ORIGINAL RESEARCH COMMUNICATION

Essential Role of NADPH Oxidase-Dependent Reactive Oxygen Species Generation in Regulating *MicroRNA-21* Expression and Function in Prostate Cancer

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Abstract

Aims: Oncogenic microRNAs (*miRs*) promote tumor growth and invasiveness. One of these, *miR-21*, contributes to carcinogenesis in prostate and other cancers. In the present study, we tested the hypothesis that NADPH oxidase-dependent reactive oxygen species (ROS) regulate the expression and function of *miR-21* and its target proteins, maspin and programmed cell death 4 (PDCD4), in prostate cancer cells. **Results:** The highly aggressive androgen receptor negative PC-3M-MM2 prostate cancer cells demonstrated high expression of *miR-21* and p47^{phox} (an essential subunit of NADPH oxidase). Using loss-of-function strategy, we showed that transfection of PC-3M-MM2 cells with anti-*miR-21*- and p47^{phox} siRNA (si-p47^{phox}) led to reduced expression of *miR-21* with concurrent increase in maspin and PDCD4, and decreased the invasiveness of the cells. Tail-vein injections of anti-*miR-21*- and si-p47^{phox}-transfected PC-3M-MM2 cells in severe combined immunodeficient mice reduced lung metastases. Clinical samples from patients with advanced prostate cancer expressed high levels of *miR-21* and p47^{phox}, and low expression of maspin and PDCD4. Finally, ROS activated Akt in these cells, the inhibition of which reduced *miR-21* expression. **Innovation:** The levels of NADPH oxidase-derived ROS are high in prostate cancer cells, which have been shown to be involved in their growth and migration. This study demonstrates that ROS produced by this pathway is essential for the expression and function of an onco-miR, *miR-21*, in androgen receptor-negative prostate cancer cells. **Conclusion:** These data demonstrate that *miR-21* is an important target of ROS, which contributes to the highly invasive and metastatic phenotype of prostate cancer cells. *Antioxid. Redox Signal.* 19, 1863–1876.

Introduction

PROSTATE CANCER is the most commonly diagnosed cancer among men in the United States, accounting for 29% of all cancer diagnoses (3). Projections for 2012 include 241,740 of new patients with prostate cancer along with 28,170 people succumbing to the disease (42). The prostate cancer mortality is second only to the cancer of lung and bronchus with the major damage caused by the metastatic disease. The 5-year survival rate for a metastatic prostate cancer is ~30% as compared with almost 100% survival seen in the localized disease (2). This disparity in the survival rate is the reflection of lack of effective treatment options, which, in turn, can be attributed to the incomplete understanding of the underlying pathogenesis of the metastatic prostate cancer.

Innovation

This study provides novel data with regard to a mechanism for the regulation of the microRNA, *miR-21*, by reactive oxygen species (ROS) in prostate cancer. We demonstrate that NADPH oxidase activity and *miR-21* expression are high in prostate cancer, where they serve as an important regulator of cell invasiveness. Inhibition of ROS by inhibition of NADPH oxidase or reducing *miR-21* expression decreased the metastatic phenotype of prostate cancer cells. Overall, the data presented provide a novel mechanism by which ROS can influence the metastatic potential of prostate cancer cells and identify discrete targets for new drug development.

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MicroRNAs *miRs* are 18~22 nucleotides long non-coding RNAs that are endogenously expressed in the cells (19). They regulate expression of the proteins by suppressing translation or causing degradation of mRNAs by binding to their 3'-untranslated region (UTR) (30). In cancer, several miRNAs are deregulated, resulting in both tumor suppression and tumor promotion (56). One of these *miRs*, *miR-21*, an onco-miR (38) is up-regulated in a number of cancers, including prostate cancer, suggesting that this *miR* could play a pivotal role in oncogenesis (49). In breast cancer, *miR-21* promoted the growth of breast cancer cells by down-regulating tumor suppressor genes, such as programmed cell death 4 (PDCD4), maspin, and tropomyosin 1 (TPM1) (41, 59). PDCD4 was also shown to be involved in the *miR-21*-regulated colon cancer intravasation and metastasis (4). In hepatocellular and ovarian cancer cells, *miR-21* increased proliferation and metastasis by decreasing phosphatase and tensin homolog deleted on chromosome 10 (PTEN) (27, 31); while in glioma cells, it increased invasion by regulating the matrix metalloproteinase regulators (16).

Prostate cancer cells exhibit high levels of reactive oxygen species (ROS), which augment cell proliferation and metastasis (43). Increased levels of ROS are observed in androgen-dependent (LNCaP) and -independent (PC3 and DU145) prostate cancer cell lines that are exposed to prostate-specific antigen and testosterone in cultures (45). NADPH oxidase appears to be the major source of ROS in these cells, whose activity is induced by extracellular signal-regulated kinase 1/2 (ERK1/2), a member of the mitogen-activated protein kinase (MAPK) family (17, 18). In DU145 prostate cancer cells, ROS derived from NADPH oxidase provided trophic support and reduced apoptosis of these cells (7). However, the mechanism(s) underlying ROS-dependent proliferation and metastasis of prostate cancer cells still remain unclear.

In the present study, we show that ROS produced by NADPH oxidase plays a critical role in the induction of *miR-21* in prostate cancer cells. The data suggest that *miR-21* could serve as a mediator of ROS-dependent progression of prostate cancer to an invasive and metastatic phenotype, and thus provide an ideal target for the development of anti-cancer therapies.

Results

miR-21 regulates the invasiveness of PC-3M-MM2 prostate cancer cells

Previous studies have shown that *miR-21* is over-expressed in prostate cancer (23, 34, 49). The androgen-dependent LNCaP cells are less invasive than the androgen-independent DU145 and PC3 cells (22). Furthermore, PC-3M-MM2 cells (derived from bone metastatic cultures) are the highly invasive variant of PC3 cells (11). To determine whether *miR-21* expression correlates with invasiveness of prostate cancer cells, we examined its expression in LNCaP, DU145, and PC-3M-MM2 cells. The levels of *miR-21* were 1.0 ± 0.09 , 6.18 ± 0.31 , and 8.03 ± 0.58 arbitrary units for LNCaP, DU145, and PC-3M-MM2 cells, respectively (see Supplementary Fig. S1A; Supplementary Data are available online at www.liebertpub.com/ars). Similarly, the levels of PDCD4, a downstream target that is negatively regulated by *miR-21*, showed the appropriate corresponding levels in these cell lines (Supplementary Fig. S1B). These data suggest a correlation between invasiveness and *miR-21* expression of different prostate cancer cell lines.

To determine the consequence of *miR-21* over-expression, we examined the effect of its down-regulation by anti-*miR-21* oligonucleotides on the invasiveness of PC-3M-MM2 cells. First, the effectiveness of the anti-*miR-21* in reducing the expression of *miR-21* was determined by quantitative real-time polymerase chain reaction (qRT-PCR) studies after transfecting cells with anti-*miR-21* for 24 h. Figure 1A shows dose-dependent reductions in the *miR-21* expression by anti-*miR-21*. The levels of *miR-21* in the presence of 12.5, 25, and 50 nM anti-*miR-21* were reduced to 0.42 ± 0.04 , 0.30 ± 0.04 , and 0.08 ± 0.02 -fold (mean $\pm$ standard error), respectively. Anti-*miR-21* also increased levels of PDCD4 and maspin, which are negatively regulated by *miR-21* (59). PDCD4 levels were increased to $180\% \pm 28\%$ and $163\% \pm 13\%$, while the maspin levels were increased to $155\% \pm 15\%$ and $149\% \pm 10\%$, at 25 and 50 nM anti-*miR-21* concentration, respectively (Fig. 1B, C). Control cells were transfected with 5 nM scrambled anti-*miR* (anti-*miR-scr*) and were assumed to be 100%. Increasing the concentrations of anti-*miR-scr* to 50 nM did not affect the levels of *miR-21* expression or the levels of PDCD4 and maspin (data not shown). We also observed high basal levels of both PDCD4 and maspin in immortalized prostate epithelial cells (RWPE-1), as compared with the PC-3M-MM2 (Fig. 1B, C). Since *miR-21* binds to the 3'-UTRs of *PDCD4* and *maspin* to down-regulate their expression, we determined the transcriptional regulation of both these proteins in the presence of anti-*miR-21* by luciferase assays (59). Cells exposed to anti-*miR-21* showed increased *PDCD4* and *maspin* luciferase activities, which are indicative of reductions in the inhibitory effects of *miR-21* by anti-*miR-21*. The activities of *PDCD4* and *maspin* 3'-UTRs were $132\% \pm 7\%$ and $124\% \pm 7\%$ of the control, respectively, in the anti-*miR-21* group (Fig. 1D). These data confirm previous observations that *miR-21* serves as a negative regulator of the expression of both PDCD4 and maspin (23, 59).

Since *miR-21* regulates the invasiveness of various cancer cells (4, 23, 27, 59), we next determined whether it has similar functions in PC-3M-MM2 prostate cancer cell lines. Cell invasion was assessed by a modified wound-healing and matrigel invasion assays. Cells were first transfected with 50 nM anti-*miR-scr* or anti-*miR-21* for 24 h and then plated for these assays. As shown in Figure 1E, anti-*miR-21* reduced migration in wound-healing assays, as compared with anti-*miR-scr* cells. The percent of wound closure was $28\% \pm 2\%$ in anti-*miR-scr*-transfected cells, while it was $12\% \pm 2\%$ in anti-*miR-21*-transfected cells (Fig. 1E). Similarly, anti-*miR-21* reduced invasion by $\sim 50\%$, compared with anti-*miR-scr* control group in matrigel assays (Fig. 1F). Thus, similar to an earlier study in DU145 and PC3 prostate cancer cells (23), *miR-21* regulates invasiveness of PC-3M-MM2 prostate cancer cells.

miR-21 regulates the in vivo metastasis of prostate cancer cells

To determine whether *miR-21* promotes *in vivo* tumor metastases, lung metastases were induced by injecting PC-3M-MM2 cells *via* dorsal tail vein into severe combined immunodeficient (SCID) mice. Twenty-four hours before injections, these cells were transiently transfected with either 50 nM anti-*miR-scr* or anti-*miR-21*. The anti-*miR-21* group exhibited significantly less lung metastatic lesions, compared with mice injected with anti-*miR-scr* cells (Fig. 2A). The

FIG. 1. Anti-miR-21 reduces miR-21 expression and function in prostate cancer cells. Androgen-independent PC-3M-MM2 cells were transfected with different doses of anti-miR-21 oligonucleotides (anti-miR-21) for 24 h. Lysates were used to prepare RNA for qRT-PCR for miR-21 (A), or were used in western blotting studies for programmed cell death 4 (PDCD4) and maspin (B). U6 was used as a control gene for qRT-PCR studies, while β -actin was used as loading controls for western blotting studies. (C) Bar charts represent mean $\pm$ SEM of at least four different experiments. * is the statistically significant difference from anti-miR-scr-transfected control cells ($p < 0.05$). (D) PC-3M-MM2 cells were co-transfected with PDCD4 or maspin luciferase reporter plasmids, and 50 nM scrambled anti-miR oligonucleotides (anti-miR-scr) or anti-miR-21. Luciferase activity was measured after 24 h using a Dual-Luciferase Reporter Assay kit from Promega. For normalization, cells were also transfected with Renilla-luciferase reporter plasmid. Results presented are mean $\pm$ SEM ($n = 3$; * denotes $p < 0.05$ with anti-miR-scr group). (E) *In vitro* migration of PC-3M-MM2 cells was assessed by modified wound-healing assays. Cells were transfected with 50 nM anti-miR-scr or anti-miR-21 for 24 h, after which a wound was created and cells were imaged (0 h). Cells were then allowed to grow for another 24 h, and images were again taken to assess the closure of the wound. Images are representative of three different experiments with similar results. Bar graph is the percent of wound closure presented as mean $\pm$ SEM ($n = 3$; asterisk (*) is $p < 0.01$ from anti-miR-scr). (F) Cells were transfected with anti-miR-scr or anti-miR-21 for 24 h, and later plated on the top chamber of the Matrigel invasion chamber. After 24 h, numbers of invaded cells were counted on the membranes stained with toluidine blue. Numbers depicted in histogram are mean $\pm$ SEM of three separate experiments. Asterisk (*) indicates a statistically significant difference ($p < 0.01$) from anti-miR-scr-transfected control cells. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

numbers of lung metastatic lesions were 165 ± 9 and 39 ± 11 in animals injected with the anti-miR-scr and anti-miR-21 cells, respectively (Fig. 2B). These findings indicate that miR-21 regulates *in vivo* metastasis of prostate cancer cells.

miR-21 expression in prostate cancer cells is dependent on high ROS generation

Prostate cancer cells exhibit high ROS generation, mediated by the NADPH oxidase enzyme system (17). We hypothesized that ROS promotes miR-21 expression in prostate cancer cells. Figure 3A shows that PC-3M-MM2 cells expressed high levels of ROS, as determined by 2',7'-dichlorofluorescein diacetate (H₂DCFDA) fluorescence. Treatment of these cells

with Diphenyleneiodonium chloride (DPI), a known chemical inhibitor of NADPH oxidase (33), reduced ROS generation from 30 ± 2 to 15 ± 1 arbitrary units (Fig. 3A bar chart). To determine whether NADPH oxidase is essential for ROS generation in prostate cancer cells, PC-3M-MM2 cells were transfected with different concentrations of small interfering RNA (siRNA) against p47^{phox} (si-p47^{phox}), an essential subunit of NADPH oxidase activity (21). Maximal inhibition of p47^{phox} levels was observed at 25 nM si-p47^{phox}, and this concentration was used for future experiments (Fig. 3B). The reductions in p47^{phox} levels by si-p47^{phox} significantly decreased ROS generation by these cells. ROS generation averaged 19 ± 1 arbitrary units in the si-p47^{phox} group, compared with 44 ± 2 arbitrary units in the scrambled siRNA (si-scr; 25 nM) group (Fig. 3C).

FIG. 2. Inhibition of *miR*-21 reduced lung metastases in xenograft model of prostate cancer. PC-3M-MM2 cells were transfected with either 50 nM anti-*miR*-scr or anti-*miR*-21 for 24 h. After 24 h, 1×10^6 cells were injected *via* dorsal tail vein in severe combined immunodeficient (SCID) mice, and 4–5 weeks later, the mice were euthanized. Lungs were isolated, and numbers of metastatic lesions were counted macroscopically. (A) Representative picture shows lungs from two mice per treatment group. Some of the metastatic lesions are highlighted with black arrows, which show a significant reduction in the number of lesions in the anti-*miR*-21 group. (B) Table shows the summary of the effect of anti-*miR*-21 against the *in vivo* metastases of the prostate cancer cells. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

We next determined whether suppression of ROS generation could affect *miR*-21 expression. Treatment of PC-3M-MM2 cells with 5 μ M DPI for 24 h reduced the expression of *miR*-21 to 0.46 ± 0.02 fold from vehicle-treated controls (Fig. 4A). In addition, *N*-acetyl-L-cysteine (NAC, 500 μ M) reduced *miR*-21 expression to 0.50 ± 0.07 fold of control cells (Supplementary Fig. S2A). However, 2,4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (C-PTIO, 100 μ M), a nitric oxide scavenger, did not produce any change in *miR*-21 expression (Supplementary Fig. S2B). These results suggest that reactive nitrogen species are not involved in regulating *miR*-21 levels in prostate cancer cells.

DPI treatment also increased PDCD4 and maspin expression. Their respective levels in the presence of DPI were $196\% \pm 24\%$ and $188\% \pm 15\%$ of vehicle-treated controls (Fig. 4B, C). DPI and NAC also decreased the expression of *miR*-21 in DU145 prostate cancer cells (Supplementary Fig. S3). Functionally, DPI decreased the wound closures, which are indicative of reduced migration of prostate cancer cells (Fig. 4D). Furthermore, DPI suppressed invasion of prostate cancer

cells in matrigel assays. The percentage of invading cells was reduced to $33\% \pm 14\%$ by DPI (Fig. 4E).

Inhibition of p47^{phox} expression by si-p47^{phox} decreased *miR*-21 expression. *miR*-21 levels were 1.00 ± 0.04 , 0.49 ± 0.05 , and 0.59 ± 0.01 fold in the presence of si-scr and 25 and 50 nM si-p47^{phox}, respectively (Fig. 5A). A similar effect was observed after knockdown of another NADPH oxidase subunit, p22^{phox}. Transfection of these cells with si-p22^{phox} (5 nM) reduced the expression of p22^{phox} to 0.59 ± 0.07 fold and the levels of *miR*-21 to 0.63 ± 0.10 fold (See Supplementary Fig. S4A, B). si-p47^{phox} also increased the levels of both PDCD4 and maspin. In the presence of 25 nM si-p47^{phox}, PDCD4 and maspin levels were $200\% \pm 27\%$ and $151\% \pm 13\%$ of si-scr group, respectively (Fig. 5B, C). Additional experiments tested the effect of NADPH oxidase in regulating the binding of *miR*-21 to PDCD4 and maspin 3'-UTR by measuring luciferase activity. In the presence of si-p47^{phox} (25 nM), the luciferase activity was $143\% \pm 13\%$ and $142\% \pm 10\%$ for PDCD4 and maspin, respectively (Fig. 5D), with si-scr (25 nM) controls as 100%. These results

FIG. 3. NADPH oxidase inhibition reduces the high reactive oxygen species (ROS) generation in PC-3M-MM2 prostate cancer cells. (A) PC-3M-MM2 cells were pretreated for 30 min with either vehicle or 5 μ M Diphenyleneiodonium chloride (DPI) and then incubated with H₂DCFDA for 20 min. After that, fluorescent images were captured with a confocal microscope. Images shown are representative of three different experiments showing identical results. Quantitative analysis of the fluorescent images by FluoView software is presented as mean $\pm$ SEM in a bar graph. (B) PC-3M-MM2 cells were transfected with different concentrations (0–50 nM) of si-p47^{phox} for 24 h, and cell lysates were used for western blotting (picture) or real-time RT-PCR (bar chart) experiments for p47^{phox}. (C) PC-3M-MM2 cells on glass coverslips were transfected with either 25 nM scrambled siRNA (si-scr) or si-p47^{phox} for 24 h, and used to measure ROS generation as described earlier. Similar to DPI, si-p47^{phox} also reduced the ROS levels in PC-3M-MM2 cells. Asterisks (*) indicate a statistically significant difference ($p < 0.05$) from vehicle and si-scr groups. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

demonstrated that *miR-21* expression and its transcriptional activity are regulated by NADPH oxidase-mediated ROS generation in prostate cancer cells.

In order to determine the functional impact of NADPH oxidase on PC-3M-MM2 cell motility, we studied the effects of si-p47^{phox} on the migration and invasion of these cells. Transient transfections of PC-3M-MM2 cells with si-p47^{phox} suppressed wound closures from 44% $\pm$ 4% (for 25 nM si-scr-transfected cells) to 17% $\pm$ 2% (for 25 nM si-p47^{phox} transfected cells) (Fig. 5E). Similarly, si-p47^{phox} also decreased prostate cancer cell invasion in matrigel assays. The number of invading cells was 45% $\pm$ 9% of the si-scr control cells (Fig. 5F). These results suggest that NADPH oxidase regulates *miR-21* expression and function in PC-3M-MM2 prostate cancer cells.

p47^{phox} siRNA suppressed lung metastases of PC-3M-MM2 cells in a xenograft model of prostate cancer

Since NADPH oxidase regulates migration and invasiveness of prostate cancer cells in cultures, and *miR-21* regulates these functions, we tested whether inhibition of NADPH oxidase reduces tumor metastasis in a xenograft model of prostate cancer. SCID mice injected (*via* tail vein) with PC-3M-MM2 cells transfected with 25 nM si-scr or si-p47^{phox} showed 20 $\pm$ 3 and 6 $\pm$ 1 metastatic lung lesions per mouse, respectively (Fig. 6A, B). These studies indicate that NADPH oxidase activity regulates metastasis of PC-3M-MM2 prostate cancer cells to the mouse lungs and, based on the previous results, might suggest a role of *miR-21* in this process.

FIG. 4. DPI suppressed *miR-21* expression in PC-3M-MM2 cells. (A) *miR-21* levels were determined in PC-3M-MM2 cells by TaqMan[®] assay after treating the cells with vehicle or DPI for 24 h. (B, C) PC-3M-MM2 cells were pre-treated with vehicle or 5 μ M DPI for 24 h; whole cell lysates were prepared; and western blotting studies were performed for PDCD4 and maspin. β -actin was used as the loading controls. Histograms are the mean $\pm$ SEM from four different experiments and (*) is the statistically significant difference ($p < 0.01$) from vehicle-treated controls. *In vitro* migration and invasion of PC-3M-MM2 cells were studied by wound-healing assays (D) and matrigel invasion assays (E), respectively. Cells were treated with vehicle or DPI for 24 h, and assays were performed as described in Materials and Methods. DPI pre-treatment inhibited wound closure as compared with the vehicle-treated controls (D), and also reduced the number of cells invading through the matrigel (E). Bar graphs are mean $\pm$ SEM of at least three different experiments with a statistically significant difference (* $p < 0.05$) between the treatment groups. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Human prostate cancer specimens demonstrate increased expression of *miR-21* and *p47^{phox}*

Human prostate cancer RNA samples were obtained from TissueScan qRT-PCR panels (Origene Technologies, Rockville, MD). Figure 7A shows that increased expression of *miR-21* was observed in samples classified as Stages I-II (localized to the prostate) and Stages III-IV (spread to nearby or distant organs), as compared with the Stage 0. The expression levels were 1.0 ± 0.53 ($n = 4$), 10.4 ± 2.6 ($n = 9$), and 13.4 ± 4.6 ($n = 5$) fold for Stages 0, I-II, and III-IV, respectively (Fig. 7A). Similarly, we also observed increased expression of *p47^{phox}* in different stages of prostate cancer, as compared with Stage 0. However, the levels of *p47^{phox}* in Stages I-II and III-IV were 29 ± 6 fold and 17 ± 4 fold, respectively (Fig. 7B). PDCD4 and maspin were next determined by immunohistochemistry in Stage IV human prostate cancer formalin-fixed, paraffin-embedded sections. Diaminobenzidine tetrahydrochloride (DAB) staining of these tissues revealed high levels of both

PDCD4 (Fig. 7C) and maspin (Fig. 7D) in the normal prostate cells, but reduced immunolabeling in the tumor cells (Fig. 7C, D). In normal conditions, PDCD4 is primarily localized in the nucleus (6). PDCD4 showed a predominant nuclear localization in normal prostate epithelial cells but not cancer cells, as indicated by the increased dark brown staining (red arrows). Similar results were found in colorectal carcinoma (32), in which reduced nuclear localization of PDCD4 was proposed as a biomarker for the disease (13).

ROS regulates *miR-21* by activating Akt

A previous study indicated that ROS could regulate invasiveness by activating Akt in prostate cancer cells (18). Akt was shown to regulate *miR-21* in cardiac myocytes (36) and prostate cancer cells (40), suggesting that it could serve as a target of ROS for regulating *miR-21*. Treatment of PC-3M-MM2 and DU145 cells with DPI or NAC led to reduced phosphorylation of Akt (p-Akt, Supplementary Fig. S5A, B).

FIG. 5. *si-p47^{phox}* reduced *miR-21* expression and activity in prostate cancer cells. PC-3M-MM2 cells were transfected with scrambled siRNA (*si-scr*) or different concentrations of *si-p47^{phox}* for 24 h. **(A)** *miR-21* levels were detected in these cells by TaqMan assays. Data presented are mean $\pm$ SEM of three separate experiments performed in triplicate. (*) is the statistically significant difference ($p < 0.05$) from *si-scr*. The expression of PDCD4 **(B)** and maspin **(C)** was determined by western blotting. Blot shown is representative of three independent experiments with similar results, and the bar graph is mean $\pm$ SEM of these experiments. Asterisk (*) denotes a statistically significant difference ($p < 0.05$) from *si-scr*. **(D)** PC-3M-MM2 cells were co-transfected with either 25 nM *si-scr* or *si-p47^{phox}*, and PDCD4- or maspin- luciferase reporter plasmids. Lysates were then used to measure the luciferase activity, which is suggestive of *miR-21* transcriptional activity. *si-p47^{phox}* significantly ($p < 0.05$) increased the PDCD and maspin luciferase activity. Data presented are mean $\pm$ SEM of three different experiments, and (*) is the statistically significant difference ($p < 0.05$) from *si-scr* transfected cells. **(E)** Wound-healing assays were performed in cells transfected with 25 nM *si-scr* or *si-p47^{phox}*. Picture shown is representative of three experiments, and bar chart is the quantitative representation of wound closure from these experiments. (*) indicates significant ($p < 0.01$) difference from *si-scr* group. **(F)** *In vitro* invasion of PC-3M-MM2 cells, transfected with 25 nM *si-scr* or *si-p47^{phox}*, was determined by matrigel invasion chamber assays. Histogram is mean $\pm$ SEM of three different experiments, and asterisk (*) denotes a statistically significant difference ($p < 0.05$) from *si-scr*. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

LY294002, a known PI3K inhibitor, also reduced the levels of p-Akt (Supplementary Fig. S5B). These data suggest that ROS activation of Akt could account for the induction of *miR-21* expression in prostate cancer cells.

Discussion

The principal finding of this study is that ROS are a major contributor to the high expression of *miR-21*, in prostate cancer cells. The main source of ROS generation is the NADPH oxidase system, whose activity is elevated in the prostate cancer cells (17). Functionally, high ROS, through its regulation of *miR-21*, contribute to increased metastatic potential of the prostate cancer cells. We also demonstrate constitutively high expression of NADPH oxidase subunit (*p47^{phox}*) and *miR-21* in different stages of human prostate

cancer. Finally, our data suggest that ROS activate Akt pathway to increase *miR-21* expression. These findings provide a better understanding of the mechanism by which oxidative stress in prostate cancer cells promotes increased invasiveness and suggest that ROS and *miR-21* could serve as useful targets for treating prostate cancer metastasis.

Our data show that the invasiveness of prostate cancer cell lines is dependent on *miR-21* expression. For example, the highly aggressive PC-3M-MM2 cells show higher levels of *miR-21* expression, as compared with LNCaP cells, which demonstrates a lower invasive phenotype and shows lower expression of *miR-21*. We previously showed that NADPH oxidase activity in prostate cancer cells is dependent on the ERK MAP kinase activity (17). Accordingly, ERK1/2 mediates phosphorylation of *p47^{phox}* subunit of the NADPH oxidase, which promotes its activation (17). In this study, we

FIG. 6. Inhibition of NADPH oxidase abrogated the lung metastases of prostate cancer cells in SCID mice. 0.5×10^6 PC-3M-MM2 cells, transfected with 25 nM si-scr or si-p47^{phox}, were injected *via* dorsal tail vein in SCID mice. After 4–5 weeks, numbers of metastatic lesions were counted macroscopically in the lungs that were isolated after euthanizing the mice. **(A)** Picture shows lungs from two mice from each treatment group, and metastatic lesions are indicated by black arrows. Summary of the *in vivo* experiment performed in **(A)** is presented in the table **(B)**, which shows the significant effect of si-p47^{phox} in reducing the number of lung metastases of the prostate cancer cells. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

further expand these findings to show that the invasive phenotype dictated by high NADPH oxidase activity is dependent on *miR-21*. This is supported by the finding that anti-*miR-21* significantly suppressed the migration of these cells in cultures and reduced the number of lung metastases.

We demonstrate that NADPH oxidase activity is essential for *miR-21* expression. p47^{phox} is a subunit of NADPH oxidase, which is critical for its activation (21). Inhibition of p47^{phox} (by siRNA) in PC-3M-MM2 cells not only reduced their metastatic potential, but also lowered the *miR-21* levels. Similarly, down-regulation of p22^{phox} by siRNA also reduced *miR-21* expression. Furthermore, treatment of prostate cancer cells with DPI, a chemical inhibitor of NADPH oxidase, or the antioxidant NAC, reduced the expression of *miR-21*. These data strongly support the role of ROS and the NADPH oxidase system in the regulation of *miR-21* expression in prostate cancer cells.

Only limited information is available with regard to the upstream regulators of *miR-21* expression. For example, interleukin-6 (IL-6) increased *miR-21* expression by stimulating signal transducers and activators of transcription 3 in multiple myeloma cells (26). Treatment of promyelocytic cells with 12-myristate 13-acetate (PMA) resulted in increased transcrip-

tion of *miR-21* *via* activator protein 1 (AP-1) (15). It is known that AP-1 is negatively regulated by downstream *miR-21* target PDCD4 (54), which itself is down-regulated by *miR-21* (14). Accordingly, AP-1-mediated activation of *miR-21* resulted in reduced PDCD4 levels, which led to increased AP-1 activity and proliferation in thyroid cells (47). In addition to these, the upstream regulators of *miR-21* also include epithelial growth factor receptor (37), nuclear factor- κ B (58), estrogen receptor (5, 51), bone morphogenic protein-6 (12), transformation growth factor- β (10), and Foxo3a (50). In prostate cancer, an androgen receptor-dependent regulation of *miR-21* has been suggested (35). These authors showed the presence of a highly conserved androgen-response element (ARE) in the *miR-21* promoter. Furthermore, they showed that apart from androgen-dependent growth, *miR-21* was also involved in the growth regulation of androgen-independent cells. Recent studies have implicated Akt in the regulation of *miR-21* in prostate cancer cells (40) and cardiac myocytes (36). The current study shows that Akt could mediate ROS regulation of *miR-21* expression in prostate cancer cells. Interestingly, *miR-21* has been shown to increase ROS generation by decreasing the activity of superoxide dismutase 3 (SOD3) or by indirectly inhibiting SOD2 levels in cancer cells (57),

FIG. 7. Expression of *miR-21*, *p47^{phox}*, *PDCD4*, and *maspin* in human prostate cancer specimens. Expression of *miR-21* (A) and *p47^{phox}* (B) was determined in the human prostate cancer RNA panel obtained from Origene Technologies. qRT-PCR studies show high expression of *miR-21* and *p47^{phox}* in advanced stages of prostate cancer. Asterisk (*) indicates a statistically significant difference ($p < 0.05$) from Stage 0 tumor samples. (C, D) Immunohistochemical studies were performed to look for the expression of *PDCD4* and *maspin* in formalin-fixed paraffin embedded prostate tumor specimens, and to compare their expression with the adjacent normal prostate tissues. Diaminobenzidine tetrahydrochloride staining shows high expression of both *PDCD4* (C) and *maspin* (D) as dark-brown nuclear staining in normal tissues as indicated by red arrows. In contrast, there is low expression of both of these proteins (C, D) in prostate tumors, which is marked by black arrows. Picture shown is representative of immunohistochemical studies performed in three different specimens. Left and right panels show 100 $\times$ - and 400 $\times$ -magnifications. Scale bar in 100 $\times$ magnified image is 100 μ m, while that in 400 $\times$ magnified image is 20 μ m. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

suggesting the existence of an ROS-driven positive feedback loop in cancer cells to promote *miR-21* expression. Overall, these data suggest that *miR-21* serves as an integrator of signals derived not only from the androgen/androgen receptor axis (35) but also from multiple factors in the tumor micro-environment and intracellular environment.

Our data showing oxidative stress-dependent regulation of *miR-21* expression in prostate cancer is supported by studies in vascular smooth muscle cells and cardiac myocytes, which showed increased expression of *miR-21* by hydrogen peroxide (H_2O_2) (8, 24). *miR-21* levels were also up-regulated in mouse macrophage cell line, RAW264.7 (48). However, our study is the first to document a role of NADPH-oxidase-mediated ROS generation in regulating *miR-21* expression and the invasiveness of prostate cancer cells.

Several *miR*s have recently been shown to be regulated by oxidative stress (44, 50). *MiR-200c*, along with other members of *miR-200* family, was up-regulated in the presence of H_2O_2 -induced oxidative stress in human umbilical vein endothelial cells (28). This H_2O_2 -mediated increase in *miR-200c* involved

p53 and retinoblastoma proteins, which further led to down-regulation of *miR-200c* target ZEB1, and increased apoptosis and senescence of the endothelial cells (28). Oxidative stress-induced up-regulation of *miR-200* family of *miR*s was also seen in various human cancer cells such as ovarian (SKOV3), melanoma (MDA-MB-435S), and breast (MDA-MB-436 and BT-549) (29). In particular, *miR-141* and *miR-200a* targeted p38 α MAPK in ovarian adenocarcinoma, resulting in its down-regulation and thus increased tumor growth (29).

The role *miR-21* plays in promoting cell survival and metastasis has been delineated in various studies. Downstream targets of *miR-21* that could explain its pro-invasive function include tumor suppressor genes, such as *PDCD4*, *maspin*, *TPM1*, and *PTEN*. *miR-21* negatively regulates the expression of these targets, thereby reducing their tumor suppressor functions (38). *PDCD4* acts by inhibiting translation of proteins through its interaction with RNA helicase eIF4A and scaffold protein eIF4G (52, 53). Thus, *PDCD4* plays an important role in carcinogenesis by regulating various proteins involved in cell transformation, cell cycle, tumor growth, and

invasion (20). Maspin, a member of the serine protease inhibitor (serpin) superfamily, inhibits cell invasion and metastasis in breast and prostate cancer cells (39, 60). In prostate cancer, maspin can suppress prostate tumor metastases by inhibiting urokinase-type plasminogen activator (uPA)/uPA receptor axis and angiogenesis (9). Additional tumor-suppressive effects of maspin in prostate cancer include increased cell-extracellular matrix (ECM) interaction by promoting cell interaction with ECM components fibronectin and laminin (1), and increased apoptotic sensitivity of cancer cells (25, 46). Maspin has also been shown to affect the redox signaling in prostate cancer (55). These authors showed that maspin interacted with glutathione *S*-transferase (GST) under basal and oxidative stress conditions in prostate cancer cells. This interaction was boosted by more oxidative stress, which, in turn, also led to protection of GST. Furthermore, maspin/GST interaction reduced ROS generation along with lower levels of vascular endothelial growth factor A (55).

In summary, we demonstrate that increased expression of *miR-21* in prostate cancer cells is dependent on the high ROS produced by NADPH oxidase. Activation of Akt appears important for the induction of *miR-21*. Since high levels of ROS are experienced in the ischemic tumor micro-environment, the induction of *miR-21* could provide a mechanism by which the tumor cells could escape to a region that is more conducive for survival. The data are corroborated by findings in human prostate cancer tissues and demonstrate high expression of both *miR-21* and *p47^{phox}* subunit of NADPH oxidase. Finally, these data provide evidence that ROS and *miR-21* are viable targets for drug development that is aimed at treating metastatic prostate cancer.

Materials and Methods

Materials

DPI, NAC, C-PTIO, and LY294002 were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies against *p47^{phox}*, maspin, and t-Akt were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); while those against β -actin, PDCD4, and p-Akt were from Sigma Chemical Co., Epi-tomics, Inc. (Burlingame, CA) and Cell Signaling Technology, Inc. (Danvers, MA), respectively. Cell culture media RPMI 1640 and penicillin/streptomycin were obtained from Invitrogen (Carlsbad, CA), while fetal bovine serum (FBS) was from Atlanta Biologicals, Inc. (Lawrenceville, GA). Human prostate cancer TissueScan qPCR panels with the clinical information, including the disease stages, were obtained from Origene Technologies (Rockville, MD), and FFPE sections for human prostate cancer were purchased from US Biomax, Inc. (Rockville, MD).

Cell cultures

The androgen-receptor negative and highly metastatic human prostate cancer cell line PC-3M-MM2 was obtained from Dr. Kounosuke Watabe (SIU School of Medicine, Springfield, IL), while DU145 and LNCaP were kindly provided by Dr. Daotai Nie (SIU School of Medicine, Springfield, IL). All these cells were cultured in RPMI 1640 media supplemented with 10% FBS, 50 units/ml penicillin, and 25 μ g/ml streptomycin. Cells were grown at 37°C in the presence of 5% CO₂ and 95% ambient air. The medium was changed every

second day, and the cells were passaged twice a week. The immortalized prostate epithelial cells, RWPE-1, were purchased from ATCC (Manassas, VA), and cultured in keratinocyte serum-free medium (K-SFM) with 0.05 mg/ml bovine pituitary extract (BPE) and 5 ng/ml human recombinant epidermal growth factor (EGF) added to it to make a complete medium. BPE and EGF were supplied with K-SFM as a part of the K-SFM kit by Life Technologies (Grand Island, NY). All the experiments were performed using confluent monolayers.

Transfection of PC-3M-MM2 cells

Transfections of PC-3M-MM2 cells with siRNA and anti-*miR*s were performed by using RNAiFectin from Applied Biological Materials, Inc. (ABM, Inc., Richmond, Canada) according to the manufacturer's protocol. Briefly, 2×10^5 PC-3M-MM2 cells were plated in 12-well cell culture plates. The next day, solution A was prepared with different concentrations (0–50 nM) of anti-*miR-21*, si-*p47^{phox}*, or si-*p22^{phox}* added to 125 μ l SFM. Solution B was made by adding 6 μ l of RNAiFectin transfection reagent (ABM, Inc) in 125 μ l SFM. Both the solutions were then mixed and incubated at room temperature for 40 min to allow the formation of transfection complexes. For control groups, 5 nM of anti-*miR-scr* and si-*scr* were used. After 40 min, complexes were diluted with 750 μ l media containing serum and added to the cell cultures by gently swirling the plates. Cells were incubated for another 24 h, and later used for Western blotting and real-time RT-PCR studies. For *in vitro* invasion chamber assay, modified wound-healing assay, and *in vivo* tumor inoculations, the same protocol was used but with 50 nM of anti-*miR-scr* and anti-*miR-21* or 25 nM of si-*scr* and si-*p47^{phox}*.

In vivo studies

To determine the role of *miR-21* and *p47^{phox}* in the spontaneous metastatic ability of PC-3M-MM2 cells, cells were transfected with 50 nM anti-*miR-scr* or anti-*miR-21*, or 25 nM si-*scr* or si-*p47^{phox}* for 24 h. After that, cells were harvested, and $0.5\text{--}1 \times 10^6$ cells in 0.2 ml of 1 $\times$ PBS were injected intravenously *via* the tail vein of 5-week-old SCID mice with an average weight of 20 g (Harlan Sprague-Dawley, Indianapolis, IN). Mice were sacrificed 4–5 weeks after the injection of the cells, and metastatic lesions on the lungs were counted macroscopically. All the animal studies were performed in compliance with the SIU School of Medicine's Laboratory Animal Care and Use Committee-approved protocols.

In vitro invasion chamber assay

The ability of prostate cancer cells to migrate through matrigel-coated membranes was measured using BD Biocoat Matrigel invasion chambers (BD Biosciences, San Jose, CA). PC-3M-MM2 cells were transiently transfected with anti-*miR-scr* or anti-*miR-21*, or si-*scr* or si-*p47^{phox}* (as described above) for 24 h. These cells were then suspended in the culture media without serum and plated on the top compartment of the invasion chamber followed by respective treatments. At the end of 24 h, the cell inserts were removed, and cells were carefully wiped from the top surface of the membrane with a cotton swab. The invasive cells adhering to the bottom surface of the membrane were stained with toluidine blue and quantified under a light microscope using a 20 $\times$ objective.

Total number of invaded cells was counted in four randomly chosen fields per treatment per insert. Each treatment group was in triplicate.

Modified monolayer scratch assay

After transient transfections of the PC-3M-MM2 cells with anti-*miRs* and siRNAs, an equal number of cells (2×10^5) was plated in each well of 12-well culture plates. After the cells reached 70%–80% confluency, a wound was created with a 1000 μ l pipette tip. The differential interference contrast (DIC) images were recorded before and just after the wound (time 0 h). The media was changed, and the cells were allowed to grow for another 24 h. DIC images were again captured by confocal microscopy, and results were quantitated by calculating the percentage of wound closure in the treatment groups where the scramble treatment group was assumed to be 100%.

Western blotting

Total cell lysates were used for western blotting studies for PDCD4, maspin, p47^{phox}, and p-Akt. The cells were homogenized in ice-cold 50 mM Tris HCl, 10 mM MgCl₂, and 1 mM EDTA in the presence of protease inhibitors cocktail (Sigma-Aldrich), and these lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Proteins were then transferred to nitrocellulose membranes, which were developed as previously described (17). The blots were visualized by LAS 4000 chemiluminescence imaging system (GE Healthcare Biosciences, Pittsburgh, PA) using ECL reagent (Pierce Biotechnology, Rockford, IL). The bands were normalized to β -actin, and quantitative analysis was performed with Multi Gauge software (version 3.0) from Fujifilm.

RNA isolation, reverse transcription, and real-time reverse transcriptase polymerase chain reaction

To measure the p47^{phox} and p22^{phox} transcripts the RNA isolation, reverse transcription and RT-PCR was performed as described earlier (17).

PCR primers

The set of primer sequences were from Sigma Genosys (St. Louis, MO):

p47^{phox}: 5'-CATGTTCTGCTTAAGTGGCA-3' (sense);
5'-AGGTGTGGGACGGGAGATC-3' (antisense).
p22^{phox}: 5'-ACCACGGCGGTCATGTACTT-3' (sense);
5'-CGCTTCACCCAGTGGTACTT-3' (antisense).
 β -actin: 5'-TGAGACCTTCAACACCCAGCCATG-3' (sense);
5'-CGTAGATGGGCACAGTGTGGGTG-3' (antisense).

TaqMan® Real-Time PCR

Total RNA was isolated from PC-3M-MM2 cells using QIAzol Lysis Reagent (Qiagen, Valencia, CA) as directed by the manufacturer. *miR-21* cDNA was generated from 200 ng of total RNA, which was reverse transcribed using *hsa-miR-21* qRT-PCR primer set (Applied Biosystems, Foster City, CA) and TaqMan MicroRNA Reverse Transcription (RT) kit (Applied Biosystems). Each RT reaction contained 1 $\times$ RT specific primer, 1 $\times$ RT reaction buffer, 0.15 μ l of 100 mM dNTPs, 50 U/ μ l MultiScribe RT enzyme, and 3.8 U/ μ l RNase inhibitor. The

15 μ l reaction mix was then incubated for 30 min at 16°C, 30 min at 42°C, and 5 min at 85°C and then held at 4°C in a PCR cycler. The real-time PCR was performed on Applied Biosystems StepOnePlus™ Real-Time PCR System using a standard TaqMan PCR kit (Applied Biosystems) protocol. Briefly, after the RT step, 2 μ l of the RT reaction was combined with 1 μ l of 20 $\times$ TaqMan MicroRNA Assay (forward primer, reverse primer and probe) and 17 μ l of TaqMan Universal PCR Master Mix in a 20 μ l final volume. The reactions were incubated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Mature *miR-21* expression was calculated using the 2^{-ddC_t} method relative to U6-snRNA and expressed as fold change. All TaqMan-PCRs were performed in triplicate.

Immunohistochemistry

FFPE human prostate tumor sections on glass slides were obtained from US Biomax. Immunohistochemistry staining was performed using DAB solution for the detection of PDCD4 and maspin. Antigen retrieval was performed by deparaffinizing, rehydrating, and immersing the tumor section containing glass slides in 10 mM sodium citrate buffer (pH 6.0) for 15 min in 90°C water bath. After cooling down to room temperature, the slides were dipped in 3% H₂O₂ for 15 min at room temperature to block endogenous peroxidase activity. The sections were then washed twice with 1 $\times$ PBS and blocked with 5% goat serum in 1 $\times$ PBS for 20 min at room temperature. The sections were then incubated with respective primary antibodies (1:200 dilution) for 12 h at 4°C after which they were incubated with horseshoe radish peroxidase tagged secondary antibody (Vectastain ABC kit, Burlingame, CA) for 45 min at room temperature according to the manufacturer's protocol. The peroxidase activity was identified by a reaction with DAB solution kit (Vector Laboratories, Inc., Burlingame, CA) for 5 min at room temperature. Sections were then counterstained with hematoxylin, dehydrated with ethanol, and, finally, mounted in Cytoseal 60 (Richard-Allan Scientific, Kalamazoo, MI). Slides were imaged using an Olympus light microscope (Olympus imaging America Inc., Center Valley, PA) using Olympus DP controller software Ver. 2.3.1.231.

Plasmid and siRNA/anti-miRs co-transfection

PC-3M-MM2 cells were simultaneously transfected with plasmid DNA (PDCD4, Maspin, and pGL3 Renilla, a kind gift from Dr. Y.Y Mo, SIU School of Medicine) using DNAfectin and siRNAs (scramble or p47^{phox}) or anti-*miRs* (scramble or *miR-21*) using RNAiFectin. Briefly, for each well of a 24-well plate, the following mixes were made and added together. (i) Plasmid DNA mix: Solution A was prepared by adding 0.4 μ g maspin or PDCD4 luciferase plasmid and 0.1 μ g of pGL3 Renilla luciferase plasmid in 32 μ l of SFM. In a separate tube, solution B was made by adding 2 μ l of DNAfectin in 32 μ l of SFM. Both the solutions were combined (about 65 μ l total for each well), gently mixed, and incubated for 20 min. (ii) Anti-*miR* (or siRNA) mix: 50 nM of anti-*miR-scr* or anti-*miR-21* were added to 32 μ l of SFM to make solution A, while solution B was prepared by adding 2 μ l of RNAiFectin to 32 μ l of SFM. Both the solutions were combined (about 65 μ l total for each well), mixed, and incubated for 20 min at room temperature. At the end of 20 min, 65 μ l of the plasmid mix and 65 μ l of anti-*miR* (or siRNA) mix were added to each well containing 130 μ l

of SFM. SFM was replaced with serum-enriched media after 8 h, cells were incubated for another 24 h, and lysates were used for luciferase assays. For siRNA transfections, a similar protocol was followed except that the concentration used for si-scr and si-p47^{phox} was of 25 nM each.

Luciferase assay

PC-3M-MM2 cells transfected with plasmid and anti-miRs (or siRNAs) were used for this experiment. Transfections were done as described earlier, and luciferase activity was assessed by Dual-Luciferase Reporter Assay kit (Promega, Madison, WI) according to the manufacturer's protocol. Briefly, the cells were harvested using the lysis buffer provided in the kit. 25 μ l of the lysate was mixed with 25 μ l of luciferase assay substrate, and luciferase activity was measured using a Sirius single-tube luminometer (Titertek Instruments, Inc., Huntsville, AL). To measure the activity of the control Renilla luciferase, 25 μ l of stop and glo substrate was then added to the lysate mix, and luminescence was again measured. Renilla luciferase was used for normalization.

H₂DCFDA assay

PC-3M-MM2 cells were cultured on glass coverslips in 6-well culture plates. 24 h later, cells were transfected with either si-scr or si-p47^{phox} for 24 h. Cells were then loaded with 5 μ g/ml of H₂DCFDA (EMD Biosciences, San Diego, CA) in 2 ml of 1 $\times$ PBS. After incubation at 37°C for 20 min, cells were washed with 1 $\times$ PBS. Images were recorded by a confocal microscope (Olympus America, Inc., Melville, NY) at 488 nm. For DPI treatment group, cells were pre-treated with vehicle or DPI (5 μ M) for 30 min before adding the dye.

Protein determination

The levels of protein in samples were determined by Bio-Rad protein detection reagent, using bovine serum albumin to prepare standard curves.

Statistical analyses

Statistical analyses were performed by using analysis of variance followed by Tukey's honestly significant difference *post hoc* test to compensate for multiple pairwise comparisons or by paired Student's *t*-test.

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Author Disclosure Statement

The authors declare that they have no conflicts of interest.

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Abbreviations Used

BPE = bovine pituitary extract
 C-PTIO = 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide
 DAB = diaminobenzidine tetrahydrochloride
 DIC = differential interference contrast
 DPI = diphenyleneiodonium chloride
 ECM = extracellular matrix
 EGF = epidermal growth factor
 ERK1/2 = extracellular signal regulated kinase 1/2
 FBS = fetal bovine serum
 H₂DCFDA = 2',7'-dichlorofluorescein diacetate
 K-SFM = keratinocyte serum free medium
 MAPK = mitogen activated protein kinase
 miRs = microRNAs
 NAC = N-Acetyl-L-cysteine
 PDCD4 = programmed cell death 4
 ROS = reactive oxygen species
 SCID = severe combined immunodeficient
 siRNA = small interfering RNA