ORIGINAL RESEARCH COMMUNICATION

Low Oxidative Stress-Mediated Proliferation Via JNK-FOXO3a-Catalase Signaling in Transplanted Adult Stem Cells Promotes Wound Tissue Regeneration

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Abstract

Aims: Stem cells exposed to pathological levels of reactive oxygen species (ROS) at wound sites fail to regenerate tissue. The molecular mechanism underlying differential levels of ROS-mediated regulation of stem cells remains elusive. This study elucidates the mechanistic role of catalase at 10 μM H_2O_2 -induced proliferation of mouse bone marrow stromal (BMSC) and hematopoietic (HSPC) stem/progenitor cells.

Results: BMSCs and HSPCs depicted an increased growth rate and colony formation, in the presence of 10 μM but not 100 μM concentration of H_2O_2 , an effect that was perturbed by Vit. C. Mechanistically, JNK activation–FOXO3a nuclear translocation and binding of FOXO3a to catalase promoter at 10 μM H_2O_2 led to an increased expression and activity of anti-oxidant gene, catalase. This was followed by an increased proliferative phenotype *via* the AKT-dependent pathway that was perturbed in the presence of catalase-inhibitor, 3-aminotriazole due to an increased ROS-mediated inactivation of AKT. Preclinically, 10 μM H_2O_2 -mediated preconditioning of BMSCs/HSPCs transplantation accelerated wound closure, enhanced catalase expression, and decreased ROS levels at the wound site. Transplantation of male donor cells into female recipient mice or GFP-labeled BMSCs or HSPCs depicted an increased engraftment and proliferation in preconditioned cell transplanted groups as compared with the wound control. Wound healing occurred *via* keratinocyte generation and vascularization in preconditioned BMSCs, whereas only neo-vascularization occurred in the preconditioned HSPCs transplanted groups.

Innovation and Conclusion: Our study suggests a distinct role of catalase that protects BMSCs and HSPCs from low ROS and promotes proliferation. Transplantation of preconditioned stem cells enhanced wound tissue regeneration with a better antioxidant defense mechanism—as a therapeutic approach in stem cell transplantation-mediated tissue regeneration. *Antioxid. Redox Signal.* 28, 1047–1065.

Keywords: ROS, JNK-FOXO3a, catalase, BMSC/HSPC engraftment, keratinocyte generation & neo-vascularization, wound tissue regeneration

Introduction

OXIDATIVE STRESS IN CELLS results from the imbalance between the generation and clearance of oxidants. Accumulation of intracellular reactive oxygen species (ROS) often leads to disease and cell apoptosis. Harmful effects caused by ROS on the cells result in DNA damage, lipid peroxidation, protein oxidation, and oxidation of co-factors, thereby inactivating specific enzymes and leading to patho-

logical consequences (42). Several disease conditions such as atherosclerosis, diabetes, neurodegenerative diseases, cancer, and chronic wounds are associated with increased ROS. In contrast to the damaging effects of ROS, recent studies also implicate a beneficial role of ROS at a lower concentration to the cellular physiology (42). Also, the source and type of ROS as well as their amount and duration of exposure in various sub-cellular localizations state their biological effect. Emerging evidence suggests that ROS act as an

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Innovation

Bone marrow-derived bone marrow stromal stem/progenitor cells (BMSCs) and hematopoietic stem and progenitor cells (HSPCs), when exposed to 10 μ M concentration of H₂O₂, regulate the survival signaling mechanisms, thereby increasing proliferation, an effect that was perturbed by exogenous antioxidants. Mechanistically, an activation of JNK-FOXO3a signaling led to an increased expression and activity of catalase that, in turn, regulated the AKT activation-mediated proliferative phenotype of these cells. Transplantation of preconditioned BMSCs/HSPCs with 10 μ M concentration of H₂O₂ efficiently regenerated wound tissue *via* enhanced engraftment, proliferation, re-epithelization, and angiogenesis. This study offers a therapeutic approach for improved wound healing by preconditioning the stem cells, which equips them with a better antioxidant defense system.

important second messenger in tightly controlled signal transduction networks (38). ROS activate various physiological processes, including growth factor-mediated proliferation, angiogenesis, which involves activation of PI3K/AKT, MAPK/ERK, and JNK pathways (40). A complex equilibrium of ROS production exists that is temporally and spatially regulated by a fine-tuned balance between ROS generation systems and antioxidant enzymes (29). Redox regulations in cells are maintained by antioxidant enzymes that are located at different cellular compartments that convert superoxide into hydrogen peroxide (H₂O₂) and finally into water and molecular oxygen (27). The function of these antioxidant enzymes is dependent on the reaction kinetics, which, in turn, depends on the rate of reaction with H₂O₂, the concentration of H₂O₂, and enzymes *in vivo*. In addition, intracellular low-molecular-weight antioxidant, Vit. C is also known to protect the cells by quenching the free radicals (28).

Usually, cells generate H₂O₂ by the mitochondrial respiratory chain and NADPH oxidase, which is regulated by the glutathione system. Adult stem cells of bone marrow such as bone marrow stromal stem/progenitor cells (BMSCs) and hematopoietic stem/progenitor cells (HSPCs) are often exposed to different physiological levels of ROS such as H₂O₂. The low concentration of H₂O₂ in adult stem cells promotes their stemness and self-renewal capability (31). In contrast to HSPCs, myeloid committed progenitor cells are known to have a higher level of H₂O₂ (31). Thus, cellular response to H₂O₂ differs from cell to cell. However, the molecular mechanisms of cellular defense system against H₂O₂ in these adult stem cells are not yet clearly defined.

In our recent study, we extensively characterized bone marrow-derived BMSCs and HSPCs by using colony-forming efficiency (CFU) assays, differentiation assays, and expression of pluripotency and stem cell genes and proteins that were comparable with embryonic stem cell lines as well as with bonafide MSCs (8). In this study, we investigated the role of low oxidative stress in modulation of the antioxidant defense system in BMSCs and HSPCs. Our results depicted that low (10 μ M) but not high (100 μ M) concentrations of H₂O₂-induced oxidative stress significantly increased proliferation in BMSCs and HSPCs. Equimolar concentration of Vit. C and its metabolized product, dehydroascorbate (DHA)

could revert the molecular and cellular changes caused by 10 μ M H₂O₂. Further mechanistically, an activation of the JNK-FOXO3a signaling pathway along with antioxidant-catalase expression and activity was observed in these cells when exposed to 10 μ M H₂O₂. Finally, GFP-expressing BMSCs or HSPCs preconditioned with 10 μ M H₂O₂ when transplanted at the wound site *in vivo* resulted in improved engraftment, increased proliferation and catalase expression with a concomitant reduction in ROS along with enhanced keratinocyte formation and vascularization-mediated tissue regeneration.

Results

Low oxidative stress promotes proliferation of BMSCs and HSPCs

To evaluate the growth pattern of BMSCs and HSPCs in the presence of H₂O₂, the cells were treated for a maximum period of 96 h as previously described (11). Cells were harvested at a regular interval, and cell count was determined. Both BMSCs (Fig. 1A) and HSPCs (Fig. 1B) depicted a noticeable increase in cell growth, with a reduction in doubling time to 48 h when treated with 10 μ M H₂O₂ as compared with their respective controls (≥ 96 h). At higher concentrations of H₂O₂ (100 μ M), growth rates of both BMSCs (Fig. 1A) and HSPCs (Fig. 1B) were markedly decreased. Vit. C or DHA at an equimolar concentration could revert the low H₂O₂-induced increased growth rate of both BMSCs (Fig. 1A) and HSPCs (Fig. 1B). Further to corroborate the growth pattern of BMSC and HSPC, CFU and bromodeoxyuridine (BrdU) cell proliferation assays were performed. As observed in growth curve kinetics of 10 μ M but not 100 μ M concentration of H₂O₂, a significant increase in CFU and BrdU incorporation was depicted in both BMSCs (Fig. 1C, E) and HSPCs (Fig. 1D, F). Equimolar concentration (10 μ M) of Vit. C or DHA could rescue the effect of H₂O₂. These results clearly suggest that the specificity of H₂O₂-mediated oxidative stress promotes the proliferative potential of BMSCs and HSPCs that could be perturbed by the exogenous supplementation of antioxidants.

Low oxidative stress promotes cell cycle progression in BMSCs and HSPCs

The earlier observation of 10 μ M H₂O₂-induced proliferation incited us to explore the cell cycle status of these sorted bone marrow cell populations. Ten micromolar H₂O₂ significantly increased cell populations in S and G₂/M phases of the cell cycle that was perturbed in the presence of Vit. C (Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/ars). To substantiate the cell cycle status, we investigated the changes in cell cycle gene expression in BMSCs and HSPCs. Expression of cyclins B1, D2, and E1 was increased by several folds with 10 μ M H₂O₂ that was attenuated by an equimolar concentration of Vit. C (Supplementary Fig. S1B). In addition, 10 μ M H₂O₂-dependent decrease in expression of p16 and p27 cyclin-dependent kinase inhibitors (CKI) and an increase in proliferating cell nuclear antigen (PCNA) expression were observed, indicating the cell passage through S-G₂/M phases of the cell cycle (Supplementary Fig. S1C). The overall increase in cyclins, PCNA, and decrease in CKI expression by 10 μ M H₂O₂ promote the bone marrow-derived BMSCs and HSPCs to undergo cell expansion, which otherwise remains in the G₀/G₁ phase of the cell

FIG. 1. Low concentration of H_2O_2 induces proliferation of BMSCs and HSPCs. Significant increase in cell growth was observed in both bone marrow-derived BMSCs (A) and HSPCs (B) as depicted by growth curve analysis. Antioxidant effects of Vit. C or DHA could significantly reduce the H_2O_2 -induced growth rate of BMSCs and HSPCs at 10 μM concentration. Similar increase in H_2O_2 -induced colony formation units was observed in BMSCs (C) and HSPCs (D) that was perturbed by exogenous Vit. C or DHA in colony formation assay. Ten micromolar H_2O_2 -induced cell proliferation was also observed in BMSCs (E) and HSPCs (F) that was reversed by Vit. C and DHA in BrdU cell proliferation assay. Results are depicted as percent proliferation of control ($n = 4-6$ replicates per isolation; $p < 0.05$ as compared with *control and #10 μM H_2O_2 -treated group; $p < 0.01$ as compared with **control). BMSC, bone marrow stromal stem/progenitor cells; BrdU, bromodeoxyuridine; DHA, dehydroascorbic acid; H_2O_2 , hydrogen peroxide; HSPC, hematopoietic stem and progenitor cells; Vit. C, vitamin C. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

cycle. The mechanism of this cellular effect by oxidative stress was further supported by the ROS quencher-induced reversal of specific cell cycle gene induction.

Cellular redox status in H_2O_2 -treated BMSCs and HSPCs

To evaluate the intracellular and extracellular ROS levels along with glutathione (GSH) content in sorted BMSCs and HSPCs exposed to H_2O_2 , DCFH-DA staining, amplex red assay, and glutathione assay were performed. Significantly high concentration-dependent intracellular ROS levels were observed after 24 and 48 h post-treatment of H_2O_2 in BMSCs (Supplementary Fig. S2A) and HSPCs (Supplementary Fig. S2B) that was attenuated by Vit. C or DHA. The extracellular ROS level (H_2O_2 concentration) depicted a concentration-dependent significant increase that was overturned by addition of Vit. C or DHA as compared with control at 24 h. Further at 48 h post-

treatment, the extracellular ROS level decreased and was comparable to the control group, indicating the utilization of H_2O_2 by BMSCs (Supplementary Fig. S2C) and HSPCs (Supplementary Fig. S2D). In line with the H_2O_2 -induced increased ROS production, a concentration-dependent significant decrease of cellular GSH content was restored in BMSCs (Supplementary Fig. S2E) and HSPCs (Supplementary Fig. S2F) by addition of Vit. C or DHA as compared with the control at 48 h post-treatment. These data suggest that even 10 μM H_2O_2 causes significant oxidant perturbation in BMSCs and HSPCs that can, subsequently, activate downstream signal transduction pathways.

Low oxidative stress-induced activation of molecular signal transduction pathways in BMSCs and HSPCs

We evaluated the role of oxidative stress on growth-promoting and proliferative signal transduction pathways in BMSCs and HSPCs using immunoblot analysis. Ten micromolar H_2O_2

FIG. 2. Role of AKT and JNK activation on H₂O₂-induced BMSC and HSPCs proliferation. (A) Expression levels of phosphorylated and total protein levels of AKT, ERK1/2, p38 MAPK, and JNK were determined by Western blot analysis. Ten micromolar H₂O₂-induced AKT and JNK activation (phosphorylation) that was reverted by 10 μ M Vit. C in both BMSCs and HSPCs. Growth curve analysis of cells exposed to 10 μ M H₂O₂ along with pathway inhibitors in BMSCs (B) and HSPCs (C) depicted a decreased growth rate in the presence of wortmannin (AKT inhibitor) and SP600125 (JNK inhibitor) treatment. PD98059 (ERK1/2 inhibitor) and SB203580 (p38 MAPK inhibitor) did not reduce the 10 μ M H₂O₂-induced growth rate of both BMSCs and HSPCs. (D) BrdU cell proliferation assay depicted a significant decrease in BrdU incorporation in both BMSCs and HSPCs exposed to 10 μ M H₂O₂ along with wortmannin and SP600125, suggesting a pathway specificity ($n=4-6$ replicates per isolation; $p<0.05$ as compared with *control and #10 μ M H₂O₂-treated group). JNK, c-Jun N terminal kinase. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

treatment in these cells led to an increased phosphorylation of AKT and JNK (Fig. 2A). Further, a sub-lethal or high concentration of H₂O₂ (100 μ M) could phosphorylate JNK but not AKT, indicating a selective activation of JNK stress kinase (Fig. 2A), which was further confirmed by densitometric analysis (BMSCs-Supplementary Fig. S3A and HSPCs-Supplementary Fig. S3B). In addition, activation of ERK1/2 and p38 MAPK under similar conditions yielded insignificant change of these signaling mediators in both BMSCs and HSPCs. To confirm whether the earlier observation holds true, we treated HSPCs and BMSCs with H₂O₂ in both the presence and absence of inhibitors such as Wortmannin (AKT), SP600125 (JNK), PD98059 (ERK1/2) (48), and SB203580 (p38 MAPK) (12). Indeed, wortmannin and

SP600125 at 100 nM and 30 μ M, respectively, could significantly decrease the H₂O₂-induced proliferation in BMSCs (Fig. 2B) as well as HSPCs (Fig. 2C) as observed in both growth curve analysis and BrdU cell proliferation assays (Fig. 2D), suggesting pathway specificity. These observations indicate that 10 μ M H₂O₂-induced proliferation is mediated by concerted action of both AKT and JNK signaling pathways.

Cellular antioxidants facilitate BMSC and HSPC proliferation at low oxidative stress

Further, we investigated the status of the redox genes that are essential to detoxify the H₂O₂ as well as its effect on the

downstream cell signaling. Among the different isoforms of mammalian SODs, an H_2O_2 -mediated concentration-dependent increase in the expression of SOD2, the mitochondrial isoform was observed in both BMSC and HSPC populations, which were decreased by an equimolar concentration of Vit. C (Supplementary Fig. S4A). Similarly, the expression of GPx2 (mitochondrial isoform of GPx) depicted a 2.9-fold high in both BMSCs and HSPCs as compared with the control in the presence of H_2O_2 , which was not abrogated by an equimolar concentration of Vit. C (Supplementary Fig. S4B). Expression of catalase was 3.75-fold higher in BMSCs and 3.9-fold higher in HSPCs than the respective control at $10 \mu M$ exposure of H_2O_2 , which was mitigated in the presence of an equimolar concentration of Vit. C, indicating that low oxidative stress induces its expression

(Fig. 3A). However, at sub-lethal H_2O_2 concentration, catalase expression was decreased in both BMSCs and HSPCs, which substantiates with the existing literature that H_2O_2 at a high concentration downregulates catalase expression by epigenetic regulation *via* methylation of CpG islands (35).

Further, in parallel with their transcript levels, we also evaluated the activity of these antioxidant enzymes SOD, GPx, and catalase in cell extracts. Although a concentration-dependent increase in activity of SOD was observed in HSPCs and BMSCs treated with H_2O_2 , an equimolar concentration of Vit. C could not reverse the H_2O_2 -induced increase in total SOD activity (Supplementary Fig. S4C). Similarly, total GPx activity depicted an insignificant change in BMSCs (Supplementary Fig. S4D) but in HSPCs a significant increase was observed only at a high concentration ($100 \mu M$) of H_2O_2

FIG. 3. Low concentration of H_2O_2 potentiates the expression of antioxidant catalase, which when inhibited reversed H_2O_2 -induced proliferative effect on BMSCs and HSCs. $10 \mu M$ concentration of H_2O_2 induced catalase mRNA (A) and protein (B) expression, whereas a high concentration did not. (C) Biochemical enzyme activity of catalase was evaluated in BMSCs and HSPCs treated with H_2O_2 , Vit. C, and 3-AT. Catalase activity was significantly high in cells treated with $10 \mu M$ H_2O_2 . Vit. C at an equimolar low concentration ($10 \mu M$) and 3-AT, a catalase inhibitor ($30 \mu M$), significantly reversed the H_2O_2 -induced catalase activity in these cell populations. A further increase to $100 \mu M$ H_2O_2 decreased the catalase activity. (D) Similarly, a decrease in H_2O_2 -induced colony-forming efficiency was observed in the presence of 3-AT. Next, H_2O_2 -induced growth pattern of BMSCs (E) and HSPCs (F) in the presence of 3-AT depicted a decrease in growth rate. Further addition of PEG-catalase reversed the inhibition shown by 3-AT treatment. (G) Addition of PEG catalase reversed the inhibitory effect imparted by 3-AT. Further, siRNA-CAT (siRNA against catalase gene) transfected BMSCs and HSPCs depicted a significant decrease in BrdU incorporation ($n=3-6$ replicates per isolation; $p<0.05$ as compared with *control and # $10 \mu M$ H_2O_2 -treated group). 3-AT, 3 Amino-triazole. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(Supplementary Fig. S4D). Increased expression of catalase translated equally with increased protein expression (Fig. 3B) and increased biochemical activity at $10\ \mu\text{M}$ H_2O_2 that was reversed by exogenous antioxidants in BMSCs and HSPCs (Fig. 3C). These observations clearly indicate the role of the antioxidant enzyme, catalase in regulating the oxidative stress caused due to $10\ \mu\text{M}$ H_2O_2 in BMSCs and HSPCs, thereby modulating cellular proliferation.

Catalase inhibition sensitizes BMSCs and HSPCs to low oxidative stress

To further investigate the role of catalase in low oxidative stress-induced cell proliferation, a pharmacological inhibitor of catalase, 3-Aminotriazole (3-AT) was used (41). A low concentration of H_2O_2 -stimulated cell proliferation was sig-

nificantly decreased by 3-AT ($30\ \mu\text{M}$), as depicted by a decrease in the percent cell population in S-G₂/M phase of the cell cycle (Supplementary Fig. S5A). At a biochemical level, catalase enzyme activity was also observed to be significantly decreased in BMSCs and HSPCs treated with $10\ \mu\text{M}$ H_2O_2 and 3-AT (Fig. 3C). Next, colony formation (Fig. 3D) and growth curve assays also depicted a decrease in cell number when treated with $10\ \mu\text{M}$ H_2O_2 in combination with $30\ \mu\text{M}$ 3-AT in both BMSC (Fig. 3E) and HSPC (Fig. 3F). A similar outcome was observed in BrdU cell proliferation assay (Fig. 3G), although 3-AT treatment alone did not depict any significant difference in cell proliferation (Supplementary Fig. S5B). The pharmacologic administration of PEG catalase rescued the inhibitory effect on cell proliferation imparted by 3-AT (Fig. 3E–G). Catalase siRNA transfection of both BMSCs and HSPCs resulting in gene silencing

FIG. 4. Low concentration of H_2O_2 led to an increased JNK activation and FOXO3a nuclear translocation. (A) Western blot analysis revealed that $10\ \mu\text{M}$ H_2O_2 -induced AKT activation was reverted by addition of 3-AT. On the contrary, catalase inhibition led to insignificant change in JNK phosphorylation. (B) Silencing the catalase at molecular level by using CAT siRNA transfection depicted similar results as 3-AT. (C) $10\ \mu\text{M}$ H_2O_2 -induced AKT kinase activity was inhibited in the presence of inhibition of catalase (3-AT) or JNK (SP600125) or positive control AKT (wortmannin) in both BMSCs and HSPCs. (D) A dose-dependent increase in H_2O_2 -mediated FOXO3a nuclear translocation was abrogated in the presence of Vit. C that sequestered FOXO3a in the cytoplasm. Pharmacological (SP600125) as well as molecular (*pCMV-DN JNK*) inhibition of JNK prevented FOXO3a nuclear translocation, whereas AKT inhibition using wortmannin or *pCMV-AktDN* retained FOXO3a into the nucleus. Cells were stained with anti-FOXO3a rabbit polyclonal antibody followed by addition of AlexaFluor 488-labeled anti-rabbit secondary antibody. Nuclei were stained with DAPI. Quantification of nuclear localization along with Western blot images was evaluated by using NIH Image J software. CAT, catalase; DAPI, 4',6-diamidino-2-phenylindole; FOXO3a, forkhead box O3a. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

(Supplementary Fig. S5C) led to a decrease in 10 μ M H₂O₂-induced cell proliferation (Fig. 3G). These results clearly suggest that an increased cell cycle progression and expansion of BMSCs and HSPCs in the presence of 10 μ M H₂O₂ but not at higher 100 μ M H₂O₂ is correlated with increased catalase expression and activity.

To determine the effects of catalase inhibition on proliferative signaling mechanisms of AKT and JNK activation, we treated both BMSCs and HSPCs with 3-AT in the presence/absence of 10 μ M or 100 μ M concentration of H₂O₂. Catalase inhibition in these cell types depicted a downregulation of the AKT but not JNK signaling pathway (Fig. 4A), as evident from the densitometric analysis (Supplementary Fig. S5D). This observation suggests that in the absence of active catalase, even 10 μ M concentration of H₂O₂ sensitizes the cells by inducing JNK stress kinases alike a sub-lethal (100 μ M) concentration of H₂O₂ (Supplementary Fig. S5D). Similarly, decreased AKT activation whereas increased JNK activation was observed with silencing of catalase using CAT-siRNA at molecular level (Fig. 4B) and densitometric analysis or repeated blots (Supplementary Fig. S5E). To confirm AKT activation only at 10 μ M H₂O₂ in both BMSC and HSPC with active catalase, we evaluated the AKT kinase activity in the presence/absence of 3-AT (10). Significantly high AKT kinase activity was observed in the presence of 10 μ M H₂O₂ that was inhibited by 3-AT or wortmannin (positive control) (Fig. 4C). Interestingly, JNK inhibition also decreased AKT activation, indicating an existence of signaling crosstalk (Fig. 4C). Further, at molecular level, JNK (23) and AKT (47) dominant negative (DN) mutants could also decrease the 10 μ M H₂O₂-induced AKT activity (Supplementary Fig. S6A). Next, we evaluated the levels of intracellular ROS in the presence of catalase inhibition. Significantly similar high intracellular ROS levels were observed in both BMSC (Supplementary Fig. S6B) and HSPC (Supplementary Fig. S6C) treated with 10 μ M or 100 μ M H₂O₂ along with 3-AT administration, suggesting an increased ROS-mediated AKT inactivation. Ten micromolar H₂O₂-induced increased AKT activation (phosphorylation) was perturbed by molecular inhibition using AKT and JNK DN mutants in both BMSC (Supplementary Fig. S6D) and HSPC (Supplementary Fig. S6E) along with cell proliferation (Supplementary Fig. S6F). Interestingly, molecular inhibition of JNK also led to decreased cell proliferation, indicating its involvement *via* catalase downregulation-mediated effect.

FOXO3a nuclear translocation induces transcriptional upregulation of catalase at low oxidative stress

In the JNK stress signaling cascade, activated JNK is known to translocate FOXO3a, a transcription factor to the nucleus for transcriptional upregulation of FOXO3a target genes (25). Therefore, we determined the concentration-dependent effect of H₂O₂-induced activation of JNK on FOXO3a nuclear translocation. As expected, 10 μ M and 100 μ M H₂O₂ depicted a concentration-induced nuclear translocation of FOXO3a (Fig. 4D and Supplementary Fig. S7A–C) whereas Vit. C led to FOXO3a retention in the cytoplasm (Fig. 4D, Supplementary Fig. S7A). Pharmacological (Fig. 4D and Supplementary Fig. S7A) as well as molecular (Fig. 4D and Supplementary Fig. S7A–C) inhibition of JNK inhibited the nuclear translocation of FOXO3a,

whereas a similar inhibition of AKT did not have any effect on FOXO3a nuclear translocation (Fig. 4D and Supplementary Fig. S7A–C). Although activation of AKT is known to impart an inhibitory effect on FOXO3a nuclear translocation, recent studies suggest that a simultaneous JNK activation overrides AKT signaling, thereby dominating the FOXO3a nuclear translocation (4). Our results too clearly suggest the mechanism of H₂O₂-induced oxidative stress-mediated JNK activation, which, in turn, leads to nuclear translocation of FOXO3a that acts as a transcriptional co-regulator of its target gene expression. In our *in silico* analysis (data not shown) as well as other studies, an FOXO3a binding site is suggested in the promoter region of catalase gene (39). To further confirm this, we evaluated the catalase promoter that contained three potential FOXO3a binding sites: one at a distal region, distal binding site (DBS) and two others at a proximal region, proximal binding site (PBS1 and PBS2) (Fig. 5A). Chromatin immunoprecipitation (ChIP) analysis revealed that at 10 μ M H₂O₂ concentration, FOXO3a binds with a relatively higher affinity to catalase promoter at DBS in HSPCs as compared with BMSCs (Fig. 5B). However, a differential binding of FOXO3a to the PBS1 and PBS2 on catalase promoter was observed at 10 μ M H₂O₂ concentration. A high binding of FOXO3a to PBS2 in BMSCs (Fig. 5C) and PBS1 in HSPCs (Fig. 5D) was observed. Increased H₂O₂ concentration consequently led to decreased FOXO3a binding to catalase promoter (Fig. 5B–D). Vit. C and JNK inhibitor, SP600125 in the presence of 10 μ M H₂O₂ concentration, decreased FOXO3a binding to catalase promoter, indicating the 10 μ M H₂O₂-mediated transcriptional upregulation of catalase *via* JNK-FOXO3a signaling. This was further confirmed by a decreased catalase gene expression in the presence of JNK inhibitor (SP600125) but not AKT inhibitor (wortmannin) in 10 μ M H₂O₂-treated BMSC and HSPCs (Fig. 5E), suggesting that although 10 μ M H₂O₂-induced activation of JNK and AKT was observed in these proliferating cells, only JNK but not AKT imparts a direct regulation on catalase expression. To further substantiate this observation, we evaluated catalase mRNA and protein expression in the presence of JNK and AKT DN mutants. Molecular inhibition of JNK but not AKT led to a decrease in catalase mRNA (Supplementary Fig. S7D) and protein (Supplementary Fig. S7E) expression in 10 μ M H₂O₂-treated BMSC and HSPC, which corroborated further with catalase activity (Supplementary Fig. S7F). These results indicate that physiological relevance of AKT activation in 10 μ M H₂O₂-treated BMSC and HSPC might have a direct regulation on cell proliferation.

AKT and catalase inhibition downregulates cell proliferation-related genes at low oxidative stress

AKT inhibition-mediated decreased cell proliferation in 10 μ M H₂O₂-treated BMSC and HSPC was confirmed by the observation of decreased expression of cyclins B1, D2, and E2 (Fig. 5F) and PCNA (Fig. 5G) along with concomitant increased expression of CKIs p16 and p27 (Fig. 5G). Molecular inhibition of AKT also led to a decreased expression of cyclins (Supplementary Fig. S7G) and increased expression of CKIs (Supplementary Fig. S7H), thereby confirming the role of AKT in cell proliferation. Interestingly, JNK molecular inhibition indirectly led to a decrease in cyclin expression and an increase in CKIs expression *via* a decrease in catalase

FIG. 5. Low concentration of H_2O_2 potentiates FOXO3a binding to catalase promoter. (A) Schematic representation of catalase promoter depicting different FOXO3a binding sites upstream to transcription start site. ChIP analysis depicted an increased binding of FOXO3a on the catalase promoter at the (B) distal binding site (DBS, -1980 to -1974), (C) proximal binding site 2 (PBS2, -798 to -792), and (D) PBS1 (-612 to -606). (E) Low H_2O_2 -induced catalase mRNA expression was abrogated in the presence of JNK inhibitor, SP600125 but not AKT inhibitor, wortmannin. (F) AKT as well as catalase inhibition significantly decreased the 10 μ M H_2O_2 -induced cyclins B1, D2, and E2 expression in both BMSCs and HSPCs. (G) 10 μ M concentration of H_2O_2 -mediated decreased expression of CKIs, p16, and p27 was markedly reversed by AKT or catalase inhibition ($n=3$ replicates per isolation; $p<0.05$ as compared with *control; #10 μ M H_2O_2 -treated group).

expression (Supplementary Fig. S7F, G). As observed earlier, downregulation of catalase led to inactivation of AKT; 3-AT treatment also resulted in decreased cyclins (Fig. 5F), PCNA (Fig. 5G) and increased CKIs expression (Fig. 5G), indicating a simultaneous induction of the JNK-FOXO3a-catalase signaling cascade along with AKT-cyclins-cell proliferation in the presence of 10 μ M H_2O_2 -induced low oxidative stress in BMSC and HSPC wherein perturbation of the former signaling regulates the latter.

Low oxidative stress preconditioned BMSCs and HSPCs enhance wound tissue repair

One of the major causes of poor endurance of stem cells *in vivo* in an injury microenvironment is the poor expansion of transplanted stem cells. Exposure to low oxidative stress

in vitro (preconditioning with 10 μ M H_2O_2) before transplantation at the wound site was hypothesized to increase the proliferation of BMSCs and HSPCs. Thus, we utilized a murine model of excisional splinting wound as described earlier (9) wherein wounds heal through granulation and re-epithelialization, a process similar to that which occurs in humans (43). Mice transplanted with preconditioned BMSCs and HSPCs showed an accelerated wound closure post-surgery day 7 as compared with mice transplanted with naive BMSCs and HSPCs only (Fig. 6A). Further temporal quantification of the regenerated wound area depicted a 75% wound closure in preconditioned BMSCs as compared with 56% in naive BMSCs at postsurgery day 7 (Fig. 6B). Similar results were observed with preconditioned HSPCs representing an increased wound closure of 71% at postsurgery day 7 (Fig. 6B). Histopathological analysis of regenerated

FIG. 6. 10 μ M H_2O_2 preconditioned BMSCs or HSPCs transplantation improves wound repair and antioxidant defense system. (A) A representative image of wound healing in C57BL/6J mouse at postsurgery days 0 (upper panel) and 7 (lower panel). (B) Quantitation of wound area at different time points postwound generation (Days 0, 3, and 7) was performed by using NIH image J software. The results were represented as percent wound closure. (C) Representative images of hematoxylin and eosin-stained section of the regenerated wound tissue at postsurgery day 7 in various groups. (D) Quantitative analysis using NIH image J software depicted a significant increase in the number of H&E positively stained cells in preconditioned BMSCs and HSPCs transplanted groups. (E) Representative images of Masson's trichrome (MT) stained sections of the regenerated wound tissues depicting the collagen deposition. (F) Quantification of the number of MT positively stained cells in various groups. (G) A representative image of DHE-stained sections to evaluate the ROS levels at the wound site. (H) Relative mRNA expression level of antioxidant enzyme, catalase, was evaluated from regenerated wound tissue by using qRT-PCR analysis. Interestingly, 10 μ M H_2O_2 preconditioned HSPCs transplanted group depicted significantly high expression of catalase. (I) Catalase activity depicted a significant increase in naive as well as 10 μ M H_2O_2 preconditioned BMSCs or HSPCs transplanted (Tx) groups as compared with the wound control at postsurgery day 7 (Wd-Cont - Wound Control, PC - Preconditioned; $n=3$ replicates per wound, $N=5$ mice/group; $p<0.05$ as compared with *wound control; #BMSC or HSPC transplanted group; ** $p<0.01$ as compared with wound control). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

wound tissue using hematoxylin and eosin staining (Fig. 6C, lower magnification images—Supplementary Fig. S8A) and the quantitation of images (Fig. 6D) clearly demonstrated an increased proliferation of cells in preconditioned BMSCs and HSPCs as compared with naive BMSCs and HSPCs and/or wound-control (nonstem cells transplanted) groups. Also, the preconditioned stem cells transplanted group demonstrated an increased collagen deposition as demonstrated by Masson's Trichrome staining (Fig. 6E) and its quantitation (Fig. 6F). Overall ROS generated at the wound site was evaluated by using dihydroxy ethidium (DHE) staining. Remarkably, preconditioned BMSCs and HSPCs transplanted groups depicted a marked reduction in DHE staining as

compared with naive BMSCs or HSPCs transplanted groups and the wound control (Fig. 6G). Concurrently, a significant increase in the expression of antioxidant enzymes such as MnSOD (SOD2) (≥ 5.5 -fold), GPx1 (~ 8 -fold), and GPx2 (~ 3.8 -fold) was observed in preconditioned HSPCs and BMSCs transplanted groups (Supplementary Fig. S8B). However, the gene expression levels did not correlate with the activity of these enzymes—SOD (Supplementary Fig. S8C) and GPx (Supplementary Fig. S8D). Interestingly, an increase in catalase gene expression in preconditioned BMSCs (10-fold) and/or HSPCs (13-fold) transplanted groups (Fig. 6H) substantiated well with catalase activity (31.9 and 31.5 μ moles/min/ μ g of protein, respectively) as compared with naive

transplanted or wound-control group (Fig. 6I). Thus, preconditioning BMSCs or HSPCs with low concentrations of H_2O_2 induced the antioxidant enzyme, catalase. These cells when transplanted at the wound site alleviated the oxidative stress, thereby promoting wound tissue regeneration.

Low oxidative stress preconditioned BMSCs and HSPCs improve engraftment at the wound site

To determine the engraftment of transplanted stem cells at the wound site, BMSCs and/or HSPCs chimera was generated by using donor cells of male *C57BL/6J* mice transplanted onto recipient female *C57BL/6J* mice. DNA isolated from the wound tissue at 7 days postsurgery was subjected to quantification of Y-linked zinc finger protein (Zfy-1) gene

and Bcl-2 (as reference control) by using real-time PCR. Preconditioned BMSCs and HSPCs transplanted groups depicted an increased engraftment of male donor cells as compared with naive BMSCs and HSPCs transplanted groups that were abrogated when these cells were also pretreated simultaneously with an exogenous antioxidant, Vit. C (Fig. 7A). Further, engraftment of transplanted GFP-expressing BMSCs and HSPCs co-expressing other stem cell markers as reported earlier (8) were also evaluated in the regenerated wound sections. At postsurgery day 7, an increased co-localization of GFP-CAT was observed in preconditioned BMSCs and HSPCs as compared with naive BMSCs and HSPCs transplanted groups, respectively, or the wound control (Fig. 7B and quantitation—Supplementary Fig. S8E), thereby suggesting an increased cellular antioxidant defense mechanism in the transplanted

FIG. 7. Low concentration of H_2O_2 -mediated preconditioning of BMSCs or HSPCs increased engraftment during transplantation. (A) Percent of male cells at the wound site when naive or preconditioned BMSCs or HSPCs were transplanted into female mice. Simultaneous treatment of Vit. C during preconditioning of BMSCs or HSPCs reversed the effect. (B) Representative images of fluorescence microscopy at lower magnification (4 $\times$) of regenerated wound tissue sections depicting increased co-staining of catalase GFP in preconditioned BMSCs and HSPCs as compared with naive BMSCs and HSPCs, respectively, transplanted (Tx) or wound-control groups. (C) Representative confocal photomicrographs of GFP-DHE co-stained regenerated wound tissue sections of various treated groups depicting an increased engraftment of preconditioned GFP-expressing BMSCs or HSPCs led to a marked decrease in DHE staining, indicating decreased ROS levels at the wound site. Similarly, Vit. C treatment to preconditioned BMSCs or HSPCs mitigated this effect. (D) Quantitation of confocal images using NIH Image J software depicted decreased DHE staining in preconditioned BMSCs or HSPCs transplanted groups that were inhibited in the presence of simultaneous pretreatment of Vit. C (Wd-Cont - Wound Control, PC - Preconditioned; $n=3$ replicates per wound, $N=5$ mice/group; * $p<0.05$ as compared with wound control; ** $p<0.01$ as compared with wound control). DHE, dihydroxy ethidium. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

cells. In an effort to understand whether preconditioned BMSCs and HSPCs transplanted groups with a high expression of catalase could decrease the oxidative stress, we co-stained the tissue sections with DHE. Indeed, preconditioned BMSCs and HSPCs transplanted groups depicted a marked decrease in DHE staining (Fig. 7C) as compared with naive BMSCs and HSPCs transplanted groups or the wound control (Fig. 7D). Simultaneous supplementation of Vit. C to $10\ \mu\text{M}$ concentration of H_2O_2 -mediated preconditioning of BMSCs and HSPCs *in vitro* when transplanted markedly decreased the engraftment of cells as evident from less GFP-positive cells and increased DHE staining. This phenomenon corroborated well with the *in vitro* observation (Fig. 7C, D). Next, the “antioxidant” nature of Vit. C as an ROS quencher was evaluated for any beneficial effects during wound repair if administered parenterally at a pharmacological dose (200 mg/kg body wt, *i.p.*, per day $\times 7$ days). We observed a significant increase in wound closure at postsurgery day 7 as compared with the wound control (Supplementary Fig. S9A) and increased collagen deposition at the wound bed (Supplementary Fig. S9B, C). However, reports also suggest that Vit. C at a pharmacological dose acts as a “pro-oxidant” by

generating ascorbate-free radicals and H_2O_2 in extracellular fluids, which diffuse into the cells and cause depletion of ATP, thereby causing cell death (6). The BMSC and HSPC transplanted groups when administered with a pharmacological dose of Vit. C (200 mg/kg body wt, *ip.*) resulted in markedly low engraftment of cells (Supplementary Fig. S9D). BMSC and HSPC transplanted wound sections depicted a similar high DHE staining as observed in the wound-control group (Supplementary Fig. S9E, Quantitation of DHE staining-Supplementary Fig. S9F), suggesting no beneficial effect of pharmacological administration of Vit. C on reduction of ROS at the wound bed during wound healing. Thus, preconditioning of BMSCs and HSPCs with low concentrations of H_2O_2 before transplantation enhances the antioxidant defense system in the cells and makes them better equipped to survive the harsh wound environment.

Preconditioned BMSC and HSPC enhance proliferation during wound tissue regeneration

Next, to evaluate proliferation of the transplanted GFP⁺ cells, we performed co-staining with Ki67 (proliferation

FIG. 8. Increased proliferation of preconditioned BMSCs and HSPCs improves wound healing. Representative fluorescence microscopy images at lower magnification ($4\times$) of wound control, naive, or $10\ \mu\text{M}$ H_2O_2 preconditioned BMSCs and HSPCs. Regenerated wound tissue section at postsurgery day 7 was co-stained with GFP and Ki67 (*Insets*). Preconditioned BMSCs or HSPCs transplanted wound tissues depicted a significantly high number of positively co-stained cells, suggesting higher proliferation of transplanted (Tx) cells as quantitated by intensity correlation analysis-NIH image J software and represented graphically (Wd-Cont - Wound Control, PC - Preconditioned; $n=3$ replicates per wound, $N=5$ mice/group; $p<0.05$ as compared with *wound control; #naive BMSCs/HSPCs). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

marker) for both BMSCs and HSPCs. At postsurgery day 7, an increased co-staining of GFP/Ki67 in preconditioned BMSCs and HSPCs transplanted groups was observed as compared with naive stem cells transplanted, respectively, and wound-control groups (Fig. 8A). Separately, we have also preconditioned both BMSCs and HSPCs with high oxidative stress ($100 \mu\text{M}$ H_2O_2) but it led to a decrease in proliferation (data not shown) at the wound site. These observations suggest the therapeutic relevance of $10 \mu\text{M}$ H_2O_2 but not high concentration-induced oxidative stress in a translational outcome of BMSC and/or HSPC-mediated wound tissue repair/regeneration.

Preconditioned BMSC but not HSPC enhance re-epithelialization during wound tissue regeneration

Further, to determine whether the transplanted stem cells at the wound site could contribute to keratinocyte generation,

a co-immunostaining with cytokeratin (CK) and CD29 (BMSC) or CD11b (HSPC) was performed. At postsurgery day 7, a significantly high number of CD29 and CK co-stained cells were observed in preconditioned BMSCs as compared with naive BMSCs transplanted groups or wound-control groups (Fig. 9A left panel, Quantitation of co-localization-Fig. 9B). However, naive or preconditioned HSPCs did not depict similar CD11b and CK co-stained cells (Fig. 9A right panel, Quantitation of co-localization-Fig. 9C). To further confirm whether transplanted cells contributed to keratinocyte generation, we transplanted GFP-labeled cells and performed a co-staining of GFP and CK. At postsurgery day 7, preconditioned BMSCs transplanted wounds depicted a significant increase in double-positive (both GFP-green and CK-red) staining as compared with BMSCs transplanted groups or wound controls (Fig. 9D, quantification-9E, lower magnification images-Supplementary Fig. S10A, quantification of co-localization-Supplementary Fig. S10B). However,

FIG. 9. Improved keratinocyte generation by preconditioned BMSCs but not HSPCs accelerates wound healing. (A) Representative confocal images depicting an increased co-staining of preconditioned BMSCs for CD29-CK (cytokeratin, a keratinocyte marker) as compared with naive BMSC transplanted (Tx) groups but not by the naive and preconditioned HSPCs transplanted groups. Quantitation of the positively stained cells depicting a significant increase in BMSC transplantation group (B) but not in HSPC transplantation group (C) as compared with the respective wound-control group. (D) GFP-expressing preconditioned BMSCs or HSPCs confirmed the observation of enhanced keratinocyte generation by PC-BMSCs as compared with naive BMSCs or the wound control, whereas HSPCs transplanted (Tx) groups did not co-stain for GFP-CK. (E) Quantification of confocal images depicting an increase in the number of positively co-stained cells in various groups using intensity correlation analysis-NIH Image J software (Wd-Cont - Wound Control, PC - Preconditioned; $n=3$ replicates per wound, $N=5$ mice/group; $*p<0.05$ and $**p<0.01$ as compared with wound control). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

preconditioned HSPCs transplanted wounds did not depict co-localization of GFP-CK-expressing cells (Fig. 9D, E). These observations suggest that BMSCs but not HSPCs contribute to an increased number of keratinocytes, thereby leading to cutaneous regeneration.

Preconditioned BMSC and HSPC enhance vascularization during wound tissue regeneration

Finally, neo-vascularization, a crucial step for tissue regeneration, was evaluated by using immunofluorescence staining of CD31 and α -SMA at the regenerated tissue sections. Both preconditioned BMSCs and HSPCs transplanted wounds depicted an increased number of endothelial cells at the wound site as compared with naive BMSCs or HSPCs as

well as the wound control at postsurgery day 7 (Fig. 10A, Quantitation of co-localization-Fig. 10B). Further, to evaluate the participation of these transplanted cells in vasculogenesis, tissue sections were co-stained with GFP and CD31. Preconditioned HSPCs transplanted groups depicted an increased co-staining of GFP-CD31 as compared with naive HSPCs or the wound control, indicating the involvement of HSPCs in neo-vascularization (Fig. 10C, Quantitation of co-localization-Fig. 10D, lower magnification images-Supplementary Fig. S10C, quantification of co-localization-Supplementary Fig. S10D). Preconditioned BMSCs transplanted wound sections depicted an increased CD31 staining as compared with naive BMSCs or the wound control but the GFP-expressing cells were not fully co-localized with CD31-expressing cells, suggesting the paracrine effect of BMSCs that leads to enhanced vascularization at the

FIG. 10. Enhanced neo-vascularization by preconditioned BMSCs and HSPCs improves wound healing. (A) Representative confocal images depicting an increased co-staining of CD31- α -SMA in preconditioned BMSCs or HSPCs transplanted (Tx) groups as compared with naive BMSCs or HSPCs transplanted groups, respectively, suggesting neo-vascularization during wound tissue regeneration. (B) Quantification of confocal images using intensity correlation analysis- NIH Image J software depicting an increase in the number of positively co-stained cells in various groups. (C) Representative confocal images of GFP-expressing PC-HSPCs depicted an increased co-staining of GFP-CD31 in preconditioned HSPCs as compared with naive HSPCs, indicating neo-vascularization at the regenerated wound tissue. GFP-expressing PC-BMSCs too depicted increased staining of CD31 as compared with naive BMSCs, but all GFP-expressing cells did not co-localize with CD31, indicating a plausible paracrine effect of BMSCs in increasing vascularity in regenerated wound tissue (Wd-Cont - Wound Control, PC - Preconditioned; $n=3$ replicates per wound, $N=5$ mice/group; * $p<0.05$ and ** $p<0.01$ as compared with wound-control). To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

wound bed. This indicates the plausible role of these transplanted preconditioned stem cells in potentiating neo-vascularization to enhance wound tissue regeneration.

Discussion

Bone marrow-derived BMSCs and HSPCs are important components of regenerative medicines because of their immense potential of self-renewal, lineage-specific differentiation, as well as trans-differentiation capability that maintain homeostasis and tissue repair (18). During tissue injury, the adult stem cells migrate from the bone marrow and often get exposed to numerous kinds of stress in the injury microenvironment (24). Among these, oxidative stress plays an important role, leading to damage of cellular biomacromolecules (42). However, as mentioned earlier, level or content of the stressor defines the effect in the cell. Thus, in this study, we have evaluated the role of 10 μ M and 100 μ M concentration of H₂O₂-induced oxidative stress in BMSCs and HSPCs physiology. Our observations described the following: (i) 10 μ M concentration of H₂O₂ significantly increased proliferation of both BMSCs and HSPCs by activation of JNK-FOXO3a survival signaling pathways, leading to upregulation of catalase; (ii) exogenous Vit. C and its metabolized product, DHA reverted this H₂O₂-induced proliferative phenotype; (iii) mechanistically, pharmacological inhibition as well as molecular silencing of catalase sensitized these cells by decreasing cell proliferation when exposed to even 10 μ M concentration of H₂O₂; (iv) 10 μ M but not 100 μ M concentration of H₂O₂, induces binding of FOXO3a to catalase promoter; and (v) in an *in vivo* model of wound tissue healing, transplantation of preconditioned BMSCs or HSPCs with 10 μ M concentration of H₂O₂ depicted an increase in proliferation of engrafted cells, re-epithelialization, and neo-vascularization, thereby potentiating wound tissue repair and regeneration.

Stress tolerance capacity varies extensively between stem cells and its differentiated counterparts. Literature suggests that BMSCs derived from human skin tissue as compared with skin fibroblasts are more susceptible to high concentrations of ROS at a millimolar range (0.5–2 mM) and depict decreased viability. This susceptibility to high ROS has been attributed due to a less efficient antioxidant defense system in the stem cells (30). These studies are in alignment with our observations of reduced expression of antioxidant-catalase gene and protein in BMSCs and HSPCs when exposed to high (100 μ M) concentrations of H₂O₂. Similarly, other studies also depicted a deleterious effect of high ROS (50 μ M) generated at the ischemic site postmyocardial infarction on the adhesion properties of BMSCs (37). Although reports vary in terms of high concentrations of oxidant used, which ranges from high micromolar to low millimolar, a harmful effect on stem cell viability is generally observed. Also, adult stem cells isolated from different tissue sources might depict different susceptibility toward ROS. In contrast, studies depicting the effect of low concentrations of ROS on physiology of these adult stem cells, BMSC and HSPC are lacking in the literature. Similar to our observation, a short-term pretreatment of BMSCs with a low dose of H₂O₂ has depicted an increased therapeutic angiogenesis in the hind limb ischemia model (19). Also, Le Belle *et al.* have revealed the highly proliferative

neural stem cell population that possesses a high amount of endogenous ROS (21). Accumulation of ROS-mediated DNA damage has been recently implicated for HSCs to exit quiescence and differentiate into hematopoietic lineages (44). Thus, the diverse cellular responses of stem cells such as proliferation, migration, differentiation, and adhesion are modulated by a low ROS concentration.

ROS such as H₂O₂ at a low concentration can act as a second messenger to regulate signal transduction (38). The signaling role of H₂O₂ is achieved when its concentration increases above the steady-state threshold level and remains elevated for a substantial period to oxidize its protein effectors. In our study, H₂O₂-induced AKT activation was limited to 10 μ M concentration only. However, oxidative stress-induced activation of JNK depicted a dose-dependent effect in mediating its vital role. By contrast, H₂O₂ at a higher concentration activates p38 MAPK to decrease viability and expansion of HSPCs, resulting in premature senescence phenotype or apoptosis (15). Our data suggest a crucial role for the AKT and JNK signaling pathways.

Cells have their own antioxidant defense mechanisms to safeguard themselves from the permissible toxic levels of free radicals generated due to oxidative stress. We and others have observed that with an increase in H₂O₂ concentration to sub-lethal levels (100 μ M), the expression of mitochondrial isoform of SOD (SOD2) was induced in both BMSCs and HSPCs (2). This corresponded equally at activity level as SOD activity was further increased at sub-lethal levels, implicating its potential antioxidant role only during extreme stress conditions. On the contrary, our results of catalase mRNA expression in both BMSCs and HSPCs depicted similar findings as published earlier where the transcriptional activation of catalase was induced at a low concentration and inhibited at the sub-lethal concentration of H₂O₂ (35). This observation along with increased proliferation at 10 μ M but not 100 μ M (sub-lethal) H₂O₂ concentration in both BMSCs and HSPCs make it more obvious to ponder on a possible role of catalase in this phenomenon.

To comprehend the underlying molecular mechanism involved in upregulation of catalase, we studied the translocation of transcription factor FOXO3a, as it is a downstream target of both AKT and JNK signaling pathways (14). FOXOs belong to the superfamily of proteins that regulate the cell fate by modulating the expression of genes involved in oxidative stress (14). A recent study depicted a direct upregulation of catalase by FOXO3a in cardiac hypertrophy (39). Although the protective role of FOXO3a in quiescent or slow cycling cells was observed by the antioxidant responsive gene, SOD2 upregulation (17), our study mechanistically suggests a catalase upregulation by FOXO3a in a transcriptional-dependent manner at 10 μ M concentration of H₂O₂. Although activation of AKT occurs along with JNK at low oxidative stress, it is imperative to consider a JNK-FOXO3a-mediated upregulation of antioxidant gene, catalase whereas AKT activation leads to cell proliferation (Supplementary Fig. S11A). However, catalase inhibition by 3-AT or CAT-siRNA at low oxidative stress leads to increased ROS level that inactivates AKT and, subsequently, cell proliferation. An increase in ROS to sub-lethal levels led to higher nuclear translocation of FOXO3a but decreased catalase expression and an increase in SOD2 level, indicating a plausible role of other mechanisms.

Stem cells are emerging as a promising tool for the regenerative therapies correcting tissue damage. However, survival of transplanted stem cells is often compromised due to the harsh injury microenvironment that reduces their therapeutic efficacy (36). Cutaneous wound repair is a multifaceted process involving inflammation, angiogenesis, granular tissue formation, fibroblast proliferation, re-epithelialization, and matrix remodeling (22). Perturbations in the earlier mentioned sequential steps of wound repair result in chronic or delayed wound healing in diseases such as diabetes. Literature suggests that topical administration of side population of hematopoietic stem cells as compared with main population of bone marrow cells on excisional wounds led to a better wound closure in diabetic mice as well as wild-type mice (5). Similarly, BMSCs have been reported to promote wound healing in incisional full-thickness wound healing by enhanced proliferation, differentiation, angiogenesis, and re-epithelialization during wound closure (46). H_2O_2 at a differential concentration for preconditioning BMSCs and cardiac progenitor cells has shown improved angiogenesis and better myocardial architecture (33). Our study too depicted that preconditioning of stem cells with $10 \mu M H_2O_2$, a concentration at which a significantly high proliferation of these cells was observed *in vitro*, was a promising strategy for increasing stem cell expansion of engrafted cells, *in vivo*. In addition, our studies are consistent with the previous literature where only BMSCs but not $CD34^+$ cells such as our isolated HSPCs that are also $CD34^+$ cells (8) contributed to keratinocytes and re-epithelialization (46). Similarly, our study also depicts a substantial fraction of GFP-expressing BMSCs but not HSPCs engrafted in the wound appearing to co-express cytokeratin, suggesting a differentiation of transplanted cells into keratinocytes. Finally, the process of angiogenesis is important for supplying the survival factors for keratinocytes and newly formed granulation tissue. As demonstrated earlier by other workers, BMSC transplantation induces neo-vascularization at the wound site in a paracrine manner by secreting angiogenic growth factors (46). Our results also corroborate with the existing literature as the preconditioned BMSCs transplanted group exhibited increased $CD31$ -expressing cells in close proximity of GFP-positive cells, whereas the preconditioned HSPCs transplanted group depicted increased GFP- $CD31$ co-expressing cells, suggesting their participation in neo-vascularization. The study suggests transplantation of preconditioned BMSCs and HSPCs with $10 \mu M H_2O_2$ -mediated low oxidative stress as a therapeutic approach for enhanced wound tissue repair (Supplementary Fig. S11B).

Materials and Methods

Exposure to H_2O_2

BMSCs and HSPCs seeded at a different seeding density depending on the assay type were treated with vehicle or 1, 10, and $100 \mu M$ of H_2O_2 /well. Separately, BMSCs and HSPCs were incubated with equimolar concentrations of ($10 \mu M$) H_2O_2 along with antioxidants, Vit. C or DHA (45). In a different set of experiments, BMSCs and HSPCs were also incubated with $10 \mu M H_2O_2$ along with signaling pathway blockers. Further, to evaluate the loss or gain function of catalase on the cells in the presence of H_2O_2 , both molecular and pharmacological interventions were applied. Cells were

treated with either 3 Amino Triazole (3-AT, a catalase inhibitor) or transfected with siRNA specific toward mouse catalase gene for evaluating the effect of catalase inhibition. Similarly, cells were additionally treated with PEG-catalase to evaluate the effect of exogenous catalase (32).

Cell proliferation by colony formation, growth curve, and BrdU assay

For assessing cell expansion, BMSCs and HSPCs were plated for colony formation assay, bromodeoxyuridine (BrdU) cell proliferation assay, and growth curves at different seeding densities as described earlier [10]. For colony formation assay, BMSCs and HSPCs were cultured in complete Mesencult and Methocult medium (StemCell Technologies), respectively (16, 26). DNA synthesis was measured by using a BrdU Cell Proliferation Kit (Roche) by following the manufacturer's protocol (20). For growth curve assay, BMSCs and HSPCs were seeded at a cell density of 3×10^3 cells per well in at least triplicate with treatments as described earlier (8, 34).

Quantitative real-time PCR

Overall, $1.0 \mu g$ of total RNA extracted from treated BMSCs and HSPCs was used for cDNA synthesis from different treatment groups by using Oligo (dT) primer of First-Strand cDNA Synthesis kit (Thermo Scientific) and mouse-specific forward and reverse primers of antioxidant and cell cycle genes (Supplementary Table S1) for quantitative real-time RT-PCR (qRT-PCR) by using Applied Biosystems Step-One Plus PCR System (Applied Biosystems) as described earlier (7). Amplification of mouse-specific eukaryotic 18S rRNA and β -Actin was used in the same reaction of all samples as an internal control. All the target gene expression was normalized to eukaryotic 18S rRNA mRNA and shown as the fold change (7).

Intracellular and extracellular ROS detection

Intracellular production of ROS was detected by fluorescence probe 2',7'-dichloro-fluorescein diacetate (DCFH-DA; Sigma-Aldrich) as previously described (49). Briefly, after treatment for 24 h or 48 h, cells were washed $3 \times$ with PBS and then incubated in $10 \mu M$ DCFH-DA for 30 min at $37^\circ C$. The fluorescence intensity of DCF (2', 7'-dichlorofluorescein) was determined on a Perkin Elmer Spectrofluorometer at excitation and emission wavelengths of 480 and 538 nm, respectively, and background fluorescence was subtracted by using appropriate controls. For extracellular ROS detection, Hydrogen Peroxide Cell-based assay kit was used according to the manufacturer's protocol (Cayman Chemicals). After 24 h and 48 h of treatment, fluorescence intensity was measured at 530 nm (excitation wavelength) and 590 nm (emission wavelength).

Total glutathione measurements

To measure the intracellular oxidative status of stem cells, the reduced glutathione (GSH) content of each cellular protein sample was measured according to the manufacturer's protocol (Cayman Chemicals).

Biochemical assay for catalase, SOD, and GPX

Antioxidant responses of BMSCs and HSPCs were evaluated by measuring the activity of antioxidant enzymes:

catalase, SOD, and GPX. SOD and GPX activity was measured according to the protocols provided in the Cayman assay kit (Cayman Chemicals). Catalase activity was measured by using a modified protocol of Aebi.

Immunoblot analysis

Cellular protein was extracted from BMSCs and HSPCs treated with H₂O₂ alone or in different treatment regimes as described earlier, was subjected to separation by using sodium dodecyl sulfate-poly acrylamide gel electrophoresis, transferred to PVDF membranes (Amersham Biosciences), and immunoblotted by using antibodies against phosphorylated and pan signaling markers: ERK-1/2, AKT, JNK, and p38 MAPK (ThermoScientific). Signals were detected by using the chemiluminescence ECL detection system (Merck Millipore), and visualization was performed by using G: Box (Syngene, Cambridge, United Kingdom). Quantification of protein bands was performed by using NIH Image J software (7).

Cellular immunofluorescence staining—FOXO3a

Immunofluorescent staining was performed according to a reported procedure (13). In brief, treated cells were washed with cold PBS, fixed with 4% formaldehyde for 10 min at 37°C, permeabilized with 0.5% Triton X-100 for 5 min at room temperature, and fixed with methanol for overnight at 4°C. After washing with PBS, the cells were blocked with 1% BSA for 1 h and incubated with primary antibody FOXO3a in 1% BSA for 2 h at room temperature. Further, secondary anti-rabbit PE conjugated antibody was added. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) contained in the mounting medium (Amresco). Confocal images were obtained with an Olympus FV1000 confocal microscope (Olympus Corp.) by using the 40×oil immersion lens. NIH Image J software was used to quantitate nuclear localization.

siRNA silencing—catalase

For catalase knockdown, cells were transfected with siCAT or scrambled negative control according to the manufacturer's instructions by using lipofectamine 2000 (Invitrogen) (7). The cells were further treated for 48 h in the presence or absence of H₂O₂ and were utilized for different assays.

AKT kinase assay

BMSC and HSPCs were cultured and treated as described earlier for 48 h at 37°C. Kinase activity was measured by using the In-Cell AKT ELISA Kit (Pierce), according to the manufacturer's protocol.

Transient transfection of DN plasmids—AKT and JNK

DN AKT and JNK mutant plasmids (Addgene) were transfected transiently by using lipofectamine 3000 (Invitrogen). Cells were cultured in low serum-containing medium for 48 h followed by various treatment regimes. After 48 h, cells were trypsinised and utilized for different assays.

ChIP assay

ChIP assay was performed according to the manufacturer's protocol (Millipore). Cells were treated with different treatment regime, washed, and cross-linked with 1% formalde-

hyde at 37°C for 10 min. Cells were lysed in buffer containing a protease inhibitor cocktail, and they were sonicated by using Bioruptor (Diagenode) followed by preclearing with Protein A Agarose/Salmon sperm DNA slurry. Immunoprecipitation was performed overnight at 4°C with 5 µg of anti-Foxo3a antibody, and this was followed by sequential washings with low-salt immune complex buffer, high-salt immune complex buffer, LiCl immune complex buffer, and, finally, with TE buffer. DNA was eluted from the beads and recovered by phenol/chloroform extraction and ethanol precipitation. The purified DNA was used as a template for PCR amplification.

BMSC or HSPC transplantation-mediated wound healing

Bone marrow-derived BMSCs and HSPCs isolated from C57BL/6J mice as described earlier (8) were used for autologous transplantation in the wound model as naive or post *in vitro* treatment using a low (10 µM) concentration of H₂O₂ (preconditioned, PC) and/or pretreatment with an equimolar concentration (10 µM) of H₂O₂ and Vit. C. In a separate set of experiments, isolated BMSCs and HSPCs were labeled with GFP lentivirus for 24 h followed by replacement with fresh medium. After 48 h, the cells were selected by "puromycin-selection" and infected cells were used as naive or preconditioned as described earlier for transplantation. An excision wound splinting model was generated in 8–10 week-old C57BL/6J mice as described earlier (43). Briefly, two symmetrical full-thickness excisional wounds were created beside the midline by using a 5-mm-diameter sterile biopsy punch. Transplantation of bone marrow-derived naive or preconditioned BMSCs and HSPCs was performed by injecting intradermally (*id.*; 0.7 × 10⁶ cells) and on the wound surface (0.3 × 10⁶ cells) as previously described (9). A silicone splint was placed so that the wound was centered within the splint. An immediate-bonding adhesive was used to fix the splint to the skin, and Tegaderm was placed over the wounds. Animal experimentation protocols were approved by the Institutional animal ethics committee (approval No. IICT/CB/AD/25/06/2014/13 and IICT/CB/AD/26/08/13/08).

Quantification of wound closure

Representative digital images of wound healing depicting wound area at different time points postwound generation (days 0, 3, and 7) in wound-control, BMSC, PC-BMSC, HSPC, and PC-HSPC transplanted groups were taken. Time to wound closure was defined as the time at which the wound bed was completely re-epithelialized and filled with new tissue. Wound area was measured by tracing the wound margin and calculated by using an image analysis program (NIH Image J software). The results were represented as percent wound closure (1), calculated in comparison with the original size of the wound as mentioned later and described earlier (3):

$$\text{Percent Wound Closure} = \frac{1 - (\text{Wound Area})}{(\text{Original Wound Area})} \times 100$$

The inside edge of the splint exactly matched the edge of the wound, so that the splinted hole was used to represent the original wound size. Mice were euthanized on postsurgery

day 7; skin samples including the wound and surrounding skin were harvested by using a 10-mm biopsy punch.

Histological and immunofluorescence analysis of the regenerated wound

Regenerated wound tissue samples from various treatment groups were fixed in 4% paraformaldehyde. Cross-sections of wound tissue mounted on slides were stained with hematoxylin-eosin, Masson's Trichrome, and DHE. The regenerated wound tissue was separately homogenized in respective assay buffers to evaluate catalase, SOD, and GPX assay. RNA extracted from wound tissue samples was utilized for gene expression of oxidative stress-associated genes by using qRT-PCR analysis. Separately, frozen wound tissue sections from the various groups were stained with specific antibodies against catalase, GFP, Ki67, CD29, CD11b, CK, CD31, and α SMA (Cell Signaling Technologies). Isotype control antibodies were used for negative controls. Sections were examined with Olympus FV1000 and FV10i confocal microscope. Co-localization of different cellular markers was evaluated by using the intensity correlation analysis of NIH Image J software, and data were represented as Pearson's coefficient value (1).

Vit. C in wound healing

Parenteral administration of Vit. C (250 mg/kg body wt, *i.p.* per day) (6) was carried out for 7 days in the excisional splinting wound model transplanted with BMSCs or HSPCs. The mice were monitored for wound healing, engraftment of transplanted cells, and histological analysis.

Statistical analysis

Data from experiments performed with repeated BMSC/HSPC isolations at least thrice were statistically analyzed by taking mean $\pm$ standard deviation (SD) or standard error of mean (SEM). To determine the difference between treatment group and their respective controls, Graph Pad prism version 6.05 was used to obtain statistical significance by using one- or two-way ANOVA followed by Tukey's multiple-comparison test. Blots and photomicrographs represent experiments reproduced at least thrice with similar results.

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Author Disclosure Statement

The authors have no conflicts to disclose.

References

1. Bolte S and Cordelières FP. A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc* 224: 213–232, 2006.
2. Burova E, Borodkina A, Shatrova A, and Nikolsky N. Sublethal Oxidative Stress Induces the Premature Senescence of Human Mesenchymal Stem Cells Derived from Endometrium. *Oxidative Med Cell Longevity* 2013: 12, 2013.
3. Canesso MCC, Vieira AT, Castro TBR, Schirmer BGA, Cisalpino D, Martins FS, Rachid MA, Nicoli JR, Teixeira MM, and Barcelos LS. Skin wound healing is accelerated and scarless in the absence of commensal microbiota. *J Immunol* 193: 5171–5180, 2014.
4. Chaanine AH, Jeong D, Liang L, Chemaly ER, Fish K, Gordon RE, and Hajjar RJ. JNK modulates FOXO3a for the expression of the mitochondrial death and mitophagy marker BNIP3 in pathological hypertrophy and in heart failure. *Cell Death Dis* 3: 265, 2012.
5. Chan RK, Garfein E, Gigante PR, Liu P, Agha RA, Mulligan R, and Orgill DP. Side population hematopoietic stem cells promote wound healing in diabetic mice. *Plast Reconstr Surg* 120: 407–411; discussion 412–413, 2007.
6. Chen Q, Espey MG, Sun AY, Lee J-H, Krishna MC, Shacter E, Choyke PL, Pooput C, Kirk KL, Buettner GR, and Levine M. Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo. *Proc Natl Acad Sci U S A* 104: 8749–8754, 2007.
7. Das A, Fernandez-Zapico ME, Cao S, Yao J, Fiorucci S, Hebbel RP, Urrutia R, and Shah VH. Disruption of an SP2/KLF6 repression complex by SHP is required for Farnesoid X receptor-induced endothelial cell migration. *J Biol Chem* 281: 39105–39113, 2006.
8. Dhoke NR, Kalabathula E, Kaushik K, Geesala R, Sravani B, and Das A. Histone deacetylases differentially regulate the proliferative phenotype of mouse bone marrow stromal and hematopoietic stem/progenitor cells. *Stem Cell Res* 17: 170–180, 2016.
9. Geesala R, Bar N, Dhoke NR, Basak P, and Das A. Porous polymer scaffold for on-site delivery of stem cells—Protects from oxidative stress and potentiates wound tissue repair. *Biomaterials* 77: 1–13, 2016.
10. Geesala R, Gangasani JK, Budde M, Balasubramanian S, Vaidya JR, and Das A. 2-Azetidinones: synthesis and biological evaluation as potential anti-breast cancer agents. *Eur J Med Chem* 124: 544–558, 2016.
11. Gülden M, Jess A, Kammann J, Maser E, and Seibert H. Cytotoxic potency of H₂O₂ in cell cultures: impact of cell concentration and exposure time. *Free Radic Biol Med* 49: 1298–1305, 2010.
12. Henklova P, Vrzal R, Papouskova B, Bednar P, Jancova P, Anzenbacherova E, Ulrichova J, Maurel P, Pavek P, and Dvorak Z. SB203580, a pharmacological inhibitor of p38 MAP kinase transduction pathway activates ERK and JNK MAP kinases in primary cultures of human hepatocytes. *Eur J Pharmacol* 593: 16–23, 2008.
13. Ho K-K, McGuire VA, Koo C-Y, Muir KW, de Olano N, Maifoshie E, Kelly DJ, McGovern UB, Monteiro LJ, Gomes AR, Nebreda AR, Campbell DG, Arthur JSC, and Lam EW-F. Phosphorylation of FOXO3a on Ser-7 by p38 promotes its nuclear localization in response to doxorubicin. *J Biol Chem* 287: 1545–1555, 2012.
14. Huang H and Tindall DJ. Dynamic FoxO transcription factors. *J Cell Sci* 120: 2479–2487, 2007.
15. Ito K, Hirao A, Arai F, Takubo K, Matsuoka S, Miyamoto K, Ohmura M, Naka K, Hosokawa K, Ikeda Y, and Suda T.

- Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells. *Nat Med* 12: 446–451, 2006.
16. Kaufman DS, Hanson ET, Lewis RL, Auerbach R, and Thomson JA. Hematopoietic colony-forming cells derived from human embryonic stem cells. *Proc Natl Acad Sci USA* 98: 10716–10721, 2001.
 17. Kops GJPL, Dansen TB, Polderman PE, Saarloos I, Wirtz KWA, Coffey PJ, Huang T-T, Bos JL, Medema RH, and Burgering BMT. Forkhead transcription factor FOXO3a protects quiescent cells from oxidative stress. *Nature* 419: 316–321, 2002.
 18. Körbling M and Estrov Z. Adult Stem Cells for Tissue Repair—A New Therapeutic Concept? *N Engl J Med* 349: 570–582, 2003.
 19. Kubo M, Li T-S, Suzuki R, Ohshima M, Qin S-L, and Hamano K. Short-term pretreatment with low-dose hydrogen peroxide enhances the efficacy of bone marrow cells for therapeutic angiogenesis. *Am J Physiol Heart Circ Physiol* 292: H2582–H2588, 2007.
 20. Lau CP, Ng PK, Li MS, Tsui SK, Huang L, and Kumta SM. p63 regulates cell proliferation and cell cycle progression associated genes in stromal cells of giant cell tumor of the bone. *Int J Oncol* 42: 437–443, 2013.
 21. Le Belle JE, Orozco NM, Paucar AA, Saxe JP, Mottahedeh J, Pyle AD, Wu H, and Kornblum HI. Proliferative neural stem cells have high endogenous ROS levels that regulate self-renewal and neurogenesis in a PI3K/Akt-dependant manner. *Cell Stem Cell* 8: 59–71.
 22. Lee DE, Ayoub N, and Agrawal DK. Mesenchymal stem cells and cutaneous wound healing: novel methods to increase cell delivery and therapeutic efficacy. *Stem Cell Res Ther* 7: 1–8, 2016.
 23. Liang Q, Bueno OF, Wilkins BJ, Kuan CY, Xia Y, and Molkentin JD. c-Jun N-terminal kinases (JNK) antagonize cardiac growth through cross-talk with calcineurin–NFAT signaling. *EMBO J* 22: 5079–5089, 2003.
 24. Lo EH, Wang X, and Cuzner ML. Extracellular proteolysis in brain injury and inflammation: role for plasminogen activators and matrix metalloproteinases. *J Neurosci Res* 69: 1–9, 2002.
 25. Mackintosh C. Dynamic interactions between 14-3-3 proteins and phosphoproteins regulate diverse cellular processes. *Biochem J* 381: 329–342, 2004.
 26. Meirelles Lda S and Nardi NB. Murine marrow-derived mesenchymal stem cell: isolation, in vitro expansion, and characterization. *Br J Haematol* 123: 702–711, 2003.
 27. Michiels C, Raes M, Toussaint O, and Remacle J. Importance of SE-glutathione peroxidase, catalase, and CU/ZN-SOD for cell survival against oxidative stress. *Free Radic Biol Med* 17: 235–248, 1994.
 28. Montecinos V, Guzmán P, Barra V, Villagrán M, Muñoz-Montesino C, Sotomayor K, Escobar E, Godoy A, Mardones L, Sotomayor P, Guzmán C, Vásquez O, Gallardo V, van Zundert B, Bono MR, Oñate SA, Bustamante M, Cárcamo JG, Rivas CI, and Vera JC. Vitamin C is an essential antioxidant that enhances survival of oxidatively stressed human vascular endothelial cells in the presence of a vast molar excess of glutathione. *J Biol Chem* 282: 15506–15515, 2007.
 29. Nathan C and Ding A. SnapShot: reactive oxygen intermediates (ROI). *Cell* 140: 951–951.e2.
 30. Orciani M, Gorbi S, Benedetti M, Di Benedetto G, Mattioli-Belmonte M, Regoli F, and Di Primio R. Oxidative stress defense in human-skin-derived mesenchymal stem cells versus human keratinocytes: different mechanisms of protection and cell selection. *Free Radic Biol Med* 49: 830–838, 2010.
 31. Parmar K, Mauch P, Vergilio J-A, Sackstein R, and Down JD. Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. *Proc Natl Acad Sci U S A* 104: 5431–5436, 2007.
 32. Paulsen CE, Truong TH, Garcia FJ, Homann A, Gupta V, Leonard SE, and Carroll KS. Peroxide-dependent sulfenylation of the EGFR catalytic site enhances kinase activity. *Nat Chem Biol* 8: 57–64, 2011.
 33. Pendergrass KD, Boopathy AV, Seshadri G, Maiellaro-Rafferty K, Che PL, Brown ME, and Davis ME. Acute preconditioning of cardiac progenitor cells with hydrogen peroxide enhances angiogenic pathways following ischemia-reperfusion injury. *Stem Cells Dev* 22: 2414–24, 2013.
 34. Peng L, Jia Z, Yin X, Zhang X, Liu Y, Chen P, Ma K, and Zhou C. Comparative analysis of mesenchymal stem cells from bone marrow, cartilage, and adipose tissue. *Stem Cells Dev* 17: 761–73, 2008.
 35. Quan X, Lim S-O, and Jung G. Reactive oxygen species downregulate catalase expression via methylation of a CpG Island in the Oct-1 promoter. *FEBS Lett* 585: 3436–3441, 2011.
 36. Sart S, Ma T, and Li Y. Preconditioning stem cells for in vivo delivery. *BioRes Open Access* 3: 137–149, 2014.
 37. Song H, Cha M-J, Song B-W, Kim I-K, Chang W, Lim S, Choi EJ, Ham O, Lee S-Y, Chung N, Jang Y, and Hwang K-C. Reactive oxygen species inhibit adhesion of mesenchymal stem cells implanted into ischemic myocardium via interference of focal adhesion complex. *Stem Cells* 28: 555–563, 2010.
 38. Stone JR and Yang S. Hydrogen peroxide: a signaling messenger. *Antioxid Redox Signal* 8: 243–70, 2006.
 39. Tan W-Q, Wang K, Lv D-Y, and Li P-F. Foxo3a inhibits cardiomyocyte hypertrophy through transactivating catalase. *J Biol Chem* 283: 29730–29739, 2008.
 40. Truong TH and Carroll KS. Redox regulation of EGFR signaling through cysteine Oxidation(). *Biochemistry* 51: 9954–9965, 2012.
 41. Ueda M, Kinoshita H, Yoshida T, Kamasawa N, Osumi M, and Tanaka A. Effect of catalase-specific inhibitor 3-amino-1,2,4-triazole on yeast peroxisomal catalase in vivo. *FEMS Microbiol Lett* 219: 93–98, 2003.
 42. Urao N and Ushio-Fukai M. Redox regulation of stem/progenitor cells and bone marrow niche. *Free Radic Biol Med* 54: 26–39, 2013.
 43. Wang X, Ge J, Tredget EE, and Wu Y. The mouse excisional wound splinting model, including applications for stem cell transplantation. *Nat Protocols* 8: 302–309, 2013.
 44. Weiss C and Ito K. DNA Damage: a sensible mediator of the differentiation decision in hematopoietic stem cells and in leukemia. *Int J Mol Sci* 16: 6183, 2015.
 45. Wilson JX. The physiological role of dehydroascorbic acid. *FEBS Lett* 527: 5–9, 2002.
 46. Wu Y, Chen L, Scott PG, and Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* 25: 2648–2659, 2007.
 47. Xie R, Cheng M, Li M, Xiong X, Daadi M, Sapolsky RM, and Zhao H. Akt isoforms differentially protect against

- stroke-induced neuronal injury by regulating mTOR activities. *J Cereb Blood Flow Metab* 33: 1875–1885, 2013.
48. Zelivianski S, Spellman M, Kellerman M, Kakitelashvili V, Zhou X-W, Lugo E, Lee M-S, Taylor R, Davis TL, Hauke R, and Lin M-F. ERK inhibitor PD98059 enhances docetaxel-induced apoptosis of androgen-independent human prostate cancer cells. *Int J Cancer* 107: 478–485, 2003.
49. Zeng W, Xiao J, Zheng G, Xing F, Tipoe GL, Wang X, He C, Chen Z-Y, and Liu Y. Antioxidant treatment enhances human mesenchymal stem cell anti-stress ability and therapeutic efficacy in an acute liver failure model. *Sci Rep* 5: 11100, 2015.

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Abbreviations Used

3-AT = 3 Amino-triazole
 BMSC = bone marrow stromal stem/progenitor cells
 BrDU = bromodeoxyuridine
 CAT = catalase
 CDK = cyclin-dependent kinases
 CFU = colony-forming efficiency
 ChIP = chromatin immunoprecipitation
 CKI = cyclin-dependent kinase inhibitors
 DAPI = 4',6-diamidino-2-phenylindole
 DCFH-DA = 2',7'-dichloro-fluorescein diacetate
 DHA = dehydroascorbic acid
 DHE = dihydroxy ethidium
 DN = dominant negative
 FITC = fluorescein isothiocyanate
 FOXO3a = forkhead boxO3
 GPx = glutathione peroxidase
 GSH = glutathione
 H₂O₂ = hydrogen peroxide
 HSPC = hematopoietic stem and progenitor cells
 JNK = c-Jun N terminal kinase
 PCNA = proliferating cell nuclear antigen
 PE = phycoerythrin
 PEG-CAT = polyethyleneglycol-catalase
 PI = propidium iodide
 ROS = reactive oxygen species
 SOD = superoxide dismutase
 Vit. C = vitamin C