

NFκB1 (p50) suppresses SOD2 expression by inhibiting FoxO3a transactivation in a miR190/PHLPP1/Akt-dependent axis

Kejun Du^{a,b,*}, Yonghui Yu^{a,*}, Dongyun Zhang^{a,*}, Wenjing Luo^b, Haishan Huang^{a,c}, Jingyuan Chen^b, Jimin Gao^c, and Chuanshu Huang^{a,c}

^aNelson Institute of Environmental Medicine, New York University School of Medicine, Tuxedo, NY 10987;

^bDepartment of Occupational and Environmental Health, School of Public Health, Fourth Military Medical University, Shanxi 710032, China; ^cZhejiang Provincial Key Laboratory for Technology and Application of Model Organisms, School of Life Sciences, Wenzhou Medical College, Wenzhou, Zhejiang 325035, China

ABSTRACT The biological functions of nuclear factor κB1 (NFκB1; p50) have not been studied as often as those of other members of the NFκB family due to its lack of a transcriptional domain. Our recent studies showed that p50 functions as an apoptotic mediator via its inhibition of GADD45α protein degradation and increase in p53 protein translation. Here we report a novel function of p50 in its regulation of superoxide dismutase 2 (SOD2) transcription via an NFκB-independent pathway. We find that deletion of p50 in mouse embryonic fibroblasts (MEFs; p50^{-/-}) up-regulates SOD2 expression at both protein and mRNA levels. SOD2 promoter-driven luciferase is also up-regulated in p50^{-/-} cells compared with wild-type (WT) MEF (p50^{+/+}) cells, suggesting p50 regulation of SOD2 at the transcriptional level. Our results also show that p50 deficiency specifically results in down-regulation of phosphorylation and increased transactivation of FoxO3a compared with WT cells. Further studies indicate that p50-down-regulated FoxO3a phosphorylation is mediated by activated Akt via up-regulation of microRNA 190 (miR190), in turn inhibiting PH domain and leucine-rich repeat protein phosphatase 1 (PHLPP1) translation. Together our studies identify a novel p50 function in the regulation of SOD2 transcription by modulating the miR190/PHLPP1/Akt-FoxO3a pathway, which provides significant insight into the physiological function of p50.

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INTRODUCTION

Oxidative stress is a hallmark of many diseases (Olmos *et al.*, 2009) and is caused by the imbalance between reactive oxygen species (ROS) and biological antioxidant systems (Miao and St Clair, 2009).

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*These authors contributed equally to this work.

The authors declare no conflict of interest.

Address correspondence to: Chuanshu Huang (Chuanshu.huang@nyumc.org).

Abbreviations used: DN, domain negative; FOXO, forkhead box class O; MEF, mouse embryonic fibroblast; miR190, microRNA 190; NFκB, nuclear factor κB; PHLPP, PH domain and leucine-rich repeat protein phosphatase; ROS, reactive oxygen species; SOD2, superoxide dismutase 2; STAT, signal transducer and activator of transcription; WT, wild type.

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On one hand, mitochondria generate ROS during cellular aerobic metabolism, including superoxide anions and hydrogen peroxide (Fang *et al.*, 2002), which play an important role in regulation of normal physiological function and pathogenesis of various diseases (Mukherjee *et al.*, 2011). On the other hand, cells synthesize protective proteins, such as superoxide dismutase 2 (SOD2), glutathione peroxidase, and catalase, to guard against the effects of ROS (Liu *et al.*, 2011).

SOD2, known as manganese superoxide dismutase (Xu *et al.*, 2008), is a superoxide scavenger enzyme located in the mitochondrial matrix that protects cells from the damage of mitochondrial ROS by detoxifying O₂ radicals via dismutation to yield molecular oxygen and hydrogen peroxide (Makino *et al.*, 2011). SOD2 is considered the primary defensive enzyme against oxidative stress within mitochondria (Chung *et al.*, 2011). As a primary antioxidant enzyme in the mitochondria, SOD2 is important for maintenance of cellular redox balance (Xu *et al.*, 2008). As a nuclear-encoded enzyme,

SOD2 is imported into the mitochondrial matrix to catalyze dismutation of superoxide radicals (Hitchler *et al.*, 2006; Hempel *et al.*, 2011). SOD2 also works in the cellular context with other antioxidant enzymes to protect cells from damage associated with exposure to ROS (Hitchler *et al.*, 2006). Numerous studies show that SOD2 is induced in various types of cells and tissues (Xu *et al.*, 2008). SOD2 expression is rapidly up-regulated in cell response to oxidative stress (Xu *et al.*, 2008). The targeted disruption of murine SOD2 causes neonatal lethality (Chung *et al.*, 2011), and a high level of SOD2 expression is correlated with nuclear factor κ B (NF κ B) activation in various cells (Xu *et al.*, 2008).

NF κ B is a transcription factor protein complex that controls the transcription of various genes (Thapa *et al.*, 2011). In mammalian cells, the NF κ B family has five Rel members: p50 (NF κ B1), p52 (NF κ B2), p65 (Rel A), Rel B, and c-Rel (Song *et al.*, 2006). The p50/p65 heterodimer is predominantly expressed in all types of mammalian cells and is involved in cellular responses to a variety of stimuli (Lin and Ghosh, 1996; Hatada *et al.*, 2003; Song *et al.*, 2006). Although p50/p50 homodimer has been identified in intact cells for many years (Yu *et al.*, 2013), physiological and pathological functions of p50 have yet to be explored. Our recent studies showed that p50 functions as an apoptotic mediator by inhibiting GADD45 α protein degradation and increasing p53 protein translation (Song *et al.*, 2006; Yu *et al.*, 2013). In the present study, we demonstrate that p50 modulates SOD2 expression at the transcriptional level by regulating the microRNA 190 (miR190)/PH domain and leucine-rich repeat protein phosphatase 1 (PHLPP1)/Akt-FoxO3a axis.

RESULTS

NF κ B1/p50 inhibits SOD2 transcription

Previous studies showed that SOD2 transcription is positively regulated by SP1 and NF κ B (Xu *et al.*, 2008). To evaluate the role of p50 in SOD2 protein expression, we compared the SOD2 expression level in p50^{-/-} cells with that in WT cells. As shown in Figure 1A, deletion of p50 resulted in marked up-regulation of SOD2 protein expression (Figure 1A). Consistent with SOD2 protein expression, SOD2 mRNA was also up-regulated in p50^{-/-} cells (Figure 1B). These results indicate that p50 negatively regulates SOD2 expression, which is in contrast to a report on NF κ B-positive regulation of SOD2 expression (Xu *et al.*, 2008). To test whether p50 regulated SOD2 expression at the transcriptional level, we transfected WT mouse embryonic fibroblast (MEFs) and p50^{-/-} MEFs cells with SOD2 promoter-driven luciferase reporter and established stable transfectants of WT SOD2 promoter luciferase and p50^{-/-} SOD2 promoter luciferase. The results showed that SOD2 promoter transcription activity is significantly up-regulated in p50^{-/-} cells compared with WT cells (Figure 1C), suggesting that up-regulation of SOD2 expression in p50^{-/-} cells occurs at the transcriptional level via an NF κ B-independent pathway.

p50 mediates FoxO3a phosphorylation and inhibits SOD2 transcription and expression

Our foregoing results indicate that p50 provides an inhibitory effect on SOD2 transcription in SOD2 promoter-driven luciferase reporter assay. Bioinformatic analysis shows that the SOD2 promoter reporter contains the binding sites for multiple transcription factors, including SP1, Stat1, Stat5, AP-1, and FoxO3a (Figure 2A). To identify which of these transcription factors is implicated in p50 inhibition of SOD2 transcription, we compared activation of pathways related to SP1, AP-1, Stat1, Stat5, and FoxO3a between WT and p50^{-/-} cells. As shown in Figure 2, B and C, knockout of p50 (p50^{-/-}) decreased

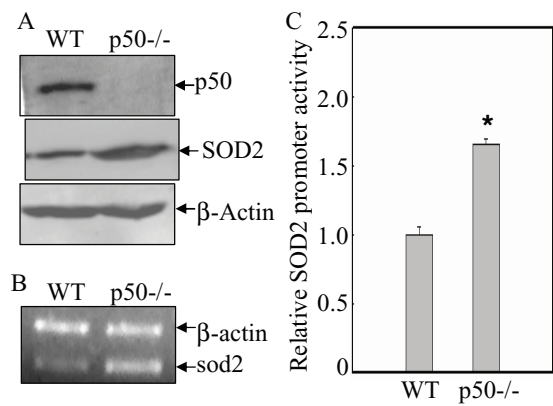


FIGURE 1: p50 expression inhibits SOD2 expression at the transcriptional level. (A) Cell extracts, as indicated, were subjected to Western blot for determination of p50 and SOD2. β -Actin was used as loading control. (B) SOD2 mRNA level in WT and p50^{-/-} cells analyzed by reverse transcription-PCR, with β -actin as loading control. (C) SOD2 promoter activity analyzed by determination of SOD2 promoter-driven luciferase activity. The asterisk indicates a significant increase compared with medium control ($p < 0.05$).

SP1 expression and phosphorylation of JNK at Thr-183/Tyr-185 and c-Jun at Ser-63/73, whereas it did not show observable alteration of p-Stat1 Tyr-701 and p-Stat5 Tyr-694, suggesting that SP1, AP-1, Stat1, and Stat5 might not be implicated in p50 inhibition of SOD2 expression. We next examined the potential contribution of FoxO3a to p50 regulation of SOD2 expression by comparing FoxO3a phosphorylation and transactivation between WT and p50^{-/-} cells. Phosphorylation of FoxO3a at Ser-318/321 and Ser-253 was markedly impaired in p50^{-/-} cells compared with WT cells, whereas total protein of FoxO3a expression showed observable up-regulation in p50^{-/-} cells compared with WT cells (Figure 2D). FoxO3a phosphorylation at Ser-318/321 and Ser-253 leads to its export from nuclear to cytoplasm, resulting in inactivation of its transcription activity (Liu *et al.*, 2005). To determine the role of p50 in regulation of Foxo3a-dependent transactivation, we transfected FoxO3a-dependent (4xDBE promoter) luciferase reporter into both WT and p50^{-/-} cells and stable transfectants; WT 4xDBE promoter-luciferase and p50^{-/-} 4xDBE promoter-luciferase were established. Consistent with FoxO3a protein phosphorylation, FoxO3a-dependent transcription activity was significantly up-regulated in p50^{-/-} cells compared with WT cells (Figure 2E). Our results provide strong indication that p50 regulates FoxO3a phosphorylation and transactivation, contributing to modulation of SOD2 transcription.

p50-activated Akt mediates FoxO3a phosphorylation and SOD2 down-regulation

Activated Akt phosphorylates FoxO3a protein at Ser-318/Ser-321 and Ser-253, which mediates FoxO3a nuclear exportation and transcription inhibition (Ferber *et al.*, 2012). To explore the mechanisms underlying p50 regulation of FoxO3a phosphorylation at Ser-318/Ser-321 and Ser-253, Akt phosphorylation at Thr-308/Ser-473 was assessed in both WT and p50^{-/-} cells. The results showed that Akt phosphorylation at Thr-308/Ser-473 was dramatically decreased in p50^{-/-} cells compared with WT cells (Figure 3A), suggesting that Akt was inhibited in p50^{-/-} cells. To evaluate role of Akt activation in FoxO3a phosphorylation, transactivation, and SOD2 expression, we transfected dominant-negative mutant of Akt (DN-Akt) into WT cells and established stable transfectant WT(DN-Akt). As shown in

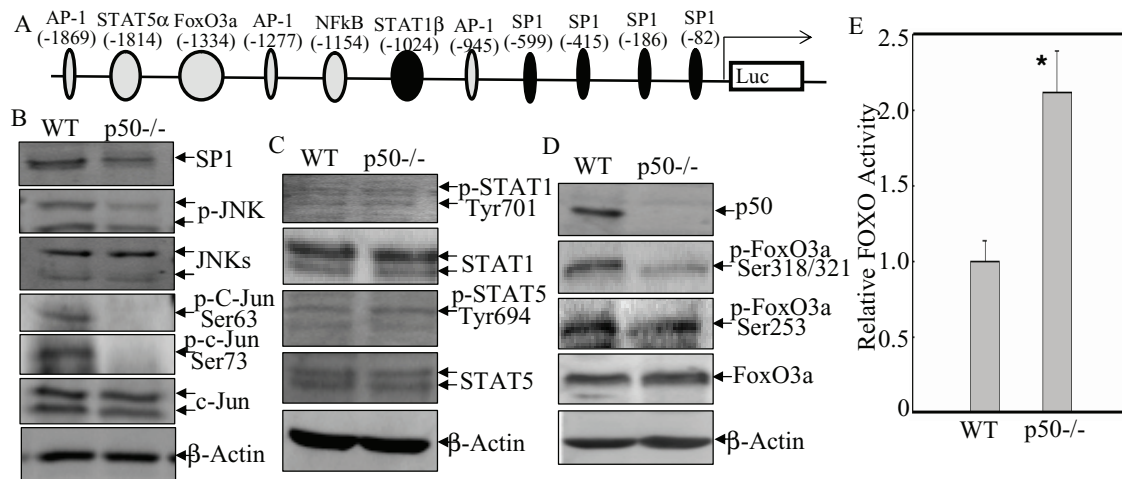


FIGURE 2: p50 mediates FoxO3a phosphorylation and inhibits SOD2 transcription and expression. (A) Scheme of the *sod2* promoter-driven luciferase reporter. (B–D) Cell extracts of WT and p50^{-/-} subjected to Western blot for determination of SP1, phosphor-JNK at Thr-183/Tyr-185, phosphor-c-Jun at Ser-63/Ser-73, phosphor-Stat1 at Tyr-701/Tyr-694, phosphor-FoxO3a at Ser3-18/321 and Ser-253, and FoxO3a. β-Actin was used as loading control. (E) The plasmids pGL-4xDBE and *Renilla* luciferase were stably cotransfected into WT and p50^{-/-} cells. FoxO3a-dependent transcriptional activity is presented as luciferase activity relative to medium control. The asterisk indicates a significant increase compared with medium control ($p < 0.05$).

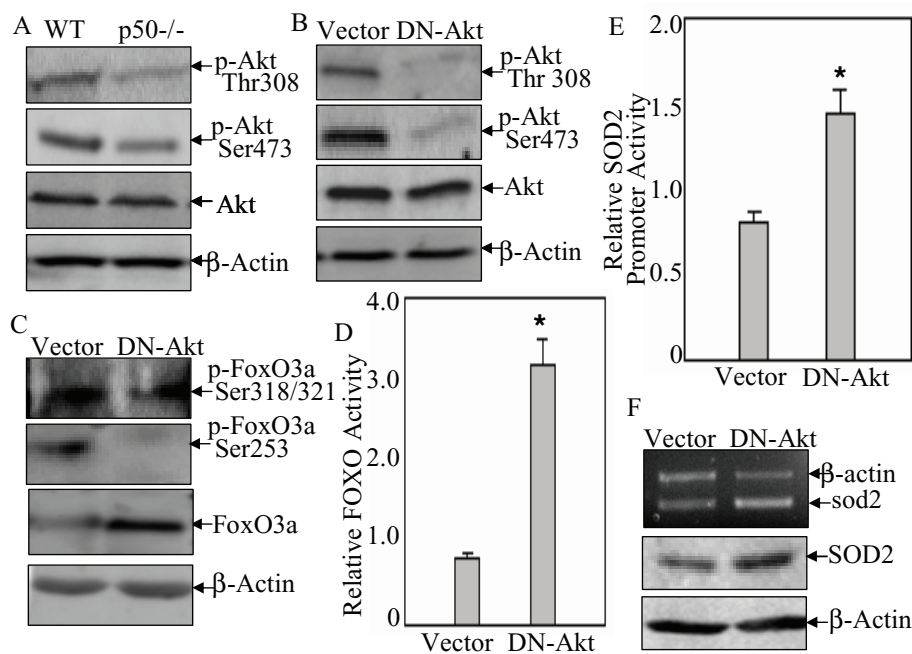


FIGURE 3: p50-activated Akt mediates FoxO3a phosphorylation and inhibits SOD2 transcription and expression. (A) Cell extracts of WT and p50^{-/-} subjected to Western blot for detection of total Akt and phosphor-Akt at Ser-473/Thr-308. (B, C) Cell extracts of WT and DN-Akt subjected to Western blot for determination of total Akt, phosphor-Akt at Ser-473/Thr-308, and total and phosphor-FoxO3a at Ser-318/321 and Ser-253. β-Actin was used as loading control. (D) The plasmids pGL-4xDBE and *Renilla* luciferase were stably cotransfected into WT and DN-Akt cells. FoxO3a-dependent transcriptional activity is presented as luciferase activity relative to medium control. The asterisk indicates a significant increase compared with vector control transfectant ($p < 0.05$). (E) SOD2 promoter reporter activity in WT and DN-Akt transfectants analyzed by determination of SOD2 promoter-driven luciferase transcription activity. The asterisk indicates a significant increase compared with vector transfectant ($p < 0.05$). (F) SOD2 mRNA level in WT and DN-Akt cells was analyzed by reverse transcription-PCR (Total), or cell extracts of WT and DN-Akt were subjected to Western blot for detection of SOD2 protein expression. β-Actin was used as loading control.

Figure 3B, introduction of DN-Akt did inhibit Akt phosphorylation at Thr-308/Ser-473. The inhibition of Akt phosphorylation at Thr-308/Ser-473 by DN-Akt impaired FoxO3a phosphorylation at Ser-318/Ser-321 and Ser-253 and increased FoxO3a-dependent transactivation (Figure 3, C and D). Consistent with the increase of FoxO3a-dependent transactivation, ectopic expression of DN-Akt also led to significant increases in SOD2 promoter-driven transcriptional activity (Figure 3E), mRNA, and protein expression in comparison to vector control transfectant (Figure 3F). Total Akt level was not increased after transfection of DN-Akt into WT MEFs (Figure 3B). This is consistent with our previous reports (Li *et al.*, 2004; Ouyang *et al.*, 2007). A possible explanation is that the negative feedback loop inhibited endogenous Akt expression upon DN-Akt ectopic expression. Our results demonstrate that p50 positively regulates Akt phosphorylation and activation, which mediates phosphorylation of FoxO3a at Ser-318/Ser-321 and Ser-253 and inhibition of FoxO3a-dependent transactivation, further leading to reduction of SOD2 protein expression.

p50 up-regulates Akt phosphorylation at Thr-308/Ser-473 and inhibits FoxO3a transactivation and SOD2 expression in a miR190/PHLPP1-dependent manner

PHLPP1 is a key Akt phosphatase and is responsible for dephosphorylation of Akt at

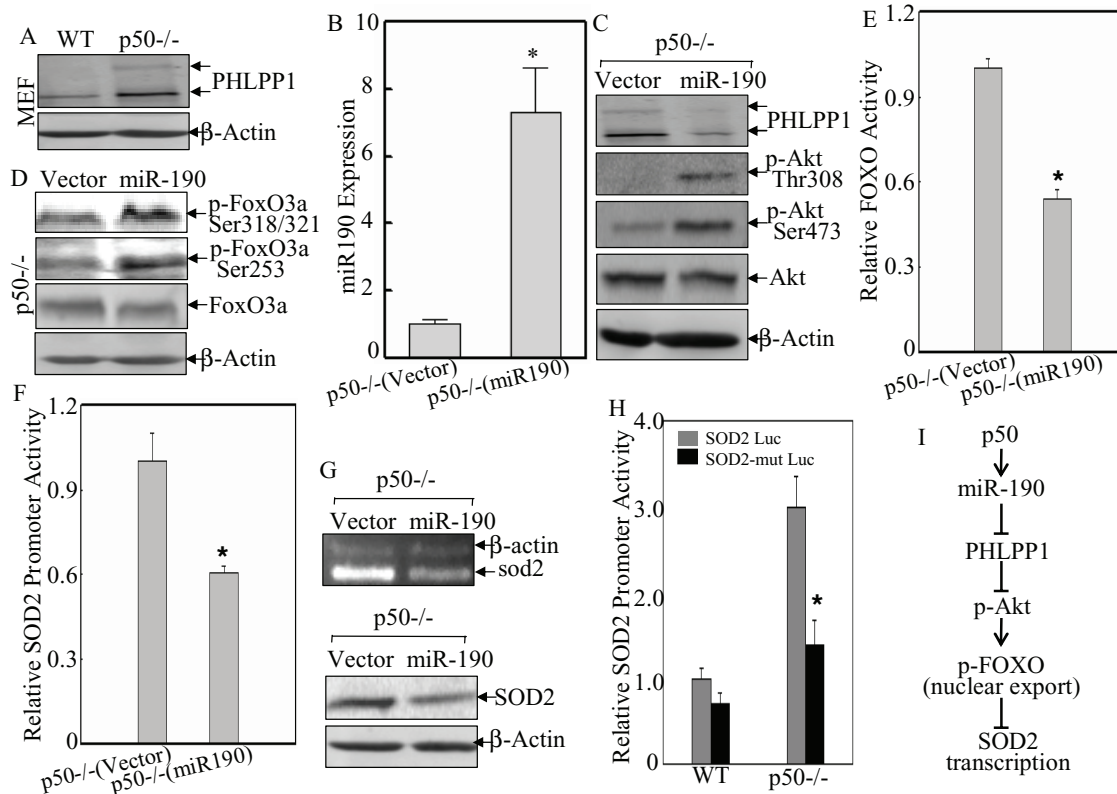


FIGURE 4: p50-regulated miR190/PHLPP1/Akt mediates FoxO3a phosphorylation, transactivation, SOD2 transcription, and protein expression. (A) Cells extract of WT and p50^{-/-} subjected to Western blot for determination of indicated protein expression, with β-actin as protein loading control. (B) miR190 level analyzed by real-time PCR, using total RNA extracted from p50^{-/-}(vector) and p50^{-/-}(miR190) cells. Data are means ± SD of five experiments. (C, D) Cell extracts of p50^{-/-}(vector) and p50^{-/-}(miR190) subjected to Western blot for determination of indicated protein expression, with β-actin as protein loading control. (E) The plasmids pGL-4xDBE and *Renilla* luciferase were stably cotransfected into p50^{-/-}(vector) and p50^{-/-}(miR190) cells. FoxO3a-dependent transcriptional activity is presented as luciferase activity relative to p50^{-/-}(vector) control. The asterisk indicates a significant decrease compared with vector control transfectant ($p < 0.05$). (F) SOD2 promoter activity of p50^{-/-} and p50^{-/-}(miR190) cells analyzed using SOD2 promoter-driven luciferase transcriptional activity. The asterisk indicates a significant decrease compared with p50^{-/-}(miR190) cells ($p < 0.05$). (G) SOD2 mRNA level in p50^{-/-} and p50^{-/-}(miR190) cells was evaluated by reverse transcription-PCR (top), and SOD2 protein expression was determined by Western blot. β-Actin was used as loading control. (H) The plasmids of SOD2 promoter-driven luciferase reporter and the SOD2 promoter-driven luciferase reporter with a point mutation resultant of loss of FoxO3a binding site (mut), together with *Renilla* luciferase, were stably cotransfected into WT and p50^{-/-} MEF cells. SOD2 promoter activity was analyzed by measuring promoter-driven luciferase activity. The asterisk indicates a significant decrease in comparison to WT SOD2 promoter reporter transfectant ($p < 0.05$). (I) Schedule of p50-regulated miR190 mediates SOD2 down-regulation.

Ser-473 and Thr-308 (Gao *et al.*, 2008), whereas miR190 is able to regulate human PHLPP1 translation and expression (Beezhold *et al.*, 2011). Our most recent studies demonstrated that knockout of p50 results in reduction of miR190 transcription, leading to up-regulation of PHLPP1 protein translation and expression (Yu *et al.*, 2013). Consistent with our previous report, p50 deficiency resulted in PHLPP1 up-regulation (Figure 4A). To address whether the defect of miR190 expression was responsible for PHLPP1 up-regulation and Akt inhibition in p50^{-/-} cells, we stably transfected miR190 expression plasmid into p50^{-/-} cells and established stable transfectant, p50^{-/-}(miR190), and identified it by real-time PCR as shown in Figure 4B. The constitutively stable expression of miR190 in p50^{-/-} cells led to inhibition of PHLPP1 protein expression and up-regulation of Akt phosphorylation at Ser-473 and Thr-308 (Figure 4C). Moreover, expression of miR190 in p50^{-/-} cells also increased FoxO3a phosphorylation at Ser-318/Ser-321 and Ser-253 and decreased FoxO3a transactivation activity (Figure 4, D and E). Consistent with the

inhibition of FoxO3a transactivation by miR190, SOD2 promoter-driven luciferase reporter activity, mRNA expression, and protein expression were also dramatically impaired in miR190-transfected p50^{-/-} cells (Figure 4, F and G). To identify the role of FoxO3a in p50 regulation of sod2 transcription, we transfected WT SOD2 promoter-driven luciferase reporter and this reporter with FoxO3a binding-site point mutation (C → G mutation at -1329) into both WT and p50^{-/-} cells. The transfectants were used to determine the role of FoxO3a in p50 mediation of SOD2 transcription. The results clearly indicate that SOD2 promoter transcription activity was significantly up-regulated in p50^{-/-} cells, whereas this up-regulation was attenuated in the p50^{-/-} transfectant of an SOD2 promoter-driven luciferase reporter with point mutation at FoxO3a binding in p50^{-/-} cells (Figure 4H). These results indicate that p50 expression inhibits SOD2 transcription and this inhibition is mediated by p50 attenuation of FoxO3a transcriptional activity. Collectively our results demonstrate that p50-regulated miR190 mediates PHLPP1

down-regulation, which in turn inhibits Akt phosphorylation, resulting in reduction of FoxO3a phosphorylation at Ser-318/Ser-321 and Ser-253 and induction of its transactivation. The transactivated FoxO3a mediates SOD2 transcription as shown in Figure 4l.

DISCUSSION

p50 homodimers translocate into the nucleus and bind to the NFκB-binding sites of its target genes, but p50 homodimers alone cannot act as a transcription factor to regulate NFκB downstream gene expression (Lerebours *et al.*, 2008; Moss *et al.*, 2008). For this reason, compared with other members of NFκB family, the biological function of p50 in the regulation of protein expression is far from understood (Karst *et al.*, 2009; Yu *et al.*, 2009). Our recent studies demonstrate that p50 plays a role as an apoptotic mediator via its inhibition of GADD45α protein degradation and increases p53 protein translation. Here we identified a novel function of p50 in regulating SOD2 transcription via NFκB-independent and FoxO3a-dependent pathways.

Accumulating evidence suggests that SOD2 may function as a new type of tumor suppressor (Dhar *et al.*, 2007). SOD2 activity is reduced in >80 different types of neoplastic cells (Xu *et al.*, 2008). Overexpression of SOD2 leads to suppression of cancer phenotypes (Xu *et al.*, 2008), and SOD2 is associated with tumor suppression (Weyemi *et al.*, 2012). Therefore understanding the molecular mechanisms of the regulation of SOD2 expression should be highly significant in cancer research. Our present study shows that SOD2 expression in p50^{-/-} cells is remarkably increased compared with WT cells. Further studies show that p50 regulates SOD2 expression at the transcriptional level. Moreover, p50 deficiency specifically results in down-regulation of FoxO3a phosphorylation at Ser-318/Ser-321 and Ser-253 and increase of FoxO3a transactivation compared with WT cells, further leading to SOD2 transcription and protein expression.

A series of transcription factors, such as specificity protein-1, activator protein-1, and signal transducers and activators of transcription (STATs), are involved in regulation of SOD2 gene expression (Belton *et al.*, 2006; Hitchler *et al.*, 2008). In this study, we showed that knockout of p50 decreases expression of SP1 and phosphorylation of JNK at Thr-183/Tyr-185 and p-c-Jun at Ser-63/73 and does not show observable effects on p-Stat1 Tyr-701 and p-Stat5 Tyr-694, suggesting that SP1, AP-1, Stat1, and Stat5 might not be implicated in p50 inhibition of SOD2 expression. Forkhead transcription factors of the O class (FoxO) proteins are identified by the winged-helix structure of their DNA-binding domain (Ferber *et al.*, 2012), and forkhead transcription factor is involved in regulation of SOD2 expression (Li *et al.*, 2006). FoxO plays an important role via its regulation of antioxidant defense in many species (Belton *et al.*, 2006), and the mammalian genome encodes four FoxO proteins (FoxO1, FoxO3a, FoxO4, and FoxO6; Ferber *et al.*, 2012). FoxO3a, also known as FKHL1, can protect quiescent human cells from oxidative stress by directly increasing quantities of SOD2 (Belton *et al.*, 2006). The FoxO3a translocation regulates its transactivation (Kops *et al.*, 2002). The FoxO3a translocation depends on Akt-mediated phosphorylation at Ser-253 and Ser-315 (Sengupta *et al.*, 2011). Phosphorylation of FoxO3a at Ser-253 in the forkhead domain leads to disruption of nuclear localization and its binding to the promoter regions of its regulated gene (Afanas'ev, 2010). After phosphorylation, FoxO3a forms a complex with 14-3-3 proteins and in turn is transported from nucleus to cytosol (Marfe *et al.*, 2010). In cytosol, the phosphorylated FoxO3a proteins are degraded by the ubiquitin-proteasome system (Liu *et al.*, 2005). Thus this shuttling system provides the cell with "double-negative" regulation of FoxO3a and

subsequent inhibition of FoxO3a-dependent transcriptional activity (Li *et al.*, 2006). Our results show that FoxO3a phosphorylation at Ser-318/321 and Ser-253 is markedly impaired in p50^{-/-} cells compared with WT cells, whereas this decreased phosphorylation in p50^{-/-} cells results in its transactivation, contributing to induction of SOD2 transcription and protein expression.

It was reported that Akt is the kinase responsible for phosphorylation of FoxO3a protein, which mediates FoxO3a nuclear exportation and inhibition; therefore FoxO3a is negatively regulated by Akt (Li *et al.*, 2006; Ferber *et al.*, 2012). Our most recent studies demonstrate that p50 expression attenuates translation and expression of PHLPP1, which is a major Akt phosphatase, after arsenite exposure (Yu *et al.*, 2013). Further studies show that p50-mediated up-regulation of miR190 transcription is responsible for inhibition of PHLPP1 translation. Therefore p50 mediates Akt phosphorylation and activation (Yu *et al.*, 2013). Consistently, our present studies show that Akt phosphorylation at Thr-308/Ser-473 is inhibited in p50^{-/-} cells, whereas introduction of miR190 impairs PHLPP1 expression and increases Akt phosphorylation at Thr-308/Ser-473 and FoxO3a phosphorylation in p50^{-/-} cells. Moreover, inhibition of Akt activation by ectopic expression of DN-Akt also attenuates FoxO3a phosphorylation and up-regulates FoxO3a-dependent transcription activity. These results strongly suggest that p50-regulated miR190/PHLPP1 expression mediates Akt activation, leading to induction of FoxO3a phosphorylation and inhibition of transactivation activity. Of importance, our results show that the p50-regulated miR190/PHLPP1/Akt/FoxO3a axis plays an essential role in SOD2 transcription and protein expression, demonstrating a novel function of p50 regulation of SOD2 expression and leading to regulation of normal physiological function of cells by mediation of antioxidative status and pathological development of tissues by modulation of antioxidative responses after environmental exposures and other disease-causing agents.

In summary, our present studies demonstrate a novel function of p50 in regulating SOD2 at the transcription level by targeting the miR190/PHLPP1/Akt-FoxO3a axis via NFκB transcription-independent mechanisms. This finding provides new insight into the physiological function of p50 and its pathological regulatory role in regulation of cellular oxidative status and responses. A more complete understanding of the role and molecular mechanisms underlying the biological function of p50 will help us to use p50 and its regulated downstream targets as targets for related disease management.

MATERIALS AND METHODS

Antibodies, plasmids, and other reagents

Antibodies specific against p-JNK(Thr-183/Tyr-185), JNK1/2, p-c-Jun(Ser-73), p-c-Jun(Ser-63), c-Jun, p-Stat1(Tyr-701), Stat1, p-Stat5(Tyr-694), Stat5, p-Stat3(Tyr-705), p-Stat3(Ser-727), Stat3, p-Akt(Thr-308), p-Akt(Ser-473), Akt, p-FoxO3a(Ser-318/321), p-FoxO3a(Ser-253), and FoxO3a were purchased from Cell Signaling (Beverly, MA). Antibodies specific against mouse p50 and SP1 were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody specific against PHLPP1 was purchased from Bethyl Laboratories (Montgomery, TX). SOD2 antibody was purchased from Epitomics (Burlingame, CA). β-Actin antibody was purchased from Sigma (St. Louis, MO). The dual-luciferase assay substrate was purchased from Promega (Madison, WI). V5-DEST-miR190 and the control constructs were kind gifts from Ping-Yee Law (Department of Pharmacology, University of Minnesota, Minneapolis, MN). pGL4-SOD2-promoter-luciferase and pGL4-4xDBE-promoter-luciferase were kind gifts from Bernd Evert (Neurologische Klinik und Poliklinik, University of Bonn, Bonn, Germany). pGL3-SOD₂-promoter-luciferase and pGL3-SOD₂-promoter-mut-luciferase (Olmos *et al.*, 2009) were

kind gifts from M. Monsalve (Fundación Centro Nacional de Investigaciones Cardiovasculares Carlos III, Melchor Fernández Almagro, Madrid, Spain).

Cell culture and transfectants

WT MEFs and their corresponding p50^{-/-} MEF cells were described previously (Song *et al.*, 2007). All MEFs and their transfectants were cultured in an incubator at 37°C with 5% CO₂ in 10% fetal bovine serum DMEM supplied with 1% penicillin/streptomycin and 2 mM L-glutamine (Life Technologies, Carlsbad, CA). WT MEFs were stably transfected with DN-Akt, and p50^{-/-} cells were stably transfected with miR190, as described in Yu *et al.* (2013). WT and p50^{-/-}, WT(vector) and WT(DN-Akt), and p50^{-/-}(vector) and p50^{-/-}(miR190) cells were stably transfected with TK reporter together with pGL4-SOD2-promoter-luciferase, pGL3-SOD2-promoter-luciferase or pGL3-SOD2-promoter-mut-luciferase reporter or Fox3a (4xDBE)-luciferase reporter, using PolyJet DNA In Vitro Transfection Reagent (SignaGen Laboratories, Rockville, MD). Stable transfectants were established by selection with puromycin (Alexis, Plymouth, PA) and identified by luciferase activity assay. Stable transfectants included WT SOD2 promoter luciferase, p50^{-/-} SOD2 promoter luciferase, WT 4xDBE promoter luciferase, p50^{-/-} 4xDBE promoter luciferase, WT(vector) SOD2 promoter luciferase, WT(DN-Akt) SOD2 promoter luciferase, WT(vector) 4xDBE promoter luciferase, WT(DN-Akt)4xDBE promoter luciferase, p50^{-/-}(vector) SOD2 promoter luciferase, p50^{-/-}(miR190) SOD2 promoter luciferase, p50^{-/-}(vector) 4xDBE promoter luciferase, p50^{-/-}(miR190) 4xDBE promoter luciferase, WT SOD2 promoter luciferase, WT SOD2 mut promoter luciferase, p50^{-/-} SOD2 promoter luciferase, and p50^{-/-} SOD2 mut promoter luciferase. These stable transfectants were cultured in selected antibiotic-free medium for at least two passages before experiments.

Reverse transcription-PCR

Cells were extracted for total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA), as described in the manufacturer's instructions (Du *et al.*, 2010; Fang *et al.*, 2012). We used 5 µg of total RNA for first-strand cDNA synthesis with oligdT(20) primer by SuperScript III First-Strand Synthesis system (Invitrogen). The primers used in this study were as follows: *sod2*, forward, 5'-gca gtg tgc ggc acc agc ag-3', and reverse, 5'-tcc ctt ggc caa cgc ctc ct-3'; and *β-actin*, forward, 5'-cct gtg gca tcc atg aaa ct-3'; and reverse, 5'-gtg cta gga gcc aga gca gt-3'. The PCR product was analyzed by 2% agarose gel.

Real-time PCR

Total RNA extraction with the miRNeasy Mini Kit (Qiagen, Valencia, CA) and then 1 µg of RNA was used for reverse transcription. Analysis of miR190 expression was carried out using the miScript PCR system (Qiagen) and the 7900HT Fast Real-time PCR system (Applied Biosystems, Carlsbad, CA). The primer for miR190 assay was purchased from Qiagen, and U6 primer provided by the kit was used as loading control. PCR conditions follow the specifications of the miScript PCR Starter Kit (Qiagen). Data were analyzed as described previously (Yu *et al.*, 2013).

Dual-luciferase reporter assay system

For determination of SOD2 promoter-driven luciferase transcriptional activity or FoxO3a-dependent transcriptional activation, stably transfectants with luciferase reporter/TK were seeded into 96-well plates. Cells were cultured in normal culture medium for 24 h and then extracted for luciferase activity assay using the Dual-Luciferase Reporter Assay System (Promega) as described (Ouyang *et al.*, 2007).

The results are presented as relative SOD2 promoter activity or relative FoxO3a-dependent transcriptional activity (Ding *et al.*, 2006).

Western blot

Cells were seeded into six-well plates and cultured until 80% confluent in normal culture medium. The cells were then extracted with cell lysis buffer (10 mM Tris-HCl, pH 7.4, 1% SDS, 1 mM Na₃VO₄, and proteasome inhibitor), and cell protein extracts were subjected to Western blot as described previously (Li *et al.*, 2004).

Statistical methods

The Student's *t* test was used to determine the significant difference of transcriptional activity between various cells and transfectants. Differences were considered to be significant at *p* ≤ 0.05.

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