Peroxisiredoxin II Is Essential for Maintaining Stemness by Redox Regulation in Liver Cancer Cells

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Key Words. Peroxisiredoxin II • Hepatocellular carcinoma • Vascular endothelial growth factor • Cancer stem cells

ABSTRACT

Redox regulation in cancer stem cells (CSCs) is viewed as a good target for cancer therapy because redox status plays an important role in cancer stem-cell maintenance. Here, we investigated the role of Peroxisiredoxin II (Prx II), an antioxidant enzyme, in association with maintenance of liver CSCs. Our study demonstrates that Prx II overexpressed in liver cancer cells has high potential for self-renewal activity. Prx II expression significantly correlated with expression of epithelial-cell adhesion molecules (EpCAM) and cytokine 19 in liver cancer tissues of hepatocellular carcinoma (HCC) patients. Downregulation of Prx II in Huh7 cells with treatment of siRNA reduced expression of EpCAM and CD133 as well as Sox2 in accordance with increased ROS and apoptosis, which were reversed in Huh7-hPrx II cells. Huh7-hPrx II cells exhibited strong sphere-formation activity compared with mock cells. Vascular endothelial growth factor (VEGF) exposure enhanced sphere formation, cell-surface expression of EpCAM and CD133, and pSTAT3 along with activation of VEGF receptor 2 in Huh7-hPrx II cells. The result also emerged in Huh7-H-rasG12V and SK-HEP-1-H-rasG12V cells with high-level expression of Prx II. Prx II was involved in regulation of VEGF driving cancer stem cells through VEGFR-2/STAT3 signaling to upregulate Bmi1 and Sox2. In addition, knockdown of Prx II in Huh7-H-rasG12V cells showed significant reduction in cell migration in vitro and in tumorigenic potential in vivo. Taken together, all the results demonstrated that Prx II plays a key role in the CSC self-renewal of HCC cells through redox regulation.

INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common cause of cancer death worldwide [1], with rising incidence in the past decades. Intensive research efforts have been directed toward the identification of novel treatment strategies and markers associated with the initiation and progression of HCC [2]. However, despite advances in the detection and treatment of the disease, mortality rates remain high because current therapies are limited by the advanced stage in which the disease is usually diagnosed, when most potentially curative therapies such as resection and transplantation are of limited efficacy [3].

Cancer stem cells (CSCs) are a rare set of undifferentiated cells that can initiate tumor formation and generate multipotent progenitors, producing a heterogeneous population of cancer cells. CSCs have now been identified in many human cancers [4]. Moreover, the discovery of a CSC population that undergoes an epithelial-to-mesenchymal transition (EMT), a

SIGNIFICANCE STATEMENT

This study demonstrates that Peroxisiredoxin II (Prx II), an antioxidant enzyme, overexpressed in liver cancer cells has high potential for self-renewal activity. Prx II expression significantly correlated with expression of epithelial-cell adhesion molecules and cytokine 19 in liver cancer tissues of hepatocellular carcinoma patients, induced expression of stem cell markers, and exhibited strong sphere-formation activity in liver cancer cells. In addition, Prx II was involved in regulation of vascular endothelial growth factor (VEGF) driving cancer stem cells through VEGFR-2/STAT3 signaling to upregulate Bmi1 and Sox2. Thus, our finding suggests that Prx II may be essential for maintenance of stemness by redox regulation in liver cancer.
driver of metastasis, has bolstered the importance of CSC research for cancer-therapy development [5]. Several studies identified CSCs in HCC. CSC self-renewal and hierarchical organization features have been experimentally validated by xenotransplantation of freshly resected HCC specimens. In HCC, CSC markers include epithelial-cell adhesion molecules.

![Figure 1](image.png)

**Figure 1.** Association of Prx II with cancer stem cell in HCC cells. (A): Representative image of EpCAM(+)/K19(+) HCC showing Prx II expression in contrast to EpCAM(-)/K19(-) HCC without expression of Prx II (magnification, × 200). (B): Comparison of proportions of Prx II expressing in EpCAM(+)/K19(+) HCCs and EpCAM(-)/K19(-) HCCs. (C): Expression levels of Sox2 in Huh7 cells transfected with siPrx II. (D): Proportions of 2',7'-dichlorodihydrofluorescein diacetate, annexin V, EpCAM, and CD133 positive in Huh7 cells transfected with siPrx II by flow cytometric analysis. The data are the mean ± SEM (n = 5 per group), *p < .05. Abbreviations: EpCAM, epithelial-cell adhesion molecules; HCC, hepatocellular carcinoma; Prx II, peroxiredoxin II; K19, cytokeratin 19.

**Table 1.** Tumor formation incidence of Huh7-H-rasG12V and Huh7-H-rasG12V-shPrx II cells, respectively, into 7-week-old athymic BALB/c female nude mice

<table>
<thead>
<tr>
<th>Cell type</th>
<th>No. of cells injection</th>
<th>Tumor incidence</th>
<th>Total (%)</th>
<th>Latency (days)</th>
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<tr>
<td>H-rasG12V</td>
<td>1 × 10^5</td>
<td>5/5</td>
<td>100</td>
<td>35</td>
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<tr>
<td></td>
<td>1 × 10^4</td>
<td>4/5</td>
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<td>1 × 10^3</td>
<td>2/5</td>
<td>40</td>
<td>35</td>
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<tr>
<td></td>
<td>5 × 10^2</td>
<td>0/5</td>
<td>0</td>
<td>35</td>
</tr>
<tr>
<td>H-rasG12V-shPrx II</td>
<td>1 × 10^6</td>
<td>4/5</td>
<td>80</td>
<td>35</td>
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<tr>
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<td>1 × 10^5</td>
<td>1/5</td>
<td>20</td>
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<td>5 × 10^3</td>
<td>0/5</td>
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Abbreviations: Prx II, peroxiredoxin II.
Vascular endothelial growth factor (VEGF) and ROS play critical roles in vascular pathophysiology [8]. VEGF-R2 (also known as KDR), as the major VEGF receptor, requires phosphorylation or activation, demonstrating many signaling capacities in the formation of vascular-mimicry channels [9]. Researchers found VEGF-R2 was expressed in tumor cells from patients with colorectal cancer and responded to VEGF stimulation with augmented VEGF-R2-mediated proliferation and tumor growth, and the activation of VEGF receptors on tumor cells could mediate tumor growth and metastasis [10]. Peroxiredoxins (Prxs), antioxidant enzymes, lost the peroxidase activity by inhibitory phosphorylation on threonine or tyrosine residue [11]. Peroxiredoxin II (Prx II) regulated the VEGF-R2 oxidation mechanism, representing a potential anti-angiogenic target [12]. In the absence of Prx II, the cellular H2O2 level markedly increases and the VEGF-R2 becomes inactive, no longer responding to VEGF stimulation in endothelial cells.

We investigated whether and how Prx II may regulate maintaining self-renewal of cancer stem cells and stimulate CSCs independent of mitogenic effects in HCC, highly prevalent in lethal human malignancies. Our data suggest that Prx II plays a key role in maintaining the self-renewal of CSCs through redox regulation in liver cancer cells.

#### Materials and Methods

### Immunohistochemical Staining

Immunohistochemistry was performed as shown in Supporting Information Method S1.

### Cell Culture and Treatments

The human HCC cell lines Huh7, Huh7-hPrx II, Huh7-H-RasG12V, SK-HEP-1, SK-HEP-1-hPrx II, SK-HEP-1-H-RasG12V were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, Waltham, MA) containing 10% fetal bovine serum (Hyclone, South Logan, UT), penicillin (100 U/ml), and streptomycin (100 mg/ml). Huh7 cells were incubated with human VEGF165 (Cell Signaling Technology, Danvers, MA) for 1 hour. Huh7-H-RasG12V-shPrx II cell lines were established by transfection of Huh7-H-RasG12V and SK-HEP-1-H-RasG12V cells with shRNA-Prx II and scrambled-shRNA. Additional information is provided in Supporting Information Methods.

### Flow Cytometry Analyses

The intracellular ROS of HCC cell lines were determined using 2’,7’-dichlorodihydrofluorescein diacetate (DCF-DA; Invitrogen, Waltham, MA). Cells were incubated with 20 mM of DCF-DA for 15 minutes at 37°C, and then washed with PBS. Apoptosis in cells was measured by washing with PBS, staining with Annexin V Binding Buffer (BD Biosciences, San Diego, CA), and labeled with Annexin V-FITC (BD Biosciences, San Diego, CA), as recommended by the manufacturer. Staining involved EpCAM (BioLegend, San Diego, CA) and CD 133 (milenyi biotec, San Diego, CA) for 20 minutes at 37°C, then washed with PBS, and analyzed by FACS Calibur (BD Biosciences, San Diego, CA) [13].

### Western Blot Analysis

We homogenized liver tissues and cell lysates in a lysis buffer (20 mM HEPES, 150 mM NaCl, 2 mM EGTA, 1 mM EDTA, 20 mM glycerol phosphate, 1% Triton X-100, and 10% glycerol with protease Sigma) and a phosphatase-inhibitor cocktail (Roche). Additional details were performed as described previously [21]. The membranes were primarily blotted with primary antibodies against Prx II and Prx II and GAPDH (Abfrontier, Seoul, Korea); VEGFR1, VEGFR2, pVEGFR2 (Tyr951), pVEGFR2 (Tyr 1059), pVEGFR2 (Tyr1175), Sox2 (Cell Signaling Technology, Danvers, MA); pSTAT3 HA (Roche); STAT3 (Santa Cruz Biotechnology, Dallas, TX); Oct 3/4 (Abcam, Cambridge, MA).

### Xenograft Mice

All mice were maintained and used for experiment according to the IACUC-approved protocols of the Lee Gil Ya Cancer and Diabetes Institute. Tumorigenicities of Huh7-H-RasG12V and Huh7-H-RasG12V-shPrx II cells were assayed by subcutaneous inoculations of 5 × 105, 1 × 106, 1 × 107 and 1 × 108 cells resuspended in mixed 100 μl matrigel of PBS into the flanks of 7-week-old athymic BALB/c female nude mice (n = 5 in four groups). Tumor size was measured using calipers (calculated volume = shortest diameter2 × longest diameter/2). Seven weeks after cell inoculation, grafts were removed and photographed.

### Data Analysis

Statistical analysis was conducted using SPSS software (version 20.0.1, SPSS Inc., Chicago, IL). Chi-square test or Fisher’s exact test was used as deemed appropriate. Statistical significance was assumed for p < .05.

### Results

#### Prx II Expression in EpCAM(+)/K19(+) HCCs and HCC Cells Are Involved in Stemness

As reported, the functional involvement of most liver CSC markers in the maintenance of liver CSC features potentially makes them a good target for the eradication of liver CSCs [7]. The expression of stemness-related markers such as cytokeratin 19, EpCAM, and CD133 in HCC is associated with aggressive biological behavior and poor clinical outcomes compared with conventional HCCs that do not express stemness-related markers [14]. Increased expressions of Prx II were reported in a variety of distinct human cancers including HCC [15]. In addition, Prx II knockdown inhibited the growth of HCC cells [14]. Thus, we first investigated whether Prx II expression may be involved in stemness in HCC. We performed clinicopathologic features (Supporting Information Table 1) and immunohistochemical staining to find the expression of Prx II in patient HCCs with EpCAM(+)/K19(+) and EpCAM(−)/K19(−). Prx II was remarkably more expressed in EpCAM(+)/K19(+) HCCs than in EpCAM(−)/K19(−) HCCs (Fig. 1A). Prx II expression was observed in seven (87.5%) of eight EpCAM(+)/K19(+) HCCs and four (44.4%) of nine EpCAM(−)/K19(−) HCCs (Fig. 1B). The protein expression of Prx II was significantly higher in EpCAM(+)/K19(+) HCCs than...
was rather lower than in Huh7 cells (Fig. 1D). The results suggest functional relevance of Prx II in the stemness of HCC cells.

**Prx II Overexpression Enhanced Stemness in HCC**

We investigated the involvement of Prx II in the maintenance of stemness by overexpressing hPrx II in Huh7 (Huh7-hPrx II) and SK-HEP-1 (SK-HEP-1-hPrx II) cells. Transfection of hPrx II led to increment of Sox2 and Oct 3/4 in western blot (Fig. 2A and Supporting Information Fig. S1A) as well as cell-surface expression of EpCAM and CD133 in Huh7-hPrx II and SK-HEP-1-hPrx II cells (Fig. 2B and Supporting Information Fig. S1B). Huh7-hPrx II cells had a significantly lower level of ROS and apoptosis expression on their cell surface than Huh7 cells. The frequency and size of sphere cells increased in Huh7-hPrx II and SK-HEP-1-hPrx II cells (Fig. 2C and Supporting Information Fig. S1C). These data suggest that Prx II overexpression enhances stemness by reducing ROS-mediated apoptosis in liver cancer cells.
Prx II Drives VEGFR2/STAT3 Signaling to CSCs Self-Renewal by VEGF Stimulation in HCC Cells

Prx II is an essential antioxidant enzyme that prevents the oxidative inactivation of VEGFR2 in vascular endothelial cells [18]. VEGF drives VEGFR2 to recruit JAK2/STAT3, leading to Sox2 promoter occupancy and induction by STAT3 to form an autocrine loop-driving CSC self-renewal [19]. To understand whether Prx II drives CSC self-renewal through VEGFR2/STAT3 signaling, we exposed VEGF to Huh7 Mock and Huh7-hPrx II cells. The auto-phosphorylation of VEGFR2 on 1175 tyrosine residues was more induced by VEGF stimulation in Huh7-hPrx II cells than Huh7 Mock cells. The expression level of Sox2 and pSTAT3 also increased in Huh7-hPrx II cells (Fig. 3A). In addition, VEGF pretreatment induced significantly increased sphere formation as well as significantly increased EpCAM and CD133 positive populations cells in Huh7-hPrx II cells (Fig. 3B, 3C and Supporting Information Fig. S2). These data suggest that Prx II may drive CSC self-renewal through VEGFR2/STAT3 signaling by VEGF stimulation in HCC cells.

Involvement of Prx II in Regulation of CSC Self-Renewal and EMT, Induced by H-ras<sup>G12V</sup> Activation in HCC Cells

Oncogenes of the RAS family regulate many cell activities, including proliferation, survival, and differentiation. Mutations in these genes are common events for many types of cancer [20]. One mechanism by which Ras proteins influence cell growth is regulation of an intracellular level of ROS: second messengers affecting a variety of cellular processes including cell proliferation [20, 21]. Ras activation in cancer cells is associated with CSCs self-renewal [22]. H-ras activation in HCC cells induced enhanced expression of Prx II (Data not shown). To understand whether Prx II overexpressed in Huh7 H-ras<sup>G12V</sup>...
cells may affect regulation of CSCs self-renewal and cancer-cell metastasis, we performed the following experiments.

As shown in Figure 4A, remarkably increased Prx II in Huh7 H-rasG12V cells induced the phosphorylation of VEGFR2 (Tyr-905, Tyr 1059, Tyr 1175) and STAT3. However, the activation was greatly reduced in H-rasG12V-shPrx II cells compared with Huh7 H-rasG12V cells. Activated VEGFR2 may enhance the EMT and metastatic potential of surviving cancer cells [23]. EMT is a process by which the primarily E-cadherin-dependent cell contacts between contiguous epithelial cells break down [24]. The intermediate filament protein Vimentin, a requisite regulator of mesenchymal cell migration, is another important marker of EMT, regulating EMT induction via oncogenic H-Ras [25]. As shown in Figure 4B, E-cadherin expression was lower, however, vimentin was increased in Huh7-H-rasG12V-shPrx II cells.

Figure 4. Cancer stem cell traits in Huh7-H-rasG12V cells. (A): The expression levels of VEGFR2/STAT3. (B): The expression levels of stemness and epithelial-to-mesenchymal transition markers in Huh7-H-rasG12V and Huh7-H-rasG12V-shPrx II cells. (C): Sphere formation in Huh7-H-rasG12V and Huh7-H-rasG12V-shPrx II cells cultured for 7 days (magnification, × 100). Bar represents 100 microns. (D): Flow cytometric analysis of DCF-DA, annexin V, EpCAM, and CD133 in Huh7-H-rasG12V and Huh7-H-rasG12V-shPrx II cells. (E): Migration assay of Huh7-H-rasG12V and Huh7-H-rasG12V-shPrx II cells. (F): Invasion assay of Huh7-H-rasG12V and Huh7-H-rasG12V-shPrx II cells. The data are the mean ± SEM (n = 5 per group), *p < .05, **p < .01. Abbreviations: DCF-DA, 2',7'-dichlorodihydrofluorescein diacetate; EpCAM, epithelial-cell adhesion molecules; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Prx II, peroxiredoxin II; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor.
H-rasG12V cells compared with mock cells. By contrast, H-rasG12V-shPrx II cells increased E-cadherin and decreased Vimentin. Recent evidence has highlighted a link between EMT and the CSCs that initiate and maintain tumors [26]. As shown in Figure 4C, sphere formation in 7 days of culture increased in Huh7-H-rasG12V cells compared with mock cells, indicating that Huh7-H-rasG12V cells possess a significant portion of cancer stem cells. In these cells, the expression of stemness-related transcription factors, Nanog, Sox2, and Oct3/4, increased. However, sphere formation and the expression of stemness markers diminished in Huh7-H-rasG12V-shPrx II and SK-HEP-1-H-rasG12V-shPrx II cells (Fig. 4B, 4C and Supporting Information Fig. S3A). Additionally, downregulation of Prx II significantly increased ROS and apoptosis compared with control cells (Fig. 4D and Supporting Information Fig. S3B). When we examined wound healing and invasion activities, knockdown of Prx II significantly reduced both activities in Huh7-H-rasG12V cells (Fig. 4E, 4F). We next investigated the tumor-initiating ability of xenograft with Huh7-H-rasG12V and H-rasG12V-shPrx II cells. Huh7-H-rasG12V cells formed tumors in two of five nude mice at 1 x 10^3 cells per injection, four of five at 1 x 10^4 cells per injection, and five of five 1 x 10^5 cells per injection. In contrast, H-rasG12V-shPrx II cells formed tumors in one of five nude mice at 1 x 10^4 cells per injection and four of five 1 x 10^5 cells per injection (Table 1). Consequentially, knockdown of Prx II significantly inhibited tumor-initiation ability (Fig. 5A). Furthermore, immunohistochemical data also showed that immunoreactivity to Sox2, Vimentin, and Prx II decreased more markedly in tumors of Huh7-H-rasG12V-shPrx II cells than those of Huh7-H-rasG12V cells (Fig. 5B). These data suggest a role for Prx II as a positive regulator of EMT and stemness.

**Prx II Regulated CSCs Self-Renewal Through VEGFR2/STAT3 Signaling by VEGF Stimulation in H-rasG12V Induced HCC Cells**

It was demonstrated that Ras signaling in VEGF2-mediated endothelial specification of vascular progenitor cells provides novel insights into temporal aspects of signaling for cell lineage specification through widely shared effector molecules [27]. We examined the expression of VEGFR1 and VEGFR2, and knockdown effect of VEGFR1 and VEGFR2 in Huh7-H-rasG12V cells. As shown in Figure 6A, VEGFR1 and VEGFR2 were expressed in Huh7-H-rasG12V cells. Knockdown of VEGFR2 greatly attenuated VEGF-mediated Prx II expression and inhibited Sox2, but not in the cells knocked down VEGFR1. Furthermore, VEGFR2 was required for VEGF-mediated sphere formation (Fig. 6B). Immunoprecipitation (IP) of VEGFR2 showed VEGF stimulated recruitment of Prx II (Fig. 6C). VEGF pretreatment showed significantly more increased sphere-cell frequency and sphere size in H-rasG12V HCC cells than VEGF nontreatment (Fig. 6D and Supporting Information Fig. S4A). Taken together, VEGF induces Prx II expression to activate VEGFR2, leading to CSCs self-renewal in H-rasG12V HCC cells.

**DISCUSSION**

Hepatocellular CSCs that are important for early detection remain poorly understood, including the precise cell(s) of origin, molecular genetics, and the mechanisms responsible for the highly aggressive clinical picture of HCC [28]. Exploration of the difference between CSCs from normal stem cells is crucial not only for understanding of tumor biology but also for development of specific therapies that effectively target these cells in patients [28]. Prx II might play an important role in hepatocarcinogenesis and might be used as a molecular target for HCC prevention and treatment [29]. Cell proliferation and clone formation clearly decreased when Prx II expression was inhibited, while flow cytometry analysis showed the percentage of cell apoptosis was enhanced. Inhibition of Prx II expression also obviously increased generation of ROS and malondialdehyde; both are products of peroxidation [30], thereby implying the important role of Prx II in
hepatocarcinogenesis, possibly through its function in regulating peroxidation and providing a favorable microenvironment for cancer-cell survival and progress [30]. Prx II actively participates in the maintenance of redox balance in preventing hepatic cancer cells from oxidative damages causing cell apoptosis and cell-cycle arrest, suggesting that Prx II may also play a role in HCC CSCs. In this study, we demonstrated that Prx II may play an essential role in HCC CSC survival. We investigated the involvement of Prx II in stemness-related positivity on HCC patients. In previous reports, EpCAM and K19-positive HCCs showed significantly increased EMT-related protein expression, suggesting they may acquire more invasive characteristics, compared with K19-negative HCCs, through the upregulation of EMT-associated genes in HCC [31]. Prx II was highly expressed in EpCAM(+) /K19(+) HCCs. We hypothesized that Prx II specifically regulates CSC self-renewal of HCC cells from ROS. Downregulation of Prx II in Huh7 cells with treatment of siRNA reduced expression of EpCAM and CD133 as well as Sox2 in accordance with increased ROS and apoptosis, which were reversed in Huh7h-Prx II cells. In addition, sphere formation was remarkably larger in Huh7-hPrx II cells than in mock cells.

It is well-established that overexpression of activated Ras proteins can increase intracellular ROS content by several mechanisms affecting their generation and detoxification [21, 32]. The Ras gene family encodes small (20–22 kDa) proteins that link cell-surface receptors to intracellular effector pathways that regulate cell growth, proliferation, differentiation, and survival [33]. H-Ras with a mutation at codon 12 is frequently observed in human cancers [34]. In human urothelial...
In these tumors, the activated mutant H-Ras protein contributes to cellular transformation and promotes tumorigenesis accompanied by aberrant regulation of the complex-signaling circuitry [34]. We confirmed overexpression of Prx II in Huh7-H-rasG12V and SK-HEP-1-H-rasG12V cells, and a role for Prx II as a positive regulator of EMT and stemness. Also, immunohistochemical data also show that immunoreactivity to Sox2, Vimentin, and Prx II greatly decreased in tumors of Huh7-H-rasG12V-shPrx II cells compared with Huh7-H-rasG12V cells. Therefore, Prx II enhanced self-renewal activity of CSC in HCC-H-rasG12V cells.

Furthermore, we demonstrated that Prx II expression is associated with CSC from VEGF signaling. VEGF has been shown to play a particularly important role in tumor angiogenesis and is the target of several anticancer medications [35]. Targeting VEGF has been shown to be useful in treatment of several cancers including colon cancer and ovarian cancer [36]. VEGFR2 is a tyrosine-kinase receptor essential for VEGF mediated physiological and pathological responses in endothelial cells [22]. Although initially thought to be exclusively expressed by endothelial cells, VEGFR2 is also expressed by different cancers and VEGF/VEGFR2 can act via both autocrine and paracrine mechanisms to drive cancer-cell proliferation and survival [19]. VEGFR2 plays an important role in cancer growth through distinct mechanisms, a direct effect on certain tumor cells that express VEGFR2 and a pro-angiogenic effect on the vasculature supplying nutrients to the tumor [37]. VEGF signaling through VEGFR2 uses ROS and is subjected to redox regulation, whereas waiting for additional experimental confirmation may help to explain pre-existing evidence of inhibition of angiogenesis by antioxidants [38]. Moreover, Prx II regulates the VEGFR2 oxidation mechanism, and represents a potential antiangiogenic target. The in vivo data clearly demonstrate that the Prx II-deficient VECs are defective in angiogenesis in aggressive tumors [18]. These studies showed that VEGFR2 expression was not changed significantly after induced by VEGF, but phosphorylations of VEGFR2 and STAT3, and the expression of stemness markers were significantly decreased in Huh7-H-rasG12V-shPrx II cells compared with Huh7-H-rasG12V cells. Therefore, downregulation of Prx II significantly increased the levels of ROS and apoptosis compared with Huh7-H-rasG12V and SK-HEP-1-H-rasG12V cells. We confirmed that Prx II is involved in sphere formation and migration by targeting VEGFR2 activation in HCC-H-rasG12V cells. In breast- and lung-cancer models, VEGF-bound VEGFR-2 recruits STAT3 to stimulate STAT3-dependent induction of Sox2 [19]. VEGF stimulates auto-phosphorylation of VEGFR2 to activate Prx II [18]. Following that, RAS activation induced Bmi-1 [22] and STAT3 signaling to Sox2 in HCC-H-rasG12V.

**CONCLUSION**

In conclusion, our data provided novel evidence that CSC self-renewal through VEGF signaling is regulated by Prx II, protecting oxidative inactivation of VEGFR2 in HCC cells. Taken together, our data suggest that Prx II plays a key role in the CSC self-renewal of HCC cells through redox regulation.

**ACKNOWLEDGMENTS**

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**AUTHOR CONTRIBUTIONS**

T.K. and D.Y.Y.: conception and design, assembly and analysis of data, manuscript writing, and final approval of manuscript; Y.B., Y.H.P., G.B.J., J.S.N., J.E.Y., Y.N.P., I.S.B., and J.M.K.: performed the experiments. All authors reviewed the manuscript.

**DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST**

The authors indicate no potential conflicts of interest.
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