor-derived chimera (CD45.2⁺) was detected by flow cytometry. Representative images (upper) and quantification (lower) are shown. Results are mean ± SD of three independent experiments (*n* = 5 per group per experiment). (B) H₂O₂ and Salidroside treatment does not alter the morphology of the cells after reconstitution. Venus⁺ cells from recipients described in (A) were subjected to ImageStream analysis. Representative images are shown. (C) The maintenance of HSC quiescence by Salidroside requires functional PARP1. One thousand Venus⁺ LSK cells from WT or *Parp1*^{-/-} mice were injected to lethally irradiated WT recipients. Recipient mice were then subjected to Salidroside and H₂O₂ treatments. Cycling donor-derived Venus⁺ cells were assessed by flow cytometry analysis by pyronin Y staining. Representative images (top) and quantifications (bottom) are shown. Results are mean ± SD of three independent experiments (*n* = 3 per group per experiment). (D) Salidroside reduces H₂O₂-induced-γ-H2AX in PARP1^{WT}-complemented *Parp1*^{-/-} cells. Venus⁺ cells isolated from recipients described in (C) were subjected to protein extraction followed by WB analysis using antibodies specific for γ-H2AX, FLAG-M2, and β-actin. (E) The enhancing effect of Salidroside on the long-term repopulation abilities of stressed HSCs requires PARP1. BMCs from primary recipients described in (B) were used for second transplantation by injecting 10 million whole BM cells to lethally irradiated WT recipients. Donor-derived cells were determined by flow cytometry analysis. Results are mean ± SD of three independent experiments (*n* = 3 per group per experiment). **p* < 0.5; ***p* < 0.01; ****p* < 0.001. BMT, bone marrow transplantation. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

responses, such as *Atm*, *Fancc*, *Fancd2*, *FoxO*, or DNA damage repair, such as *Lig4*, *Dna-pk*, *Ku80*, *Xpd*, *mTR* genes, has been reported (28, 43, 46). We further show that the phenylpropanoid glycoside from medicinal plants, Salidroside, prevents LSK expansion and excessive stem cell cycling in WT mice, but fails to protect *Parp1*^{-/-} HSCs from oxidative stress. This finding was confirmed by BMT using Salidroside-treated *Parp1*^{-/-} LSK cells. These results pro-

vide genetic evidence that the antioxidant effect of Salidroside on the function of HSCs requires PARP1.

Poly(ADP-ribose) polymerase-1 (PARP1) is an enzyme that catalyzes post-translational modifications with ADP-ribose polymers, which play critical roles in multiple cellular processes, including gene transcription, DNA damage detection/repair, and cell death signaling (35). The molecular architecture of PARP1 has been a subject of intensive

investigation (17, 23, 27). Among the structural domains, the function of WGR domain is less known. Recent study indicates that the WGR domain is a central component of the complex, interacting with Zn1, Zn3, CAT, and DNA, forming a network of interdomain contacts that links the DNA damage interface to the CAT domain (23). Structurally, a key Zn1-WGR contact is a salt bridge formed between Asp⁴⁵ of Zn1 and Arg⁵⁹¹ of WGR domain. Arg⁵⁹¹ also interacts with the HD domain of PARP1 (23). In this study, we determined the binding of Salidroside to a set of truncated PARP1 peptides by *in vitro* microscale thermophoretic analysis and found that Salidroside specifically bound full-length PARP1 or the truncated proteins containing the WGR domain. Furthermore, mutagenesis of R591K in the WGR domain disrupted the binding (Fig. 3 and Supplemental Fig. S3), suggesting that the WGR domain is required for Salidroside binding. Functionally, Salidroside stimulated PARP1 activation and ODDR in *Parp1*^{-/-} HSCs expressing only the PARP1 protein containing a functional WGR domain under oxidative stress (Fig. 4). This stimulation also enhanced the repopulating capacity of the stressed HSCs in irradiated recipients (Fig. 5). Taken together, our results indicate that the WGR domain of PARP1 is the structural determinant for Salidroside-PARP1 binding and PARP1 activation in the context of stressed hematopoiesis.

Another interesting finding of our study is that *Parp1*^{-/-} HSCs display increased cycling under oxidative stress, probably due to accumulation of unrepaired DNA damage. Genomic DNA integrity is a limiting factor in the maintenance of a functional HSC (28). Recent studies using mouse models deficient for homologous recombination, nonhomologous end-joining, nucleotide excision repair, and telomere maintenance pathways have shown the importance of DNA repair pathways in maintaining the HSC function (9, 11, 37, 39). Although studies have begun to connect the molecular functions of PARP1 to specific physiological and pathological outcomes (27, 35), the role of PARP1 in HSC maintenance remains to be elucidated. Our present results suggest that oxidative stress compromises the self-renewal capacity of *Parp1*^{-/-} HSCs (Fig. 1D), which is associated with sustained DNA damage accumulation (Fig. 4B), increased HSC cycling (Fig. 1E, C). In addition, by employing the FA mouse model, a blood disease associated with oxidative stress, our present study accentuated the therapeutic potential of Salidroside in HSC maintenance. Our findings are also consistent with recent studies that an intact DNA repair network is required for oxidative stress-induced DNA damage repair and HSC maintenance (28, 30, 34, 46). Although further studies are required to reveal the molecular mechanisms of PARP1 activation in protecting HSCs from oxidative stress-induced DNA damage, it is intriguing that Salidroside-stimulated PARP1 activity contributed to the accelerated repair of oxidative DNA damage in the stressed HSCs and the enhanced repopulating capacity in the recipients. In this context, our data lend support to the notion that DNA repair is essential for the maintenance of HSC self-renewal capacity (9, 11, 37, 39).

Materials and Methods

Mice

129S*Parp1*^{tm1zgw/J} (The Jackson Laboratory, Bar Harbor, ME) was backcrossing with WT C57BL/6 mice for eight

generations before interbreeding heterozygous *Parp1*^{+/-} mice to generate *Parp1*^{-/-} mice. *Fanca*^{-/-} and *Fancc*^{-/-} mice were generated by interbreeding the heterozygous *Fanca*^{+/-} mice (C57BL/6) (provided by Dr. Madeleine Carreau at Laval University) (44) or *Fancc*^{+/-} mice (provided by Dr. Manuel Buchwald at Hospital for Sick Children, University of Toronto) (6), respectively. All the animals, including BoyJ mice, were maintained in the animal barrier facility at the Cincinnati Children's Hospital Medical Center.

Chemicals

For *in vivo* experiments, mice were injected with Salidroside (75 µg/g body weight; PhytoLab, Vestenbergsgreuth, Germany), or saline vehicle, intraperitoneally (i.p.) followed by H₂O₂ (0.25 µmol/g body weight; Sigma-Aldrich, St Louis, MO) (26). All experimental procedures conducted in this study were approved by the Institutional Animal Care and Use Committee of Cincinnati Children's Hospital Medical Center.

For thermophoresis analysis, Salidroside or 1-(2-(glucopyranosyl)-phenyl)-ethanone (Sigma-Aldrich), which shares a similar chemical construct with Salidroside, was titrated (3–50,000 nM) to a constant amount of labeled truncated PARP1 proteins (100 nM).

Molecular cloning and materials

Sequences corresponding to different PARP1 domains were cloned into the pET32a vector (a gift from Dr. Masahiko Satoh at Laval University Medical Center) (12). Proteins were expressed in HMS174 (DE3) pLysS (Stratagene, Santa Clara, CA) and purified using nickel-nitrilotriacetic acid agarose gel chromatography (Qiagen, Valencia, CA). In addition to His tag, FLAG tag was also attached at the N-terminus of each construct.

For lentiviral vector construction, FLAG-tagged full-length PARP1 cDNA was cloned into the pRRL-SIN-cPPT-MNDU3-MCS-IVW (TMND-IRES-Venus) vector (a gift from Dr. Punam Malik at the Cincinnati Children's Hospital Medical Center), which is a HIV-based self-inactivating (SIN) lentiviral vector containing the central polypurine and termination tract (cPPT). The expression of PARP1 is controlled by a modified MNDU3 promoter. The vector also carries the internal ribosome entry site (IRES) followed by Venus and woodchuck hepatitis virus post-transcriptional regulatory element (WPRE).

Mutagenesis was carried out using the QuickChange Lightning Site-Directed Mutagenesis Kit (Stratagene).

Microscale thermophoretic analysis

Purified different truncated PARP1 proteins were first labeled with Alexa-647 fluorescence dye (NanoTemper, München, Germany). Salidroside or 1-(2-(glucopyranosyl)-phenyl)-ethanone were titrated (3–50,000 nM) to a constant amount of labeled truncated PARP1 proteins (100 nM). The reaction was performed in 50 mM HEPES, 50 mM NaCl, 0.01% Tween20, and 2 mM MgCl₂. Then, the samples were incubated in room temperature for 1 h before analyzing by microscale thermophoresis. A NanoTemper Monolith Instrument (NT.015; NanoTemper) was used for measuring thermophoresis. In this instrument, an infrared laser

(IR-laser) beam couples into the path of light (*i.e.*, fluorescence excitation and emission) with a dichroic mirror and is focused into the sample fluid through the same optical element used for fluorescence imaging. The IR-laser is absorbed by the aqueous solution in the capillary and locally heats the sample with a 1/e² diameter of 25 μ m. Up to 24 mW of laser power was used to heat the sample without damaging the biomolecules (42).

To analyze the thermophoresis of a sample, 4 μ l was transferred in a hydrophilic-treated glass capillary (Nano-Temper). Thermophoresis of the protein in the presence of varying concentrations of compound was analyzed for 30 s. Measurements were performed at room temperature and standard deviation was calculated from three independent experiments. Binding data were analyzed using Graphpad Prism to estimate K_d values. Data were normalized to either $\Delta F_{\text{norm}} [\%]$ ($10 \times (F_{\text{norm}}(\text{bound}) - F_{\text{norm}}(\text{unbound}))$) or Fraction bound ($\Delta F_{\text{norm}} [\%]/\text{amplitude}$).

PARP1 enzyme activity

The PARP1 enzyme activity was detected following the established protocol (PARP1 Enzyme Activity Assay; Millipore, Billerica, MA) and the previous publications (21, 26) for the measurement of cellular poly(ADP-ribosylation) capacity. Briefly, cells (2×10^5 per data point) were centrifuged at 326 *g* for 5 min and resuspended in 100% ethanol and left at -20°C for at least 20 min. Cells were then permeabilized in 10 ml of buffer A (10 mM Tris-HCl pH 7.8, 1 mM ethylenediaminetetraacetic acid [EDTA], 4 mM MgCl_2 , and 30 mM 2-mercaptoethanol). Then, cells were centrifuged again at 815 *g* for 10 min and resuspended in buffer A again and transferred to a V-shaped 96-well plate on ice for at least 5 min. Then, 20 μ l of $3 \times$ reaction buffer (with or without NAD^+) plus 13 μ l of 15 mM NaCl incorporating were added to the reaction mix followed by a 37°C incubation for 10 min. Controls (cells with a reaction buffer in the absence of NAD^+) were always included in all the experiments to define the background. Then, second fixation was done by adding 60 μ l of 4% formaldehyde/phosphate-buffered saline (PBS) for 20 min at room temperature. Sixty microliters of 100 mM glycine in PBS was then added to quench the reaction. Cells were then centrifuged at 815 *g* for 10 min and resuspended in 100 μ l of primary antibody (anti-PARP1 antibody; Acris, San Diego, CA) diluted in a fluorescence-activated cell sorter (FACS) buffer (BD Biosciences, San Jose, CA) and incubated at 37°C for 1 h or overnight at 4°C . Then, the cells were washed and resuspended in 100 μ l of diluted secondary antibody (PE-conjugated goat anti-mouse; Invitrogen, Grand Island, NY) followed by a 37°C incubation for 30 min in the dark. Cells were washed and resuspended for flow cytometry analysis. Similar levels of background were detected between samples.

Flow cytometry and cell cycle analysis

The lineage marker (Lin) mixture (BD Biosciences) for BM cells from treated or untreated mice included the following biotinylated antibodies: CD3 ϵ (145-2C11), CD11b (M1/70), CD45R/B220 (RA3-6B2), mouse erythroid cells Ly-76 (Ter119), and Ly6G and Ly-6C (RB6-8C5). Other conjugated antibodies (BD Biosciences, San Jose, CA) used for surface staining included CD45.1 (A20), CD45.2 (A104),

Scal (D7), c-kit (2B8), CD48 (HM48-1), and CD150 (9D1). Biotinylated primary antibodies were detected by incubation of antibody-coated cells with streptavidin-PerCP or fluorescein isothiocyanate (FITC) (BD Biosciences) in a two-step staining procedure. The percentage of apoptotic cells was determined by Annexin V and 7AAD staining (BD Biosciences) according to the manufacturer's instructions. For some of the experiments, pacific blue-conjugated CD45.2 (A104; BioLegend, San Diego CA) was used to determine donor-derived cells. For cell cycle analysis, cells were surface stained, fixed, and permeabilized in the BD Cytotfix/Cytoperm Buffer (BD Biosciences), then stained with 5 μ g/ml Hoechst 33342 and 150 ng/ml Pyronin Y (Sigma-Aldrich). Cells were then subjected to Flow Cytometric analysis (BD Biosciences).

BrdU incorporation assay

Subject mice were injected with BrdU (150 μ l of 10 mg/ml) (40) *i.p.* followed by BM cells isolation 14 h later. BrdU-incorporated cells (S phase) were analyzed with the FITC BrdU Flow Kit (BD Biosciences), following the manufacturer's instructions. Briefly, cells were surface stained, then fixed, and permeabilized by the BD Cytotfix/Cytoperm Buffer. After 1 h of incubation with DNase at 37°C , cells were stained with the APC-conjugated anti-BrdU monoclonal antibody. 7-AAD was added to each sample right before the flow cytometry analysis (BD Biosciences).

CFU assay

LSK cells isolated from *Parp1*^{-/-} mice were transduced with lentivirus-expressing indicated construct. One hundred of transduced cells were seeded in duplicate and cultured in a cytokine-supplemented methylcellulose medium (MethoCult 3434; Stem Cell Technologies, Vancouver, Canada) in 35-mm gridded dishes. Colonies were counted on day 7.

Bone marrow transplantation

One thousand sorted LSK cells from H_2O_2 -treated mice (CD45.2⁺) pretreated with or without Salidroside, along with 1 million BM cells (depleted of c-Kit⁺ cells to provide short-term hematopoiesis after irradiation using CD117 MicroBeads (Miltenyi Biotec, Auburn, CA) obtained from congenic WT mice (CD45.1⁺) were injected into lethally irradiated (11 Gy) congenic recipients (CD45.1⁺) by tail vein injection. Hematopoietic reconstitution in recipient mice by donor (CD45.2⁺) cells at 8 and 16 weeks post-transplantation was determined by the CD45.1-PE and CD45.2-FITC marker staining followed by flow cytometry FACS Canto I (BD Biosciences) analysis. For second transplantation, 10×10^6 bone marrow cells (BMCs) from the primary transplants (16 weeks after transplantation) were injected into lethally irradiated congenic (CD45.1⁺) recipients.

Preparation of cell extracts, immunoblotting

To prepare whole cell extracts, cells were washed with ice-cold PBS and resuspended in the ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 0.1% NP40, and 1 M NaCl supplemented with protease and phosphatase inhibitors (10 μ g/ml aprotinin, 25 μ g/ml leupeptin, 10 μ g/ml pepstatin A, 2 mM PMSF, 0.1 M NaP_2O_4 , 25 mM NaF, and 2 mM

sodium orthovanadate) for 30 min on ice. Cell lysates were resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblots were analyzed with antibodies for the active form of γ -H2AX (Millipore, Billerica, MA), FLAG-M2, or β -actin (Sigma-Aldrich). Each lane contains protein from 30,000 cells. Signals were visualized by incubation with anti-mouse or anti-rabbit secondary antibodies followed by ECL chemiluminescence (Amersham Biosciences, Piscataway, NJ).

Measurement of oxidative stress

Surface marker-stained low-density bone marrow cells (LDBMCs) were incubated with CM-H₂DCFDA (Molecular Probes, Eugene, OR; Invitrogen) in the dark for 15 min at 37°C. After washing, the cells were analyzed by flow cytometry using a FACSCanto (BD Biosciences). Data were analyzed using the FlowJo program (Tree Star, Ashland, OR).

Lipid peroxides were directly quantified using an ELISA assay that measures conversion of ferrous ions to ferric ions with the Lipid hydroperoxidase Assay Kit (Cayman, Ann Arbor, MI). Briefly, WBCs isolated from H₂O₂-treated mice were resuspended with Extract R (Lipid hydroperoxidase Assay Kit) saturated methanol. Then, 1 ml of cold chloroform was added to the test tube followed by centrifugation at 1500 g for 5 min at 0°. Then, the bottom chloroform layer was collected by a syringe needle along the side of the test tube and transferred to another tube. The chromogen was prepared by mixing equal volumes of FTS Reagent 1 and FTS Reagent 2 in a test tube and vortex. Then, 50 μ l of the freshly prepared chromogen was added to each assay tube and mixed well by vortexing followed by room temperature incubation for 5 min. Then, 300 μ l of samples from each tube were transferred into the 96-well plate for the absorbance reading at 500 nm using SpectraMax Absorbance Microplate Reader (Molecular Devices, Sunnyvale, CA).

ImageStream analysis

LDBMCs isolated from *Parp1*^{-/-} mice or donor-derived Venus⁺ cells from recipient mice, pretreated with or without H₂O₂ in the presence or absence of Salidroside, were stained for surface markers using antibodies against lineage markers (the lineage marker [Lin] mixture; BD Biosciences), Sca1, and c-kit (BD Biosciences) followed by ImageStream analysis (Amnis Corporation, Seattle, WA). The data were analyzed by IDEADS software (Amnis Corporation).

Statistical analysis

Paired or unpaired Student's *t*-test was used for two-group comparison and one-way ANOVA for more than two-group comparison. Values of *p* less than 0.05 were considered statistically significant. Results are presented as mean $\pm$ SD. * Indicates *p* < 0.05; ** indicates *p* < 0.01; *** indicates *p* < 0.001.

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Authors' Contributions

XL, designed research, performed research, analyzed data, and wrote the article; OE, LL, QY, AW, performed research; WD, designed research, analyzed data, and wrote the article.

Author Disclosure Statement

The authors declare no conflicts of interest.

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Abbreviations Used

7AAD = 7-aminoactinomycin D
AD = automodification domain
BM = bone marrow
BMC = bone marrow cell
BMT = bone marrow transplantation
BrdU = bromodeoxyuridine
CAT = the catalytic domain
CFU = colony-forming unit
cPPT = central polypurine and termination tract
DBD = DNA-binding domain
EDTA = ethylenediaminetetraacetic acid
FA = Fanconi anemia
FACS = fluorescence-activated cell sorting
FITC = fluorescein isothiocyanate
HD = the helical subdomain
HSCs = hematopoietic stem cells
i.p. = intraperitoneally
IPTG = isopropyl- β -D-1-thiogalactopyranoside
IRES = internal ribosome entry site
LDBMC = low-density bone marrow cell
LPO = lipid peroxide
LSK = Lin[−]c-kit⁺Sca1⁺
LT-HSCs = LSK-CD150⁺CD48[−]
ODDR = oxidative DNA damage repair
PAR = poly(ADP-ribose)
PARP1 = poly(ADP-ribose) polymerase-1
PBS = phosphate-buffered saline
ROS = reactive oxygen species
SD = Salidroside
SDS-PAGE = sodium dodecyl sulfate–polyacrylamide gel electrophoresis
WBM = whole bone marrow
WGR = tryptophan–glycine–arginine-rich
WPRE = woodchuck hepatitis virus post-transcriptional regulatory element
WT = wild type