**MASTER's THESIS**

# Targeting Peroxiredoxins enhances anti-cancer drug effects by suppressing stemness properties

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# Targeting Peroxiredoxins enhances anti-cancer drug effects by suppressing stemness properties

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# SUMMARY

Different natural plant extracts, extracted from herbal plants, are gaining global attention as successful therapeutic strategies for several types of cancers as Understanding the underlying mechanisms of cancer progression and resistance to drugs may provide new insights into successful clinical management of colon cancers by overcoming this challenge.

Therefore, first we concluded that Shikonin, one of natural plant extracts, induces colon cancer cell apoptosis by inhibiting mTOR signaling and overexpression of Prx V can abrogate the apoptosis-inducing effects of Shikonin by regulating mTOR signaling. Even though these effects of Prx V need to be studied deeply in detail in future studies, our results provide new insight into understanding the underlying mechanisms which may allow advantage in targeting colon cancer cellsfor abetter clinical management of colon cancer.

Also, we summarized that stemness properties of A549/GR CSCs can be critically affected by the overexpression of Prx II. Also, miR-122 can abolish the stemness characteristics induced by Prx II by directly targeting Prx II. Thus, the approaches to restore miR-122 may effective for therapeutic management of NSCLCs

# 초록

약초식물에서추출한다른천연식물추출물은암진행의기본메커니즘을이해하고약물에대한내성을 이해함으로써이러한도전을극복함으로써대장암의성공적인임상관리에대한새로운통찰력을제공 할수있기때문에여러유형의암에대한성공적인치료전략으로서세계적으로주목을받고있습니다.

따라서, 먼저천연식물추출물중하나인Shikonin이 mTOR 신호전달을억제함으로써결장암세포아 pop 토시스를유도하고 Prx V의과발현은 mTOR 신호전달을조절함으로써Shikonin의아 pop 토시스 -유도효과를제거할수있다고결론지었다. 비록 Prx V의이러한효과가향후연구에서심층적으로연구 될필요가있지만, 우리의결과는대장암세포의표적화에있어대장암의더나은임상관리를가능하게할 수있는기저메커니즘을이해하는새로운통찰력을제공합니다.

또한, 우리는 A549 / GR CSCs의 stemness 속성 Prx II의 overexpression에의해결정적으로영향을받 을수있습니다요약. 또한, miR-122는 Prx II를직접표적화함으로써 Prx II에의해유도된줄기특성을 제거할수있다. 따라서, miR-122를회복시키는접근법은 NSCLC의치료관리에효과적일수있다

# 1. CHAPTER 1

# Overexpression of Peroxiredoxin V reverses apoptotic effects of Shikonin on colon cancer cells

## 1.1 ABSTRACT

Colon cancer is ranked as the second most common cause of deaths among deadly malignancies worldwide.Clinical management of colon cancer may improve with a better understanding of its mechanismsbehind the resistance to present drugs. At current, various natural plant extracts with herbal value is gaining global attention as the development of new drugs against almost all cancers including colon cancers. Shikonin, a naturally extracted naphthoquinone pigment,considers as one of those extracts. In this study, it showed the apoptotic effects of Shikonin on colon cancer cells and the abrogation of those effects by Prx V overexpression via a series of in vitro experiments. Prx V over expressed colon cancer cell line was constructed to observe the involvement of Prx V in Shikonin-induced. In the results, colon cancer cell apoptosis was induced by Shikonintargeting rapamycin signaling whereas those effectswere abrogated by overexpression of PrxV. Therefore,the targeting of PrxV might provide new way for a successful clinical management of colon cancer by sensitizing colon cancer cells to drugs and thereby inducing cell apoptosis.

## 1.2 INTRODUCTION

The resistance of colon cancers to existing therapies depends on their metastatic stage. Due to this resistance, it has become the second number two among cancer-related death worldwide.Therefore, successful clinical management of colon cancers requires an urgent development of more efficient therapeutic strategies (1).Understanding the underlying mechanisms of colon cancer progression and resistance to drugs may provide new insights into successful clinical management of colon cancers by overcoming this challenge.Different natural plant extracts, extracted from herbal plants, are gaining global attention as successful therapeutic strategies for several types of cancers (2, 3). Shikonin, purified from *Lithospermumerythrorhizon*, is a natural naphthoquinone pigment compound with medicinal value (4). Shikonin is already known for itsanti-cancer activities against numerous cancer types (5), including non-small cell lung cancer (4), nasopharyngeal carcinoma (6), and ovarian carcinoma (7).Moreover, the effects of Shikonin against cancers have been studied previously oncolon cancer cells by inducing apoptotic cell death (8-11).Also, Shikoninis a regulator of various signaling pathways including PTEN/AKT/mTOR signaling in endometrioid endometrial cancer cells (12), JNK and STAT3/AKT pathways in non-small cell lung cancer(13) and ERK/JNK/MAPK/AKT pathway in leukemia cells (14).Other than that, as shown in previous studies, Shikonin is a natural inducer of ROSwhich leads the apoptotic cell death in cancer cells (8).Even though, it has identified that Shikonin is inducing the apoptotic cell death, the underlying mechanisms are yet to be studied.Therefore, in our present study, we have focused on the antioxidant enzyme system of colon cancer cells in order to reveal possible mechanisms behind Shikonin-induced apoptotic cell death.

## 1.3 MATERIALS AND METHODS

### 1.3.1 Creation of required stable cell lines and maintenance

DMEM medium (Invitrogen, Carlsbad, CA, USA) containingpenicillin (100 U/ml), streptomycin (100 mg/ml),and10% fetal bovine serumwas used to maintain the all cell lines including HCT116, HT29, HT29 Mock, and Prx V-overexpressing HT29 cells (HT29 His-PrxV). An incubator at 37˚C with 5% humidified CO2 was used to maintain the cells. The establishment of HCT116 Mock, HCT116 shPrxV and HCT116 His-PrxV cell lines were similar to the process which has been described in our previous study (17).

### 1.3.2 Apoptosis assay and cell viability assay

$5\times10^3$of colon cancer cells/wellwas seeded in 96-well plates. EZ-Cytox Kit(DoGenBio, Korea) was utilized to study the cell proliferation of colon cancer cells including HCT116, HT29, HT29 Mock and HT29 His-PrxV after 24hrs treatment with Shikonin (0, 0.1, 0.5, 1, 5, 10, 15 and 20 μM) . The protocol was followed as the instructions mentioned by manufacturer. The absorbance was measured at 450 nm. The Annexin V Detection Kit with propidium iodide was used for the detection of apoptosis,as the manufacturer mentioned. Data was analyzed by FACS analysis (FACSCalibur; BD Biosciences).

### 1.3.3 Western blotting

HT29 and HCT116 cells treated with Shikonin or cells remained untreated as controls were lysed in protein lysis, RIPA, buffer containing 1% Nonidet p-40, 50 mMTris,150 mMNaCl, , Ph8.0, and a protease inhibitor (PI) cocktail. Same concentration of protein from all the samples was separated by sodium

dodecyl sulfate–PAGE. Those proteins were then transferred to nitrocellulose membranes (Bio-Rad, California,USA). All western blots were performed with mouse or rabbit antibodies against PrxI, PrxII(AbFrontier, Seoul, South Korea), PrxIII, PrxIV, PrxV, PrxVI, mTOR, pmTOR, AKT, pAKT, pERK, p38, pP38, and His-Prx V. GAPDH was utilized as the internal control of our western data. Super Signal West Pico PLUS Chemiluminescent Substrate was used to observe the expression levels of required proteins (Thermo Fisher, CA, USA).

**DCF-DA (1.3.4 2',7'-Dichlorofluorescin diacetate), DHE (dihydroethidium) and JC-1 assays**

HT29 and HCT116 cells treated with Shikonin in a dose-dependent manner or not-treated were used to study the changes in ROS levels by Shikonin treatment using DCF-DA (Invitrogen). Treated and not-treated cells were separatelyincubated with 20 mM of DCF-DA, DHE and JC-1at 37˚C for 15 min. 1× PBSwas used to wash cells before taking the images by microscopy. Fluorescence intensities were noted qualitatively.

### 1.3.5 Transwell invasion and migration assays

24well Transwell chambers with 8.0-μM pore polycarbonate membraneswere used for these assays with (invasion) or without (migration) Matrigel. HCT116 and HT29 cell suspensions in medium containing 0.5% FBS 200-μl were added separately to the upper chambers (1×105 cells/chamber). The bottom chambers were filled with 800 μl of medium, supplemented with 20% FBS (or not) as chemo-attractant. All the chambers with cells were then incubated at 37˚C for 24 h in5% CO2 incubator. Cells which passed through membranes (coated or non-coated)werefixed with 4% paraformaldehyde. Finally, migrated or

invaded cells werestained with 0.1% crystal violet for 1 h before capture the images under a microscope. Images were captured and the cell numbers were counted.

### 1.3.6 ICC assay

Various concentrations of Shikonin were added to cells or kept untreated as controls. After treated for required time period, 3.7% Formaldehyde was used to fix cells at room temperature for 10 min. Fixed cells were then blocked with 1X PBS containing 1% bovine serum and 0.5% Triton ×-100 at room temperature for 60 min.Primary antibody against mTOR and pmTOR incubation was longed for 18 hrs. Next day, the cells were washed with 1× PBS containing Tween 20.Then cells were incubated with secondary antibodies for 2 h in a dark place at room temperature. 20 minutes of DAPI staining was used to visualize Nuclei. Nuclear Cells were then observedunder a microscope. Fluorescence intensities of cells from treated and non-treated groups were compared.

### 1.3.7 Colony-formation assay

1×103 cells/well HT29 and HCT116 cells were cultured in 6 well plates. Those cultured cells were treated with Shikonin in concentration-dependent manner. Treated or non-treated cells were maintainedfor 7 days in a 37˚C incubator with 5% CO2. After 7 days, cells were then fixed with 3.7% formaldehyde for 10 min after washing with 1×PBS. Those cells were then permeabilized with 100% Methanol for 20 min. Crystal violet was used to stain cells for 30 min.Images were captured after plates washing3 times with 1× PBS before capturing images. Counts of colonies in treated and non-treated groups were compared.

### 1.3.8 Sphere-formation assay

2×103 cells/well of HCT116 and HT29 were plated in six-well Ultra Low Cluster plates. Those cells kept culture 10 days in suspension of serum-free DMEM/F12 with B27 supplement, 20ng/ml epidermal growth factor, and 0.5% BSA. After 10 days, images of spheres were taken by inverted microscope and sphere number was counted. Number of colonies per input cells ×100% shows the efficiency of sphere-formation. Spheres were subjected to ICC in required casesasdescribed above in the section ofICC.

### 1.3.9 Statistical analysis

Data isshowed as the mean±SEM. Prism 4.0 software was used to perform Student's t-test, and significantly different values were *$p<0.05$.

## 1.4 RESULTS

### 1.4.1 Shikonin down regulates themalignancy and proliferation of colon cancer cells

In our study, first,, we confirmed the effects of Shikonin on colon cancer cells as mentioned in previous studies. According to literature, Shikonin leads ROS-based mitochondria-induced apoptosis of colon cancer cells (11).Therefore;we aim to study the effects of Shikonin on colon cancer cells.First HCT116 and HT29 cells were treated with Shikonin. Those cells were then used forthe studying of cell viability. As shown in Figure 1.1, cell viabilities of both colon cancer cells were decreased with dose-dependent Shikonin treatment. Effect of Shikonin was more prominent and observable in HT29 cells compared tothe HCT116 cells.

After observing the Shikonin effects in cell viability assay, we have designed our further experiments related to four different concentrations of Shikonin including 0, 5, 10 and 20 μM.As shown in Figure 1.2C, Sphere-forming ability was inhibited by Shikonin treatment in both colon cancer cell lines in dose-dependent manner. Similarly, the colony forming ability also was reduced with Shikonin treatment as observed by colony formation assay. Therefore, those results showed that Shikonin inhibited the cell proliferation of colon cancer cellswithincreasing concentration (Figure 1.2D). Also, as Figure 1.2E and F shows, Shikonin diminished the migration and invasion abilitiesof colon cancer cells. Taken together, our data confirmed that Shikonin inhibits colon cancer cell properties in a dose-dependent manner.

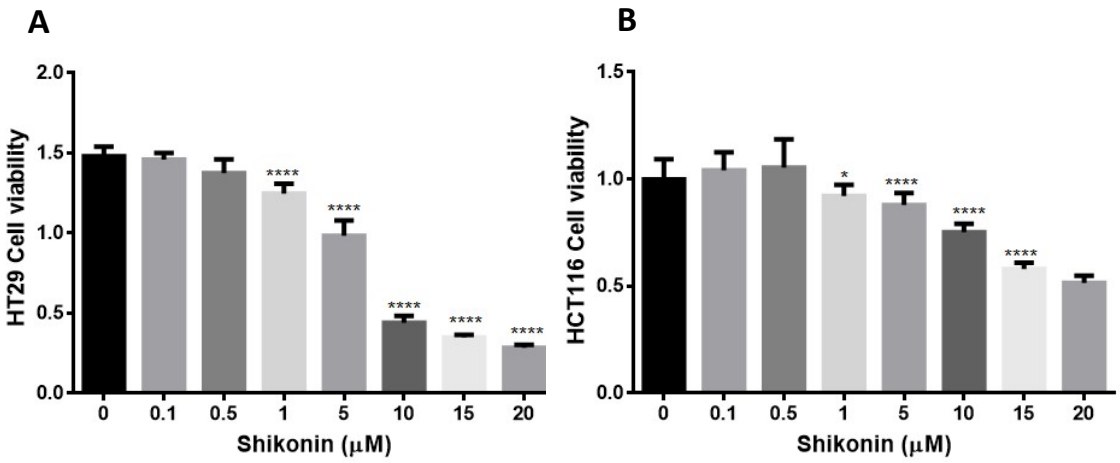


**Figure 1.1.concentration dependent effects of Shikonin on two colon cancer cell lines**

(A) Cell viability of HT29 colon cancer cells (B) Cell viability of HCT116 cells after treated with different concentrations of Shikonin treatment.

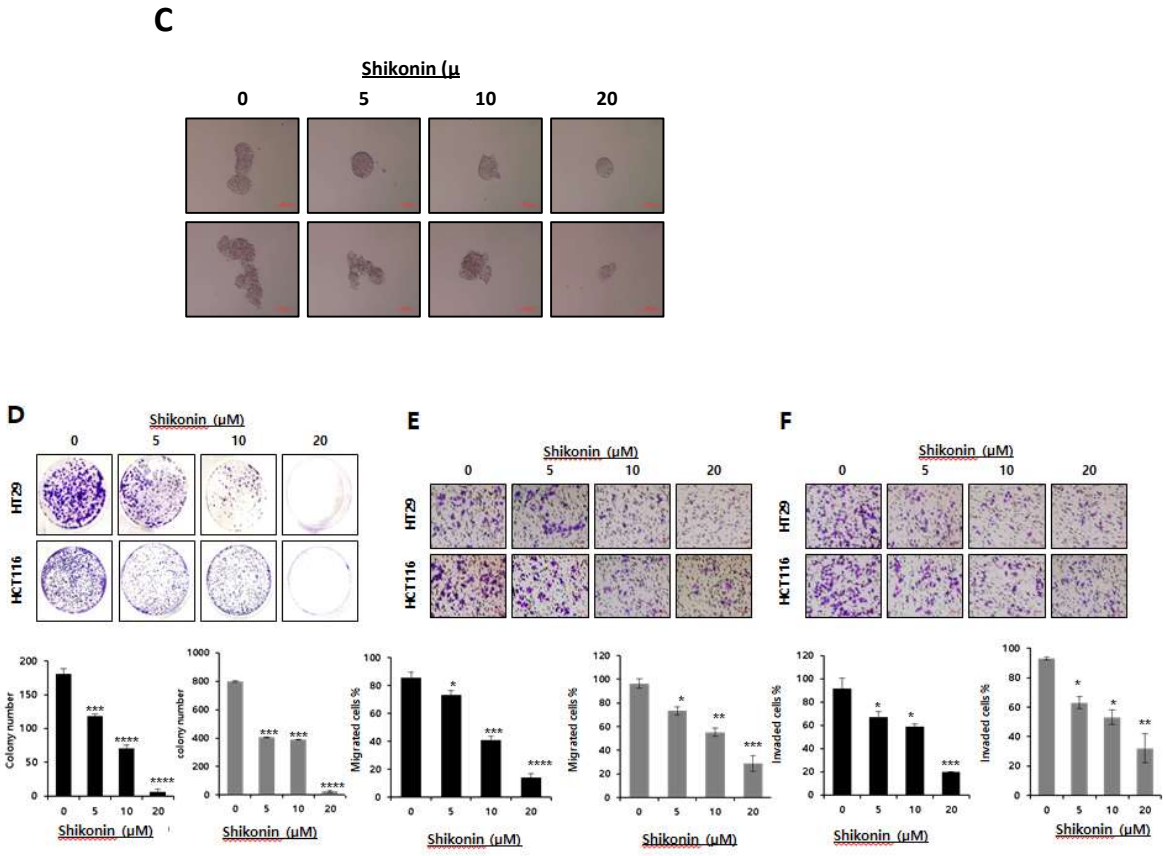


**Figure 1.2. Shikonin shows inhibitory effects on colon cancer cells**

(C) Ability to form spheres in HT29 and HCT116 cells was reduced by increasing dose of Shikonin. (D) Changes in colony forming ability of HT29 and HCT116 cells after treated with different concentrations of Shikonin. (E) Migration and (F) invasion assays which show the effects of Shikonin on cell mobility.Differences with the values of *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 bars represent 100 microns. p<0.05 values were considered statistically significant. Data is presented as the mean ± standard error.

### 1.4.2 Shikonin controls theoxidative state and antioxidant enzyme system of colon cancer cells

Then, we have focused about the effects of Shikonin on oxidative stress. Similarly, we studied the relationship betweenShikonin and antioxidant enzymes in colon cancer cells. According to previous studies, Shikonin is a natural plant extract which enhances the cisplatin-induced colon cancer cell apoptosis as a natural inducer of ROS [8]. Moreover, Shikonin has been known for its involvement in oxidative stress of various cells including cancer cells (18-20). First, we have conducted JC-1 staining as shown in Figure 1.3A,to check Shikonin's effects on colon cancer cell apoptosis, mitochondrial potential and mitochondrial ROS level. Also, we performed DCF-DA staining (Figure 1.3B) to observe the effectsof Shikonin on ROS level in colon cancer cells. JC-1 isa membrane-per meant dye which widely uses to identifyhealthy mitochondria, mainly in apoptotic studies. Disruption of mitochondria is considered to be a one of first stages of apoptosis. Also, the changesin oxido-reductive state of cells by altering the ROS considers as another stage of apoptosis. JC-1 staining is capable toidentify those changes in cells.Thus, our results implied that Shikonin inducesROS generation in a dose-dependent manner thereby inducing colon cancer cell apoptosis via induced oxidative stress.

Then,we also studied the Shikonin effects on antioxidant enzymes of colon cancer cells. Peroxiredoxin(Prx) family was selected as the candidate for further experiments. Prxs are already known as an important antioxidant enzyme in different cancer types, including colon cancer (21). Figure 1.3C, western blotting, revealed the non-observable effects of Shikonin on the expressions of some of Prx family members including PrxII, PrxIII and PrxVI. Interestingly, the PrxI expression was increased and PrxV expression was decreased withincreasing dose of Shikonin. These effects were significant in HT29 cells compared to HCT116 cells. Therefore, HT29 cells were selected for further experiments. Prx V expression in colon cancer tissues is higher than the expression of other Prx family members, as we showed before(16). We have designed our experiments with keeping this knowledge n our mind, to focus

on the effects of Prx V in Shikonin's effects on colon cancer cells.

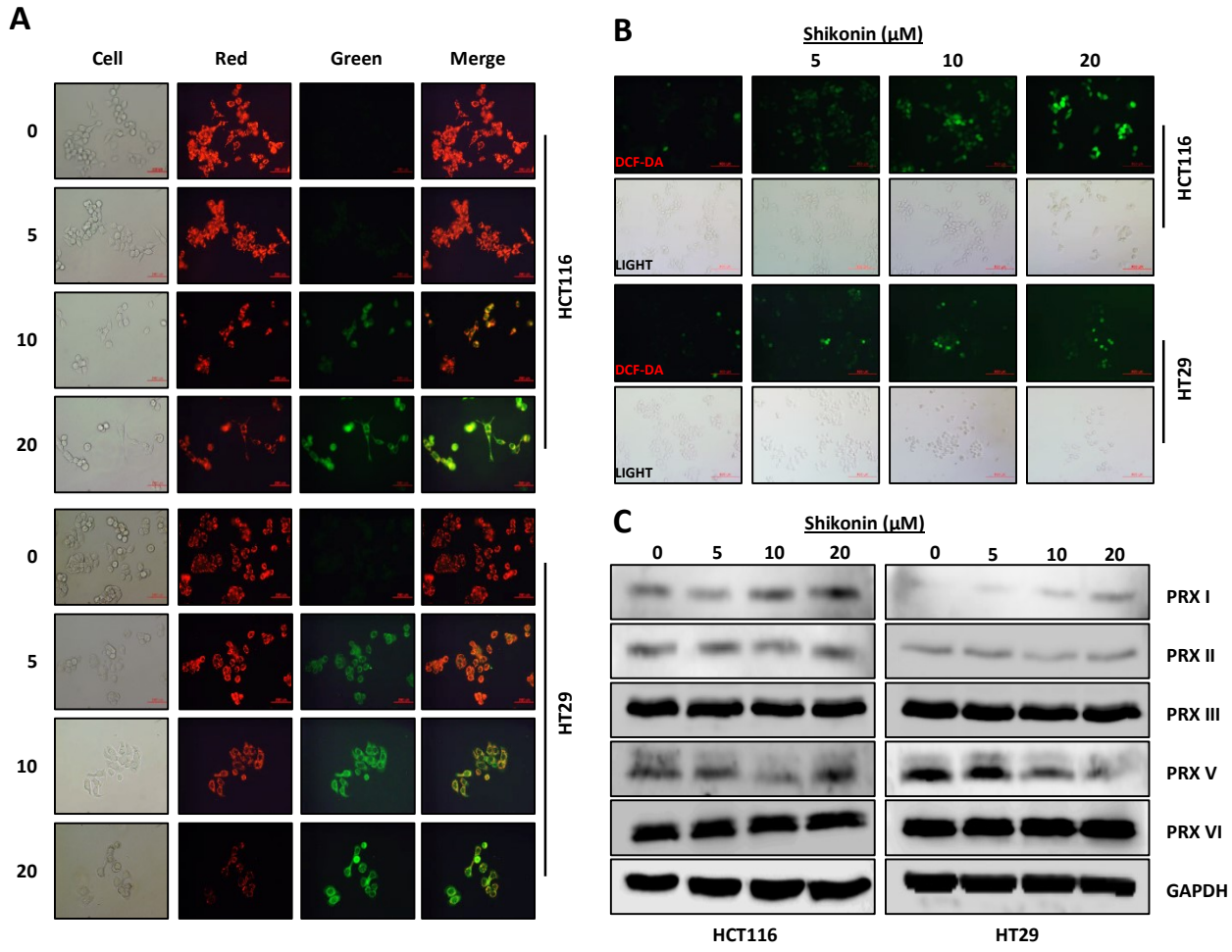


**Figure1.3. Shikonin modulates the oxidative status of colon cancer cells**

**(A)**Mitochondrial ROS level in HT29 and HCT116 cells with and without Shikonin treatment was compared by JC-1 staining.**(B)** 2',7'- Dichlorofluorescin diacetate (DCF-DA) staining further showing the effect of Shikonin on ROS levels of colon cancer cells. **(C)**Immunoblotting to show the dose-dependent effects of Shikonin on the expression of Prx family members in colon cancer cells.

### 1.4.3 MTOR signaling in colon cancer cells is regulated by Shikonin

Induced mTOR signaling has been reported to involve in the enhanced colon cancer cell progression (22). On the other hand, literature showed that the down regulation of mTOR signalinginhibits colon cancer cell growth (23). However, to our knowledge, the effects of Shikonin incolon cancer cells via modulating mTOR signaling cascade has not been studiedyet deeply.

Therefore, our main focus was about changes in mTOR signaling of colon cancer cells after treatment with Shikonin. Further, we have evaluated whether and how the inhibition of PrxV expression by Shikonin is related to mTOR signaling in colon cancer cells asshown in Figure 1.4A, western blotting data of HT29 cells treated with 0, 5, 10 and 20 μM of Shikonin.Though, the expression of mTOR was not reduced significantly, pmTOR expression was down-regulated significantly with increasing concentration of Shikonin.

Parallel, the expression of mTOR signaling pathway-related factors was observed by a series of western blotting. According to our results, Shikonin reduced pAKT and pERK expressions. But, the AKT expression was not changed with increasing treatment of various Shikonin concentrations. In Figure 1.4B, an ICCassay showed thatpmTOR expression of HT29 cells is reduced with increasing Shikonin concentration. These resultssuggestedthat Shikonin affects mTOR signaling in colon cancer cells. By strengthen our findings, Prx V overexpression of reverses Shikonin-induced colon cancer cell apoptosis and ROS level through the regulation of mTOR signaling. Previously, the results in Figure 1.3C in this study showed that Prx V level was reduced with treatment of Shikonin. Thus, we have examined whether it was related to Shikonin-induced colon cancer cell apoptosis. First of all, we established stable HT29 colon cancer cell lines with PrxV overexpression (His-PrxV) and control (HT29 Mock) as described in our previous studies (16, 17). Expression of Prx V in established cell lines was confirmed by immuno-blotting as shown in Figure 1.5A.

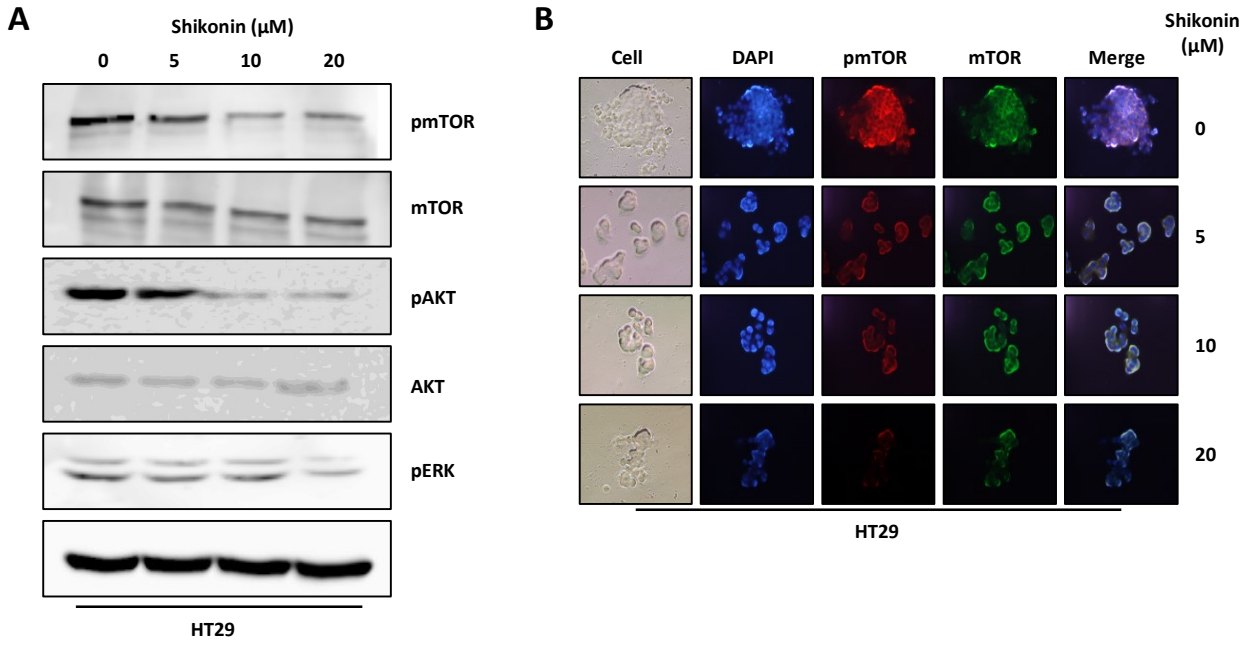


**Figure 1.4. Shikonin controls the mTOR signaling in HT29 cells**

(A) Western blotting shows dose-dependent Shikonin effects on mTOR and AKT signaling pathway-related factors. (B) ICC assay was conducted to confirm Shikonin effects on the mTOR and (p)mTOR expressions. Also, the expressions of AKT, ERK and GAPDH as internal control

According to MTT assay in Figure 1.5B, viability of Prx V over expressed cells was increased by with increasing concentrations of Shikonin compared to HT29 Mock cells. Moreover, Figure 1.5C and 1.5D, showed that Prx V overexpression reduces the apoptotic cell population by FACS analysis of cell apoptosis and DHE staining.In Figure 1.6E, immuno blotting data showed the reversed effects of Shikonin on the expression of pmTOR level in HT29 cells by Prx V over expression, while no detectable changes were observed in mTOR expression. Moreover, the Prx V overexpression did not influence significantly to the expression of other mTOR signaling-related factors. Altogether, Results un-wrapped that Shikonin induces HT29 cell apoptosis through the regulation of mTOR signaling pathway. Interestingly, the over expression of Prx V can reverse those effects.

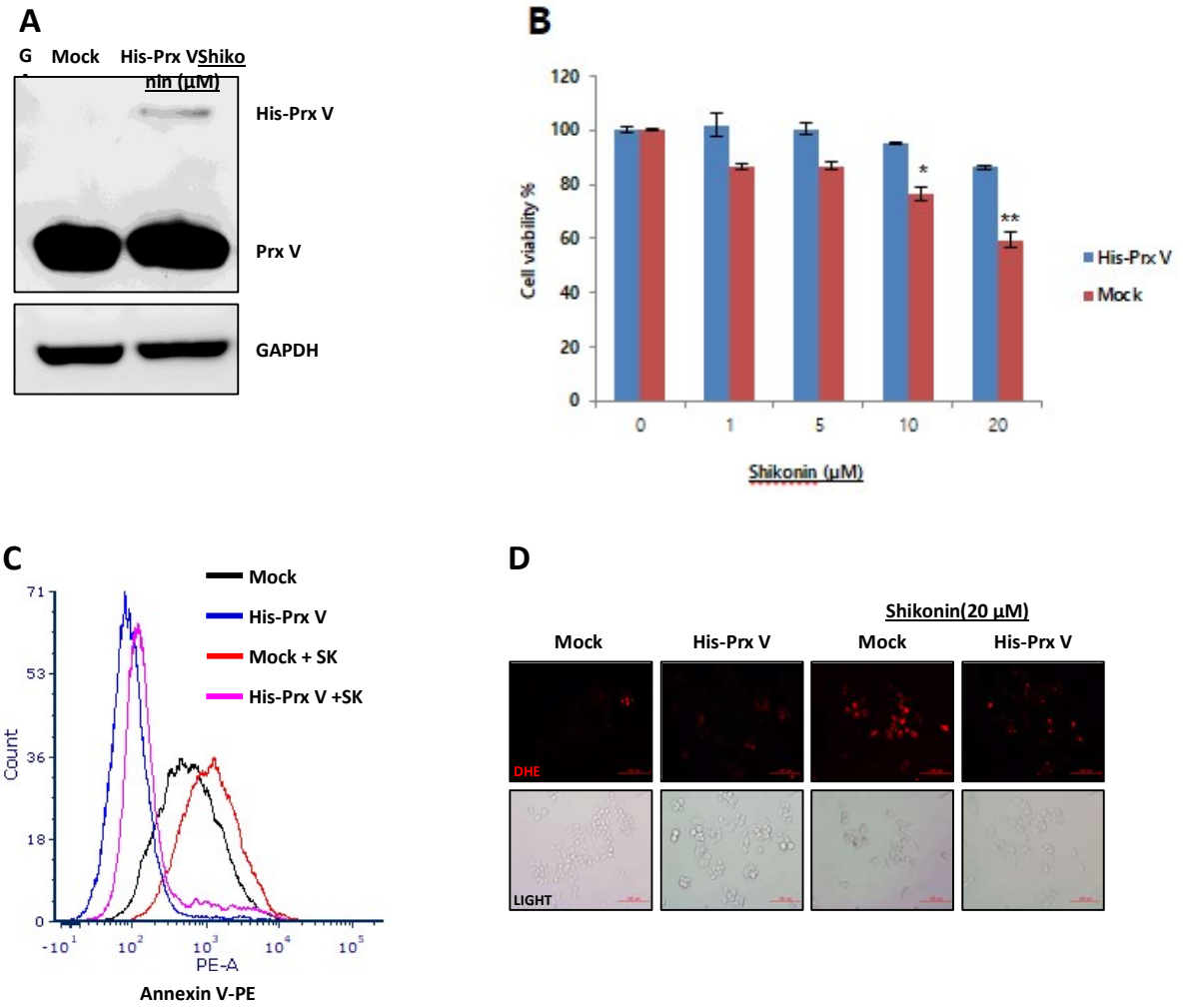


**Figure 1.5.Prx Vover expression inversely relates with Shikonin-induced cell apoptosis**

(A) Confirmed the expression levels of Prx V in stably constructed HT29 cells by western blot analysis. (B) Compared the cell viabilities of His-Prx V cells and Mock HT29 cells by MTT assay. (C) FACS analysis of cell apoptosis. (D) Checked the effects of Prx V on colon cancer cell apoptosis induced by ShkoninvaDihydroethidium (DHE) staining.

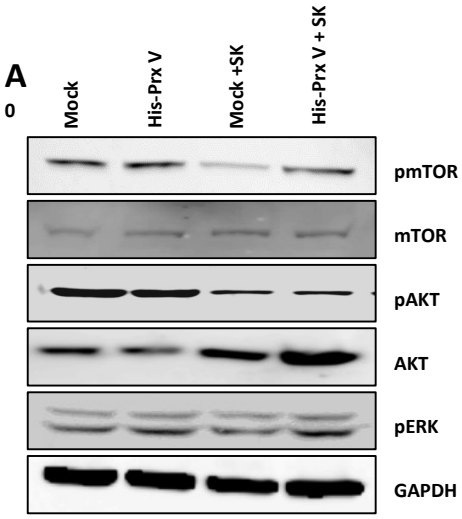


**Figure 1.6.Prx Vover expression directly targetsthe mTOR signaling in colon cancer cells.**

**A**: Highly expressed Prx V effects were observed onWestern on mTOR signaling and related factors, AKT, ERK, and GAPDH by western blotting.

## 1.5 DISCUSSION

During past several decades, the colon cancer was ranked as one of the predominant cancer types in the world, mainly in Western countries as their mortality rate was higher than other countries (24). At present, it isstayed in third place among the common malignancies worldwide (11).To date, the surgical resection is the main treatment method for colon cancers. But, the efficiency of this treatment method isreduced as a result of poor prognosis (9). Thus, on behalf of successful management of colon cancers, better and efficient therapeutic strategies should be identified.

Naturally extracted plant herbal compounds are gaining the global attention as agentswith effects against cancers. Those compounds already have been studied for various effects against several cancers (25). Shikonin is a natural naphthoquinone derivative, which has been already proved for its anticancer effects against several cancers (26). According to the literature, Shikonin sensitized colon cancer cellsradiotherapies by induced-generation of ROS. Therefore, Shikonin is considered as a natural inducer of ROS in cells (26).Furthermore, it also reported that colon cancer cell apoptosis can be induced by Shikonin viadifferent pathways which are having relationship with endoplasmic reticulum (ER) and mitochondria (9). Shikonin Otherresearch groups have confirmed that Shkonin induces colon cancer cell apoptosis via ROS-induced mitochondrial pathways (10, 11). According to a previous study, Shikonin had anticancer effects on thyroid cancer cells (27). When considering the effects of Shikonin on apoptosis of melanoma cells, it was reported that this involved ROS-mediated endoplasmic reticulum stress(28). Therefore, Shikonin is widely known to induce apoptosis of various cancer cells, including colon cancer.We also confirmed these effects of Shikonin on colon cancer cells through a series of in vitro experiments (Figures 1 and 2). The underlying mechanism of Shikonin induced apoptosis in colon cancer had not been studied.

Therefore, we focused on the antioxidant enzyme system in colon cancer cells by targeting the

Prx family. As we showed in our previous study, PrxV is expressed highly in colon cancer cells compared to normal colon tissues. Moreover, the expression level of PrxV is higher in colon cancer cells when compared to the expression of other Prx family members (17). In our data we found PrxV was downregulated by increasing concentrations of Shikonin. Therefore, we focused on PrxV to determine the underlying mechanism related to Shikonin-induced colon cancer cell apoptosis. Our previous study also showed that PrxV reduced apoptosis of colon cancer cells (16).

According to our results in this study, Shikonin inducedcolon cancer cell apoptosis via effects on mTOR signaling.It has been reported that the mTOR signaling pathway can induce colon cancer tumorigenesis (29). Furthermore, several studies have showed that antitumor effects can be enhanced by inhibiting mTOR signaling pathway in colon cancer cells (30, 31). Similarly, our results showed that Shikonin induced colon cancer cell apoptosis by down-regulating activation of mTOR signaling.

The role of PrxV was studied using HT29 His-PrxV and HT29 Mock cell lines with the treatment of Shikonin. Our results showed that the effects of Shikonin in inducing colon cancer cell apoptosis were abrogated by induced overexpression of PrxV.

## 1.6 CONCLUSION

We conclude that Shikonin induces colon cancer cell apoptosis by inhibiting mTOR signaling and overexpression of PrxV can abrogate the apoptosis-inducing effects of Shikonin by regulating mTOR signaling. Even though these effects of PrxV need to be studied deeply in detail in future studies, our results provide new insight into understanding the underlying mechanisms which may allow advantage in targeting colon cancer cellsforabetter clinical management of colon cancer.

# 2. CHAPTER 2

# Targeting Peroxiredoxin II sensitizes resistant lung cancer stem cells to Gefitinib treatment

## 2.1 ABSTRACT

Peroxiredoxin II is essentialfor A549 cell survival which is resistant to Gefitinib (A549/GR). Thus, we have utilized A549/GR cells for studying Prx II role in the stemness of NSCLCs. First, we explored the stemness related characteristics and then observed the involvement of Prx II in those characteristics;acell population,A549/GR CD133+, with stem-like cell features was successfully isolated from A549/GR cells. Those cells are withstemness related characters of cancers such as metastasis, self-renewal ability, angiogenesis, and the higher expression of stemness and EMT related markers. But, Prx II knockdowncanreversethose characteristics. Prx II in A549/GR CD133+ cells was directly targeted by microRNA 122.It results thediminishedin vitro and in vivo stemness characteristics. Then, we checkedhow Prx II and Prx-II-induced stemness characters are associated with the over expression of microRNA122. MiR-122 negatively regulated the A549/GR stemness by suppressing Wnt/β-catenin,Notchand Hedgehog signaling. Altogether, our results implied that Prx II induces A549/GR cellsstemness. Also, the targeting of either miR-122 or Prx II or both mayprovide a potent strategy for stem cell therapy of NSCLCs.

## 2.2 INTRODUCTION

Peroxiredoxins (Prxs) is an important family of antioxidant enzymes. Family members of Prx family can be divided into 3 sub-classes according to their conserved Cys residue number which participate in redox reaction. They are typical 2-Cys Prx (Prx I–IV), atypical 2-Cys Prx (Prx V) and atypical 1-Cys Prx (Prx VI) [32, 33]. Prx family members are frequently upregulated in several cancers as reported before, including lung, brain, mesothelioma, breast, prostate, cervical, colorectal cancer [34–39]. Among all the family members, Prx I, Prx II, Prx IV, and Prx VI are irregularly expressed in lung carcinomas. They have different effects on lung tumor progression, which considers as the main reason of deaths related to cancers worldwide [40]. In our previous project, the role of Prx II was showed in non-small cell lung cancer (NSCLC) cell line which is resistant to Gefitinib called (A549/GR). A549 cell line was repeatedly exposure to Gefitinib for establishing that cell line [38].

NSCLC considers as one of lung cancer histological subtypes and accounts for most lung cancer cases than other subtype [41]. Prx II has been reported to express aberrantly in NSCLCs. As reported, Prx II also associates with induced cell proliferation and tumor cell growth by activation of pJNK [38]. Moreover, other evidence has proved that stem-like properties of cancer can be maintained by Prx II. Further, Prx II induces colon cancer cell growth by activating Wnt/β-Catenin and Hedgehog (HH) pathways [42–44]. Prx II can involve in redox regulation thereby maintaining hepatocellular carcinoma (HCC) stem cells' stemness [45].

Cancer stem cells (CSCs) are responsible for the cancer metastasis, resistance to existing therapies and the progression of cancers [46]. In present study, we focused about the Prx II expression in GR resistant A549 cells. Also, our focusing was mainly directed towards the stemness properties of A549/GR stem cells

which can be modulated by Prx II. MicroRNAs (miRNAs), a group of small non-coding RNAs, have the capability to modulate the expression of tumor suppressors, oncogenes and various other genes thereby involve in cancer development [47]. At present, many researchers are willing to elucidate the functions of miRNAs in cancers. As a result, several studies have been focused on identification systems of miRNAs and their target genes related to cancer [48]. Among the screened miRNAs, miR-122 has been identified as one of tumor-suppressor genes in some cancers [49]. Furthermore, according to recent studies miR-122 targets oncogenes including Bcl- 2 and cyclin G1 thereby are diminishing the ability of tumor proliferation [49, 50]. MiR-122 also associates with the radio-sensitization and chemo sensitization of NSCLC cells as it showed that miR-122 overexpression can induces both sensitizations. Moreover, overexpression of miR-122 induces apoptosis and cell cycle arrest in NSCLC cells [50, 51]. Therefore, it can be concluded that studies in literature has already showed the possible miR-122 applications for NSCLC treatment. Importantly, Prx II in HCC can be directly targeted by miR-122 as shown in one study. According to that study, Prx II expression is downregulated by MiR-122 via binding to Prx II in HCC thereby inducing cell apoptosis and inhibiting cell growth [52]. Here, we investigated the expression of Prx II and the mechanistic links which can explain the role of Prx II in controlling CSC characteristics, such as cell proliferation, metastasis, angiogenesis and stemness in A549/GR CSCs. Also, we have showed how miR-122 inhibits the expression of Prx II. Therefore, our findings may open new approaches of the miR-122- mediated reduction of A549/GR CSC properties by inhibition of Prx II.

## 2.3 MATERIALS AND METHODS

### 2.3.1 Cell culture, generating stable cell lines and transfections

All the cell lines mentioned in the study were grown in RPMI 1640 supplemented with streptomycin (100 mg/ml), penicillin (100 U/ml) and 10% fetal bovine serum. Abovementioned same complete medium supplemented with 20 ng/ml basic fibroblast growth factor (hFGF) and 10 ng/ml of human epidermal growth factor (hEGF) was used to culture all the stem cell lines. pCMV-Prx II vector transfection and the establishment of shCON and shPrx II cell lines were as described in previous studies [38, 53]. As manufacturer described, Lipofectamine 2000 was used to transfect a DNA plasmid to express MiR-122. Cells which were positive for GFP selected for further experiments. CSCs were separated using MACS by labeling with an anti- CD133 antibody as manufacturer mentioned. Western blot was used to detect the purity of CSCs sorted by MACS.

### 2.3.2 Cell proliferation and apoptosis assays

5×103 of colon cancer cells/well was seeded in 96-well plates.EZ-Cytox Kit (DoGenBio, Korea) was utilized to study the rolesof miR-122 and Prx II in the proliferation ofA549/GR CSC.The protocol was followed as the instructions mentioned by manufacturer. The absorbance was measured at 450 nm. The Annexin V Detection Kit with propidium iodide was used for the detection of apoptosis, as the manufacturer mentioned. Data was analyzed by FACS analysis (FACSCalibur; BD Biosciences).

### 2.3.3 Western blot analysis

Cells were lysed in protein lysis, RIPA, buffer containing 1% Nonidet p-40, 50 mMTris, 150

mMNaCl, ,Ph8.0, and a protease inhibitor (PI) cocktail. Same concentration of protein from all the samples was separated by sodium dodecyl sulfate–PAGE. Those proteins were then transferred to nitrocellulose membranes (Bio-Rad, California, USA). All western blots were performed with mouse or rabbit antibodies against β-Catenin, Prx II,Gli-1, CD133, Vimentin, Hes-1, Nanog, Sox2, VEGFR2, E-cad, Shh, Notch 1, CXCR4, STAT3,pSTAT3 (Tyr 705), and pSTAT3 (Tyr-727).Super Signal West Pico PLUS Chemiluminescent Substrate was used to observe the expression levels of required proteins (Thermo Fisher, CA, USA).

### 2.3.4 DCF-DA assay

A549/GR shPrx II and shCON cells and cells transfected with miR-NC or miR-122 were used to study the changes in ROS levels.Cells seperately incubated with 20mM of DCF-DA at 37 °C for 15 min.1× PBSwas used to wash cells before taking the images by microscopy. Fluorescence intensities were noted qualitatively.

### 2.3.5 Transwell assays

24well Transwell chambers with 8.0-μM pore polycarbonate membraneswere used for these assays with (invasion) or without (migration) Matrigel.Cell suspensions in medium containing 0.5% FBS 200-μl were added separately to the upper chambers (1×105 cells/chamber). The bottom chambers were filled with 800 μl of medium, supplemented with 20% FBS (or not) as chemo-attractant. All the chambers with cells were then incubated at 37˚C for 24 h in5% CO2 incubator. Cells which passed through membranes (coated or non-coated)werefixed with 4% paraformaldehyde. Finally, migrated or invaded cells

werestained with 0.1% crystal violet for 1 h before capture the images under a microscope. Images were captured and the cell numbers were counted.

### 2.3.6Sphere-formation and ICC assays

2×103 cells/well of were plated in six-well Ultra Low Cluster plates. Those cells kept culture 10 days in suspension of serum-free DMEM/F12 with B27 supplement, 20ng/ml epidermal growth factor, and 0.5% BSA. After 10 days, images of spheres were taken by inverted microscope and sphere number was counted. Number of colonies per input cells ×100% shows the efficiency of sphere-formation. Spheres were subjected to ICC in required cases asdescribed above in the section ofICC.

### 2.3.7ICC assay

3.7% Formaldehyde was used to fix cells at room temperature for 10 min. Fixed cells were then blocked with 1X PBS containing 1% bovine serum and 0.5% Triton ×-100 at room temperature for 60 min.Required primary antibody incubation was longed for 18 hrs. Next day, the cells were washed with 1× PBS containing Tween 20.Then cells were incubated with secondary antibodies for 2 h in a dark place at room temperature. 20 minutes of DAPI staining was used to visualize Nuclei. Nuclear Cells were then observed under a microscope. Fluorescence intensities of cells from treated and non-treated groups were compared.

### 2.3.8Colony-formation assay

1×103 cells/wellA549/GR CD133+ or A549/GR CD133shPrx II, shCON, miR-NC or miR-122transfected cells were cultured in 6 well plates. cells were maintainedfor 7 days in a 37˚C incubator with 5% CO2.

After 7 days, cells were then fixed with 3.7% formaldehyde for 10 min after washing with 1×PBS. Those cells were then permeabilized with 100% Methanol for 20 min. Crystal violet was used to stain cells for 30 min. Images were captured after plates washing3 times with 1× PBS before capturing images. Counts of colonies in treated and non-treated groups were compared.

### 2.3.9Xenograft model and optical imaging

The protocol of mice maintenance and experiments was approved by the Institutional Animal Care and Use Committee of Jeju National University (Jeju, South Korea).1 × $10^5$ of all the required cells were inoculated subcutaneously as a mixture with1X PBSand100 µlofMatrigel (Sigma-Aldrich).8-week-old athymicBALB/c female nude mice (n = 5/ group) were used for all the in vivo experiments.IRDye® 800CW 2-DG was used to observe tumor growth after 35 days of inoculation by optical imagingof xenograft models.

### 2.3.10Data analysis

Five times repeated all experimentdata areshowed as the mean±SEM.Statistical Package for the Social Sciences was used for statistical analyses. Fisher's least significant difference test and one-way ANOVA were used to determine the significant difference among each treatment groups.Significantly different values were *$p<0.05$.

## 2.4 RESULTS

### 2.4.1 Prx II mediatesGR NSCLC cell stemness

Prx II is not express A549/MOCK cells whether is highly expressed in generated A549/GR cells [38]. To verify this already known fact, we have conducted western blot.It also used to observe the expression of Prx II in GR NSCLC cell lines. Prx II was only expressed in GR cells.The results were as expected.Prx II expressions were almost similar in other NSCLC cell lines and their parent MOCK cell lines.As shown in Fig.2.1a, CD133, a CSC marker was highly expressed in GR NSCLC cells than MOCK cells.As shown in Fig.2.1b, Prx II and CD133 both were highly expressed in A549/GR cells asconfirmed by ICC assays.

We established cell lines for our further experiments by expressing short hairpin RNAs against Prx II and control to check how Prx II involves in induced stemness of drug resistant NSCLCs.Therefore, control (GR-shCON) and Prx II knockdown (GR-shPrxII)cell lines were generated using A549, HCC827 and H460 lines, as mentioned in our previous study [38].Successful knockdown was shown through a series of western blotting.Also, we observed the inhibited stemness of GR NSCLC cells by knockdown of Prx,as showed through the inhibited expressions of CD133 and other genes related withstemness-includingSox2, Oct3/4 and Nanog GR-shPrxII, than GR-shCON cells as shown in Fig. 2.1c.A549/GR cells were chosen for further experiments, because of its significantly different expression of Prx II in parental A549 cells and GR resistant A549 cells compared to other selected NSCLC cells.We have conducted DCF-DA assay to observe the changes in ROS level while conducting Annexin V staining to observe apoptotic cell populations.These assays showed the induced level of DCF-DA and Annexin V positive A549/GR-shPrx II cells, by showing that the knockdown of Prx II can induce ROS level and cell apoptosis as shown in fig. 2.1d, and e.Furthermore, Knockdown of Prx II diminished the ability of A549/GR cells to self-renew in a sphereformationassay as shown in Fig. 2.Altogether, data showed thatPrx II is a mediator of A549/GR cell stemness.

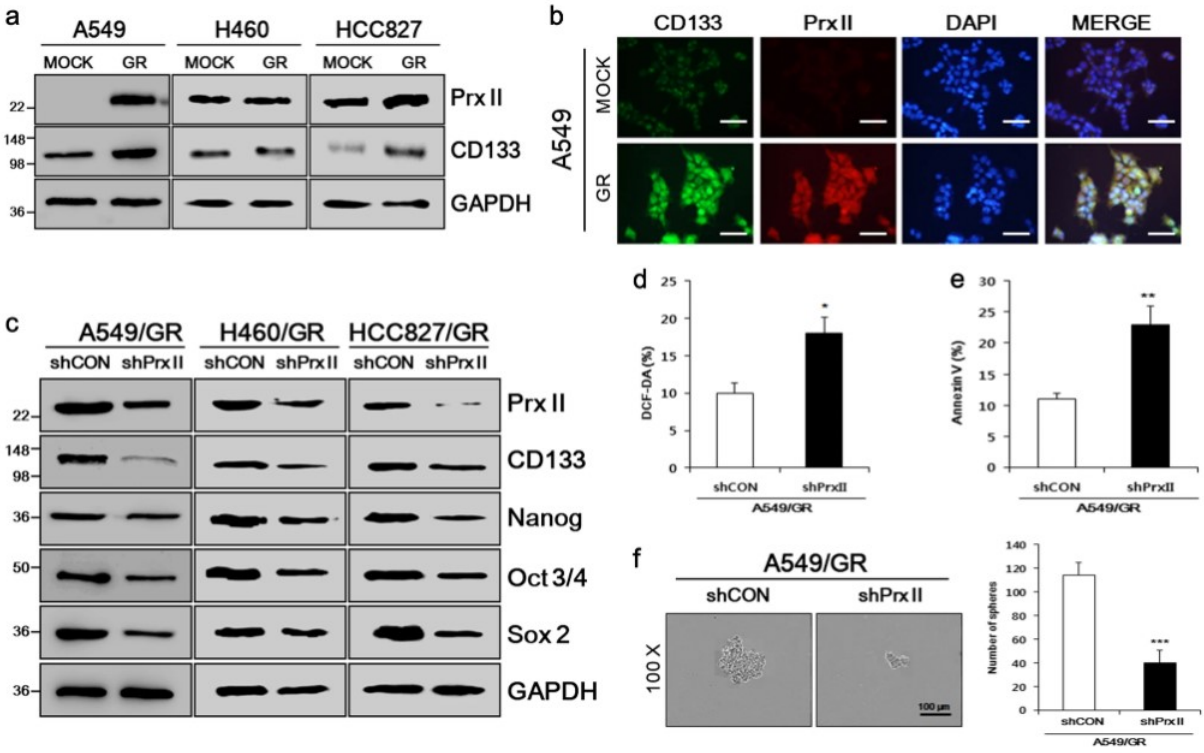


**Figure 2.1.Significance of Prx II expression in Gefitinib resistant NSCLC**

**a**Western blotting to observe Prx II and CD133 expressions in selected NSCLC and their Gefitinib resistant derivatives.bICC assays to confirm the expressions of CD133 and Prx II in A549/GR and A549 cells. cWestern blotting to check the Prx II knock down effects on the expressions of the genes related to stemness in NCLSCs. dROS levels in A549/GR shPrx II and A549/GR shCON cells were detected by DCF-DA assays. eAnnexin V staining to observe dead cell population.fPrx II knockdown effects on sphere formation were observed by sphere-formation assays in A549/GR cells.Data is represented as mean ± SEM (n = 5 per group). *P < 0.05, **P < 0.01, ***P < 0.001.

### 2.4.2 A549/ GR CD133$^{+}$ cells showed higher expression of Prx II and stemness characteristics

CD133 is a surface marker of cells which can be used to isolate CSCs [54].Therefore, CD133+ cells A549/GR cells were separated as asubpopulation (A549/GR CD133+) for further experiments.Separated CD133+ cells expressed higher level of Prx II compared to CD133– cells as observed by western blotting in Fig. 2.2a and immunocytochemistry in Fig. 2.2a, and b.As shown in Fig. 2.2a, CD133 and other gene expressions related with stemness such as Sox2, Oct3/4 and Nanog were enhanced in CD133+ cells than CD133- cells. Therefore, according to these data the induced stemness is positively co-related with high expression of Prx II.

Higher ability to metastasis, differentiation or self-renewal considers as the characteristics of CSCs [55]. Thus we have observed properties of A549/GR CD133+ cells in detail. MTT assay and Annexin V staining were used to check the cell proliferation of CD133+ cells. According to our results, the proliferation was increased due to the reduced apoptosis in Fig. 2c, d. Induced cell proliferation in CD133+ cells also has confirmed by colony forming assay as shown in Fig. 2.2e. CD133+ cells formed big and many spheres by showing those cells ability to self-renew and differentiate than CD133- cells inFig. 2.2f.

Moreover, the enhanced metastatic ability of CD133+ cells was revealed by wound-healing assays and transwell assays as shown in Fig. 2.2g and Fig. 2h [56]. Altogether, these dataverified that Prx II highly expressed CD133+ cells are having characteristics similar to CSCs.

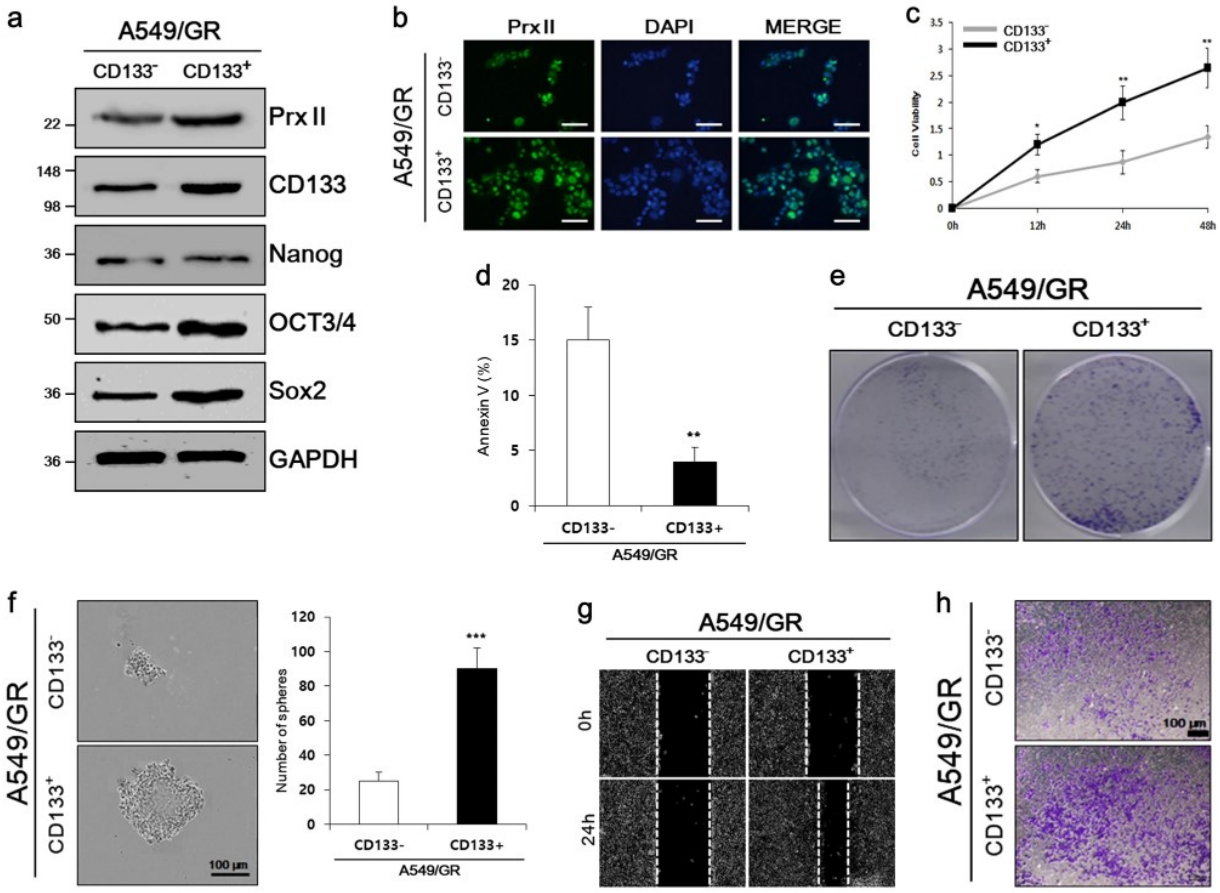


**Figure 2.2.Stemness characteristics in A549/GR CD133+ cells**

**a**Sox2, Oct3/4, Prx II, Nanog and CD133 expressions were compared inA549/GR CD133+ cells and A549/GR CD133− by western blotting b ICC showing high Prx II in A549/GR CD133+ cells compared with CD133– cells.**c**MTT assay compared cell proliferation. **d** Annexin V staining compared the apoptotic cell populations. **e**Colonyformation assays compared cell proliferation. **f**Sphereformation assay to compare ability to form spheres.**g** Wound-healing assays **h** Transwell assays to compare metastatic abilities.Data is represented as mean ± SEM (n = 5 per group). *P <0.05, **P < 0.01, ***P < 0.001.

### 2.4.3 Knockdown of Prx II weakens the stem-cell related characteristics in A549/GR CSCs

Next, our target was to investigate the involvement of Prx II in stemness properties of A549/GR CD133+ cells. Therefore, we generated control (shCON) andPrx II-knockdown (shPrx II) from A549/GR CD133+, as mentioned before [38].Those cells were used to study Prx II knockdown effects on A549/GR CSCs. As shown in Fig. 2.3a, b Successful Prx II knockdown was first confirmed by both western blotting and ICC. CD133 was expressed lower level in shPrx IIcells than shCON cells, by indicating Prx II knockdown-inhibited CD133+ CSC population. ROS and apoptotic cell percentage washigher in shPrx II cells thanshCON cells in Fig. 2.3c, d. MTT assay together with colony formation assay showed the reduced cell proliferation of A549/GR CSCs after knockdown Prx II (Fig. 2.3e, f). Therefore, those date implied the negative relationship between Prx II and CSC proliferation.

Next, we have performed transwell assays to check how Prx II knockdown affect to cancer cell metastasis as shown in Fig. 2.3g. Cell mobility was reduced by Prx II knockdown as observed in migration and invasion assays.It also showed that knockdown of Prx II inhibits EMT as sown in induced expression of E-cadherin and Vimentin in Prx II knocked down cells compared to control cells in Fig. 2.3h. Thus, all results together were able to prove that Prx II knockdown abolishes induced metastatic capacity of A549/GR stem cells.

Next we have focused on angiogenesis. To study the role of Prx II knockdown on the angiogenesisof A549/GR, we first got the help of STRING software as shown in Fig. 2.3i. Depend on STRING results,we have investigated the Prx II knockdown effect on VEGFR2, phosphorylated VEGFR2, CXCR4,STAT3, and pSTAT3. All our data implied the reduced expression of all above mentioned genesin shPrx II cells. But, Prx II knockdown not affected thephosphorylation at theSer 727 site as shown in Fig. 2.3j. In Fig. 2.3k, l,ICC assays verified above western blot results.Sphere ICC assay in Fig. 2.3m further added the value to our results.

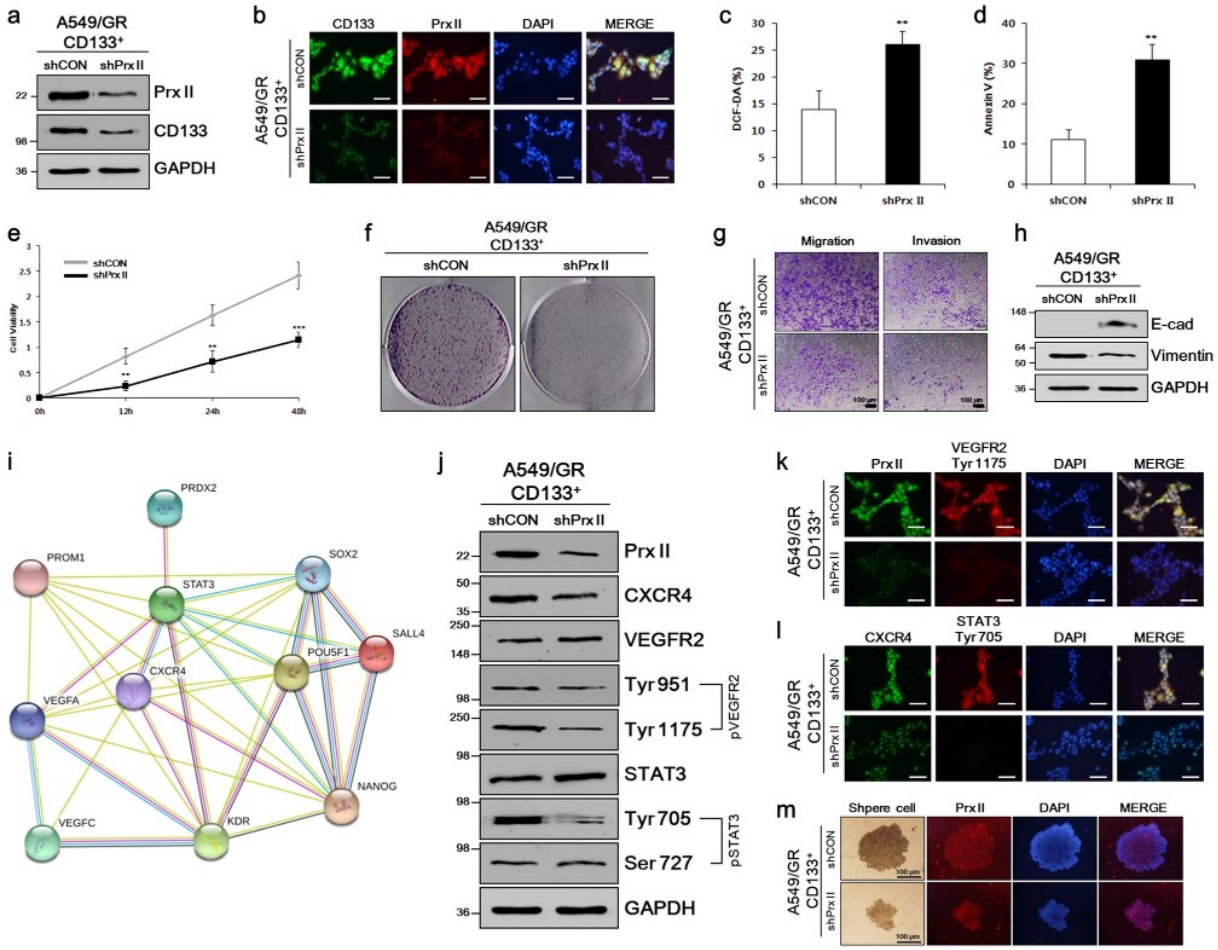


**Figure 2.3.Stem cell propertiesof A549/GRCSCs associates with Prx II**

A,b Western blot and ICC to validate Prx II knockdown c and d Compared ROS level by DCF-DA assays and apoptotic cell population by Annexin V staining assayin Prx II knockdown cells with control cells. eCell proliferation. f Colony forming ability g cell mobility were compared in shPrx II and shCON cells.h Western blotting to check E-cadherin and vimentin which relate with metastasis. iSTRING software to study interactions of Prx II with stemness and angiogenesis related genes.J western blot l ICC to check the levels of genes related to angiogenesis (STAT3, pVEGFR2, VEGFR2,CXCR4 and pSTAT3).mSphere ICC to check the expression of CXCR4. Data is represented as mean ± SEM (n = 5 per group). **P < 0.01, ***P < 0.001.

### 2.4.4 Transfection of miR-122suppresses stem cell-like characteristics in A549/ GR CSCs by Prx II inhibition

In HCC, miR-122 directly targets Prx II [52]. Thus, we checked whether it is same in drug resistant A549 NSCLCs. First, we observed lower base line miR-122 expression in CD133+ cells by real-time PCR in Fig. 2.4a. Next, we prepared stable cell lines with miR-122 over expressing and control. Successful transfection of miR-122 was validated by real time PCR after 3 days of transfection as shown in Fig. 2.4b. As shown in Fig. 2.4c, percentage of GFP-positive cell also confirmed the transfection.

Next we observed whether miR-122 can inhibit Prx II in A549/GR stem cells. As we thought, the Prx II expression was reduced with miR-122 transfection as observed by western blotting and ICC inFig. 2.4d, e. To study more in depth about how miR-122 can target Prx II in A549/GR stem cells to affect stem cell properties. ROS and apoptotic cell population were increased by miR-122 (Fig. 2.4f, g)while reducing the cell viability in Fig. 2.4h. These results revealed that miR-122 can induce NSCLC cell death by targeting Prx II.Transfection of miR-122 abolishes the stem cells' abilities to form colonies and migrate or invade (Fig.2.4i, and j).

Also the ability to form spheres was reduced by miR-122 (Fig. 2.4k). As similar to the knockdown of Prx II, the transfection of miR-122 reduced the expressions of CXCR4,VEGFR2 (Tyr 1175) and STAT3 (Tyr 705) phosphorylation.It proved that angiogenesis also inhibited (Fig. 2.4l). Western blotting also showed thehigher E-cadherin and lower vimentin by the transfection of miR-122 by showing inhibited EMT in A549/GR CSCs (Fig. 2.4m).Altogether, these resultsimplied that miR-122 suppresses CSC properties of A549/GR cells by targeting Prx II.

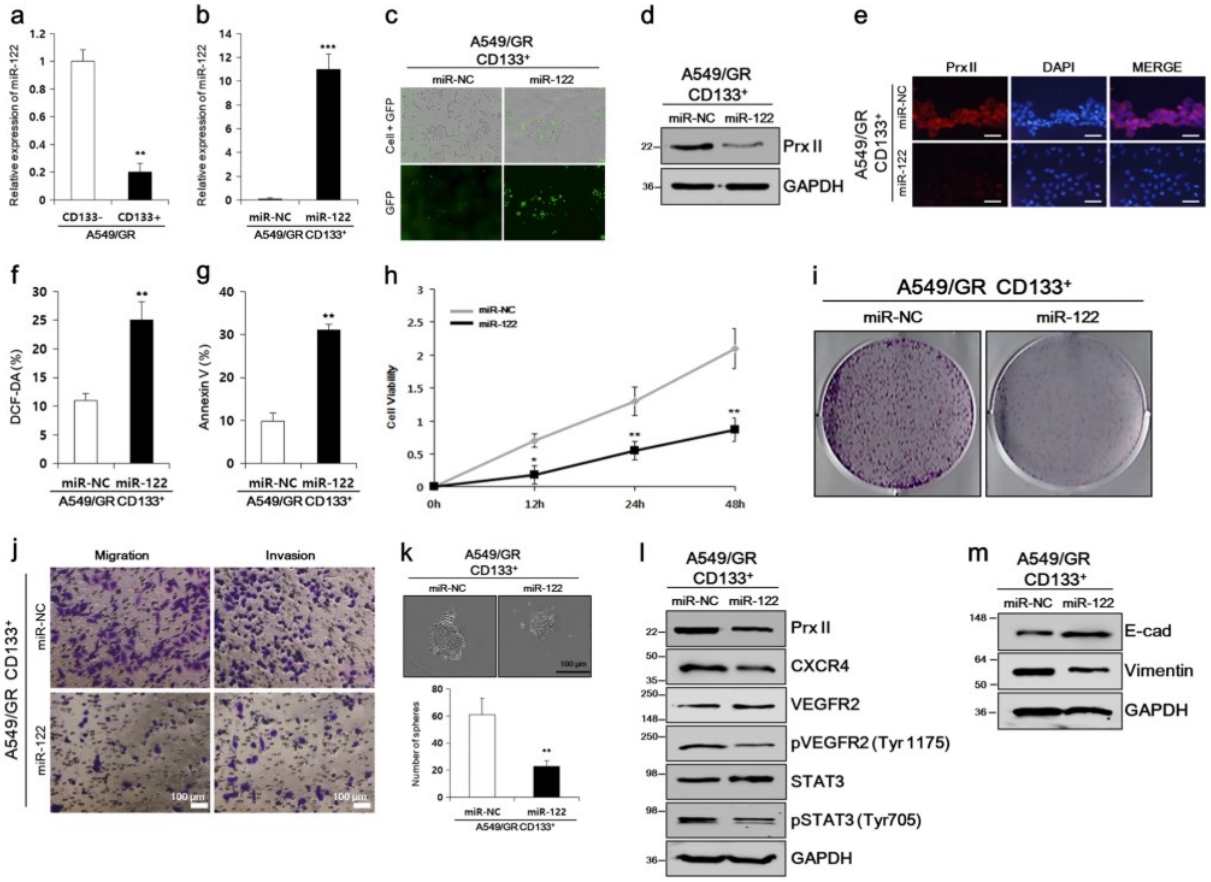


**Figure 2.4.Both Prx II and Prx II-induced stem cell properties can be targeted by miR-122 in A549/GR CSCs**

aReal time PCR to check basal miR-122 b Real time PCR to confirm successful transfection of miR-122.cScreening of GFP-positive cells. d, e Checking of Prx II expression by Western blotting and ICC assay. fInduced ROS level was observed in miR-122-transfected A549/GR cells by DCF-DA assay. gAnnexin V staining to observe apoptotic cell population.hMTT assay to observe cell proliferation. iConfirmed colony-formation ability by colony-formation assays.jMobility of cells was confirmed by Transwell migration and invasion assays. k sphere-formation ability was observed by sphere forming assay. l andm compared genes expressions related to angiogenesis and EMT in both miR-NC and miR-122 transfected cells by western blot analysis. Data is represented as mean ± SEM (n = 5 per group). *P < 0.05, **P < 0.01.

### 2.4.5 miR-122 transfection inhibitstumor growth in vivo through the inhibition of Prx II

Effects of miR-122 transfection on stem cells were further studied using in vivo system more in depth.Small size of tumors in miR-122-transfected cells as shown in Fig. 2.5a proved the suppressed tumor growth by miR-122. Figure 2.5b and c shows that miR-122 can reduce the expressions of Prx II and Vimentin in tumors.

Next, our target was to identify the molecular mechanism behind these targeting of Prx II by miR-122. For that we used Prx II 3′-UTR luciferase reporter assay. Fig. 2.5d shows the direct target sites of Prx II mRNA by miR-122.But, the mutation at Prx II 3′-UTR reverses these functions of miR-122. Then, we generated an A549 pCMV6 PrxII cell line, which express Prx II. The establishment of A549 pCMV6-Prx II cell line was as described as previously [53]. Fig. 2.5e shows that transfection of Prx II is successful as it shows the higher Prx II expression in Prx II-transfected cells compared to control cells.

However, Fig. 2.5f showed that Prx II reduced in miR-122-transfected cells compared control cells, by indicating that miR-122 inhibits the expression of Prx II. Therefore, our in vivo data validated our in vitro data by showing direct target of Prx II by miR-122.

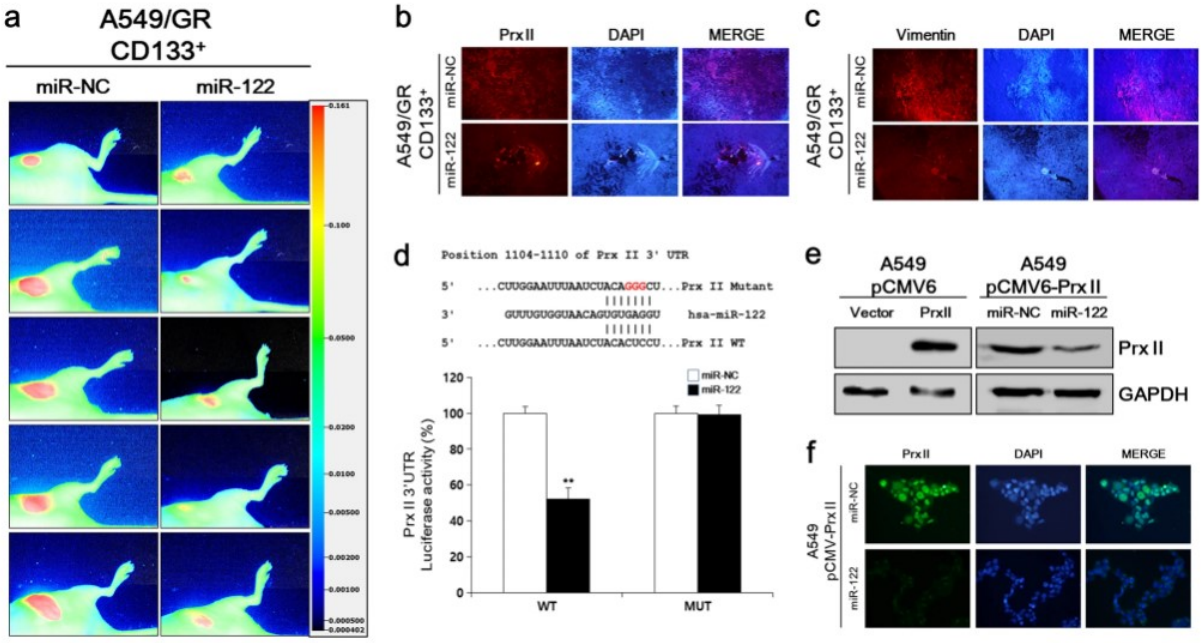


**Figure 2.5.Prx II is a direct target of miR-122**

**a**IRDye® 800CW 2-DG based optical imaging of xenograft models to compare tumor growths in miR-122 treated group and control group.**b, c**Compared the expressions of Prx II and Vimentin two tumor groups by Immunofluorescence analysis. **d**Characterized the miR-122 binding site in Prx II mRNA by Luciferase assays. **e, f**Western blotting and ICC assays to observe the Prx II expressions. Data is represented as mean ± SEM (n = 5 per group). **P < 0.01. 100 microns are represented by Bar

## 2.5 DISCUSSION

NSCLC, 80% of all lung cancers, considers one of histological forms of lung cancers [57].In one of our previous studies, we showed Prx family members expression in various NSCLCs, which are resistant to EGFR-TKIs [38]. Six family members of Prx, has been expressed stably in NSCLCs, except A549 cells, because of the significantly different Prx II in parental and resistant A549. Difference in A549 cells was caused by the methylation of upstream of Prx II gene, 5'-CCGG-3' site [38].Gefitinib, is a drug which interrupts various signaling in target cells via EGFR[58].First, we confirmed expressions of Prx II in three different NSCLC cell lines along with their DR variants. Only the A549 cell line showed a difference in Prx II expression among all cell lines. Therefore, our experiments targeted A549 cell line. Also, we showed that Prx II knock down can down regulates the NSCLC cell proliferation and stemness.

CSCs are responsible for the formation of NSCLC and failure of NSCLC sensitization to existing therapies such as Gefitinib [59, 60].Thus, we focused on the involvement of Prx II in A549/GR CSC characteristics. First of all, stem cells from A549/GR were isolated as a small subpopulation by MACS separation system, using CD133 as CSC marker. After isolation, we named it as A549/GR CD133+ cells. CD133, a glycoprotein in cell-surface transmembrane, uses to identify stem cells from both normal and tumorous [61, 62].First, we observed the higher Prx II level in stem cells compared to CD133- cells. Successful isolation of CSC was confirmed clearly via several experiments by showing aberrant CSC-like properties such as metastasis, self-renewal, cell proliferation and stemness. For all the experiments, A549/GR shCON was used as the control. It was established by transfection of scrambled shRNA. After validating the successful Prx II knock down in A549/GR CSCs, we observed that CD133 also reduce similarly. Furthermore, ROS level and apoptotic cell population were increased while the EMT, invasion, migration and cell proliferation abilities were inhibited with the knock down of Prx II. It implied the reduced potential of metastasis, because EMT is the underlying cellular program which facilitated the

tumor cell metastasis [63].VEGFR2 and STAT3 phosphorylation were inhibited by Prx II knockdown, by showing the Prx II-induced VEGF in A549/GR CSCs.VEGFR2 not responds to VEGF stimulation and become inactivate by Prx II absence[64].

Anti-angiogenic role of NSCLCs can be regulated via inhibiting STAT3/VEGFR2/VEGF signaling, as reported in previous reports[65].According to our previous study, this signaling cascade was associated positively with Prx II in HCC, thereby indicating the association with angiogenesis [39, 45]. Therefore,according to our results,knockdown of Prx I can inhibit the angiogenesis of A549/GR stem, considering the dominant role ofVEGF itself and related signaling in angiogenesis [66]. Angiogenesis considers as animportant phenomena in tumor metastasis, proliferation and development [56].

Prx II is well known as a direct target of miR-122.It canpromote the cell apoptosis while inhibiting the HCC cells proliferation by Prx II inhibition[52].Therefore, our attempt was to determine whether the association of miR-122 relate with high expression of Prx II in A549/GR CSCs.Base line miR-122 expression was lower in CD133+ cells compared to CD133– cells.Thus, our hypothesis was that higher Prx II expression may relate to miR-122 expression in A549/GR stem cells.We have prepared miR-NC or miR-122 transfected CD133+ cells, to test ourhypothesis.Our next experiments showed that over expression of miR-122 inhibits Prx II in A549/GR CSCs. Moreover, miR-122-mediated inhibition of Prx II can abolish Prx II driven stem cell properties, according to our results.Those properties includeangiogenesis, self-renewal ability, invasion,migration,cell proliferation andEMT.In vivo results also added a value to our hypothesis by showing reduced Prx II in miR-122 transfected cell tumors.

## 2.6 CONCLUSION

As a conclusion, stemness properties of A549/GR CSCs can be critically affected by the overexpression of Prx II.Also,miR-122 can abolish the stemness characteristics induced by Prx II by directly targeting Prx II. Thus, the approaches to restore miR-122 may effective for therapeutic management of NSCLCs.

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