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UCP2 Upregulation Promotes PLC γ -1 Signaling During Skin Cell Transformation

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Abstract

Uncoupling protein 2 (UCP2), whose physiological role is to decrease mitochondrial membrane potential and reactive oxygen species (ROS) production, is often overexpressed in human cancers. UCP2 upregulation has recently been proposed as a novel survival mechanism for cancer cells. However, until now, how exactly UCP2 promotes tumorigenesis remains inconclusive. Based on a widely used skin cell transformation model, our data demonstrated that UCP2 differentially regulated ROS. UCP2 upregulation decreased superoxide whereas it increased hydrogen peroxide production with concomitant increase in the expression and activity of manganese superoxide dismutase (MnSOD), the primary mitochondrial antioxidant enzyme. Furthermore, hydrogen peroxide was responsible for induction of lipid peroxidation, and PLC γ -1 activation in UCP2 overexpressed cells. Additionally, PLC γ -1 activation enhanced skin cell transformation, and pharmacological, and siRNA mediated inhibition of PLC γ -1, markedly reduced colony formation, and 3D cell growth. Moreover, hydrogen peroxide scavenger, catalase, suppressed lipid peroxidation, and dampened PLC γ -1 activity. Taken together, our data suggest that (i) UCP2 is an important regulator of mitochondrial redox status and lipid signaling; (ii) hydrogen peroxide might mediate UCP2's tumor promoting activity; and (iii) pharmacological disruption of PLC γ -1 and/or hydrogen peroxide may have clinical utility for UCP2 overexpressed cancers.

Keywords

UCP2; skin cell transformation; oxidative stress; PLC γ -1

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Introduction

Mitochondria, the power house of the cell plays a crucial role in maintaining bioenergetic status of the cell. Decades ago, it was recognized that mitochondria are responsible for reduction of molecular oxygen to generate superoxide [1], and a mounting body of evidence suggests superoxide, along with other forms of ROS (hydrogen peroxides (H₂O₂), hydroxyl radical (*OH), nitric oxide (NO), singlet oxygen (¹O₂), hypochlorous acid (HOCl), and peroxynitrite) modulate various cell functions, cell growth, and variety of cell signaling processes [2,3].

ROS are chemically reactive molecules. Although moderate levels of ROS may promote cell growth, and cell signaling, overproduction of ROS causes damage to lipids, proteins, and DNAs, leading to human disease including cancer [4–7]. Thus, well balanced ROS levels are essential for maintaining normal cell growth, and survival. Cells are equipped with ROS-scavenging systems such as anti-oxidant enzymes (SOD, catalase, GPx, etc.) which are located in both mitochondria, and cytosol. Balancing ROS generation, and ROS elimination to maintain ROS homeostasis is a critical regulator for cell functioning, and cell survival. Not surprisingly, this balance is often dysregulated in cancer cells, leading to abnormal tumor growth [8–10].

ROS, and oxidative stress are intricate processes in cancer. Due to persistent oxidative stress, cancer cells become well-adapted, and develop enhanced resistance, and simply adding ROS-generating agents may not always be sufficient to kill tumor cells [11]. To cope with enhanced oxidative stress, and such an insult to the cell, cancer cells also activate their ROS-scavenging system [12]. This could lead to tumor resistance, and thus, a failure to respond to conventional chemotherapeutic drugs.

Recently, mitochondrial uncoupling protein 2 (UCP2), one of the mitochondrial anion transporter protein, is receiving more attention as a crucial regulator of mitochondrial ROS. UCP2, unlike other members in this family, is ubiquitously expressed, and plays a critical role as an anti-oxidant defense mechanism [13–14]. Recent studies from others' and our lab have shown that UCP2 is upregulated in several human cancers [15]. In cancer cells, UCP2 are thought to be activated by superoxide, and in turn, elevated levels of UCP2 decreases superoxide production via uncoupling, providing growth advantage to tumor cells [16–18]. In addition, UCP2 may also contribute to cancer progression via regulating cancer metabolism [19]. Besides being an anion transporter, UCP2 has been shown to be able to transport TCA cycle C4 metabolites [20]. However, there was no direct evidence supporting the tumor promoting role of UCP2, using genetically modified mice. Using UCP2 knockout, and wild-type mice, our lab showed that knockout of UCP2 reduced skin papilloma, and carcinoma formation in mice, linking UCP2, and tumor promotion [21]. However, till date, the tumor promoting role of UCP2 remains questionable. In the present study, we examined the role of UCP2 in redox regulation, and lipid signaling using a skin cell transformation model, and studied how UCP2 overexpression tuned redox balance, and how it affected downstream lipid signaling.

Materials and Methods

Cell culture and Treatments

Murine skin epidermal JB6 P+ (CL-41) cells (purchased from American Type Culture Collection) were used to study tumor promotion. Cells were routinely monitored for mycoplasma contamination. Human UCP2-containing, and empty pCMV6 vectors purchased from OriGene (catalog number SC320879, and PS100001, respectively), were transfected into JB6 P+ cells, and stable clones were established. The tumor promoter 12-*O*-tetradecanoylphorbol 13-acetate (TPA, purchased from Sigma) was prepared as 1 mM stock solution in DMSO. The stock solutions were diluted in culture media to a final concentration of 5 nM.

Cells were treated with catalase (2000 units/ml, Sigma) to inhibit hydrogen peroxide levels, and PLC γ -1 was inhibited by 5 μ M U73122 (Item No 70740, Cayman Chemicals).

Western blot analysis

Whole Cell Lysates were isolated using RIPA lysis buffer (Santa Cruz Biotechnology). The concentration of the protein samples was determined using Bradford assay. 75 μ g of Whole Cell Lysates were separated by SDS-PAGE gels, and transferred onto PVDF membranes, then were probed with antibodies against PLC γ -1, pPLC γ -1 or UCP2 (Cell Signaling Technology), or MnSOD or β -actin (Santa Cruz). Proteins of interest were detected with HRP-conjugated secondary antibodies (Jackson ImmunoResearch), and visualized with the Pierce ECL Western blotting substrate (Thermo Fisher).

Cell transfection and gene knockdown

Target-specific siRNAs and siRNA transfection reagent (sc-36265, Santa Cruz) were used to inhibit PLC γ -1 expression. Healthy and subconfluent UCP2 overexpressed, and control pCMV cells were transfected for 48 hour before further analysis. A non-targeting siRNA (Santa Cruz) was used to monitor the transfection efficiency. The expression of target genes was further examined by Western blot analysis with specific antibodies.

Anchorage independent assay

Anchorage independent growth assay or the soft agar assay was performed using PLC γ -1 siRNA and control siRNA cells. 0.5% Agar mix (40 ml melted 1.25% agar solution, 40 ml 2 $\times$ EMEM, 10 ml FBS, 10 ml PBS, 1 ml glutamine, 50 μ l penicillin & streptomycin) was prepared, and kept in a 50°C water bath. Bottom agar was prepared by adding DMSO and/or TPA to the 0.5% agar mix. Top agar was prepared by diluting 1 fraction of 1 $\times$ 10⁵ cells/ml single cell suspension with 2 fractions of 0.5% agar mix, and treatments. 2.5 ml of bottom agar and 0.75 ml of top agar was laid into each well of the six-well plates. Cells were incubated in a humidified 37°C, 5% CO₂ incubator for two weeks. Cells were then stained with 0.25 mg/ml neutral red overnight, and the number of colonies were counted, and plotted.

Detection of oxidatively modified proteins (protein carbonyls)

The Oxyblot protein oxidation detection kit (EMD Millipore, S7150) was used to perform the assay. The reaction procedures were conducted according to the manufacturer's instructions. Ten percent SDS-PAGE gels were used for the separation of the mixture of the Whole Cell Lysate (n=8 per group).

Lipid peroxidation measurement

The TBAR assay kit (Item No. 10009055, Cayman Chemicals) was used for measuring lipid peroxidation. The TBARS assay is a well-recognized, established method for quantifying lipid peroxides. The measurement was performed according to the manufacturer's protocol. Samples absorbance was measured using a spectrophotometer at 532 nm. (n=4 per group).

Calorimetric determination of hydrogen peroxide production

Amplex Red hydrogen peroxide assay kit (1679128, Thermo Fisher) was used to measure H_2O_2 production. The measurement was performed according to the manufacturer's manual. The absorbance was measurement at 560 nm using a 96-well plate reader. The measurements with the Amplex Red kit were performed at least three times.

Detection of mitochondrial superoxide generation

Five thousand JB6 cells were seeded in 96-well plates. Mitochondrial superoxide generation was assayed using MitoSOX Red (Cat. M3600, Thermo Fisher). After 24 hours of incubation, cells were treated with DMSO or TPA as indicated above. Twenty-four hours after the treatment, cells were washed with PBS, and incubated in fresh medium containing 10 μ M mitoSOX red dye. The fluorescence intensity was measured at excitation/emission of 530/590 nm using a fluorescence spectrometer.

Measuring the enzymatic activities of MnSOD, GPx and Catalase

MnSOD activity was measured using the MnSOD Activity Assay Kit from Cayman Chemical (Lot 706002). Whole Cell Lysate was prepared similarly to what has been described above except that 50 mM phosphate buffer (pH 7.8, containing proteinase inhibitors) was used to replace the RIPA Buffer. The measurement was performed according to the manufacturer's protocol. MnSOD activity was determined by performing the assay in the presence of potassium cyanide to inhibit CuZn SOD. For measuring GPx activity, the assay buffer contained 1m Tris HCL, 500 mM EDTA, 100 mM NaN_3 , 100 mM GSH, 20mM NADPH, GSH reductatse, and distilled water in a total of 10 mL. Reaction was started by adding substrate buffer containing H_2O_2 . The absorbance was read at 340 nm against suitable reagent blank. Catalase activity was assayed spectrophotometrically according to the method described by Claiborne and Fridovich [22]. The rate of decrease in the absorbance caused by the disappearance of H_2O_2 was recorded for 2 minutes at 240 nM. The reaction mixture contained 20 mM H_2O_2 , 50mM KH_2PO_4 and an appropriate amount of enzyme in 50 mM potassium phosphate buffer (pH 7.0). The reaction is initiated by the addition of H_2O_2 . The absorption coefficient at 240 nm for H_2O_2 was taken to be $43.6 M^{-1} cm^{-1}$.

Cell proliferation assay

JB6 cells were seeded at a 3,000 cells/well density into a 96-well plate. The cells were subjected to overnight incubation in the IncuCyte Essens Bioscience Incubator (Birmingham, UK) under 5% CO₂ and at a temperature of 37°C. The cells were imaged every four hours and the proliferation rates based on confluency were determined using the IncuCyte software.

IP3 and DAG measurements

The measurements of IP3 and DAG were conducted using IP3 and DAG ELISA kits (Cat No. MBS010120 and MBS2603600 respectively, MyBioSource) Control pCMV and UCP2 cells were isolated using ice-cold PBS and sonicated three times for 20 seconds each with at least 30 seconds rest on ice. The cell lysates were incubated on ice for 30 min and then centrifuged at 14,000 rpm for 20 min at 4 °C. The final supernatant was used and the assays were performed according to manufacturer' instructions.

3D spheroid formation

Cells were harvested by trypsinization, counted and 5,000 cells per well were seeded in a 96-well round bottom clear cell repellent plate (Greiner Bio-One) in normal culture media supplemented with 1 % matrigel. Culture plates were centrifuged at 300 × g for 5 min and incubated in XL-3 IncuCyte incubation chamber at 37 °C. Spheroid formed over three days was analyzed using the IncuCyte zoom software.

Confocal microscopic analysis of intracellular calcium

JB6 cells were seeded in 96-well plates at 40,000 cells per 100 µL per well in a black wall/ clear bottom plate for 24 h. Cells were then treated with DMSO and TPA for 24 h. Post treatment, growth medium was removed, and cells were incubated with 100 µl of 4 µM Fluo-8 AM (ab142773, Abcam) at 37 °C for 1 h. Cells were then washed twice with PBS and then imaged with a fluorescence microscope.

Fatty acid oxidation (FAO) assay

FAO rates were measured in real time by the XF24 Extracellular Flux Analyzer (Seahorse Bioscience) according to Harwood et al. [23]. 50,000 cells/well were seeded in unbuffered MEM medium supplemented with 2.5 mM glucose and 0.5 mM L-carnitin, and incubated for 30 min at 37°C in a CO₂-free incubator. Bovine serum albumin alone or conjugated to palmitate (Seahorse Bioscience) was added to each well just prior the assay, to a final concentration of 33 and 200 µM, respectively. Oxygen consumption rates were measured for basal state and following the sequential injection of oligomycin (1 µM), carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) (0.4–0.8 µM), and a mix of antimycin A (1 µM) and rotenone (1 µM).

Statistical analysis

All experiments were repeated at least three times and each experiment group included at least three samples. Multiple group comparisons were analyzed by two-way analysis of variance (ANOVA). Data were presented as mean ± standard error (S.E.M.). Statistical

software SAS 9.4 (SAS Institute Inc) was used for all data analysis. p value < 0.05 was regarded as statistically significant.

Results

UCP2 differentially regulated ROS production

It is well known that elevated levels of ROS are implicated in cancer, and uncoupling proteins are associated with mitochondrial ROS production. Superoxide is shown to activate UCP2, and in turn, elevated UCP2 decreases superoxide via a negative feedback loop [24–25]. In this study, we examined whether upregulation of UCP2 could decrease ROS production using a skin cell transformation model - JB6 P+ cells. JB6 cells were stably transfected with a UCP2 expression vector or the empty base vector (pCMV6) (unpublished data). We measured the levels of mitochondrial superoxide, and hydrogen peroxide generation in these cells. As shown in Figure 1, measurement of mitochondrial superoxide by the fluorescent probe MitoSox Red (A), and hydrogen peroxide by Amplex Red (B) revealed that UCP2 upregulation decreased superoxide levels whereas it increased hydrogen peroxide levels. To understand this observation, we measured MnSOD, catalase and GPx activities in UCP2 over-expressed and the pCMV vector control cells. As shown in the Figure 1C–F, both MnSOD expression (C) and activity levels (D) were increased in UCP2 overexpressed cells. This increased MnSOD expression and activity suggests an increased superoxide dismutation, which is supported by the elevated hydrogen peroxide levels. However, since GPx and catalase (Figure 1E&F) activities were not significantly different between the control pCMV vector and UCP2 overexpressed cells, which might be indicative of a shifted antioxidant response in UCP2 overexpressed cells and may, therefore, be a major contributing factor for UCP2's tumor promoting activity. In Figure 1D, the tumor promoter TPA treatment increased MnSOD expression but not activity in the control cells, which is consistent with what we have described previously [26]. Therefore, our study provides direct evidence in support of the role for UCP2 as a regulator of mitochondrial redox balance and these data suggest that UCP2 differentially regulates the levels of mitochondrial superoxide and hydrogen peroxide in favor of tumorigenesis.

UCP2 enhanced lipid and protein oxidation

Hydrogen peroxide is highly stable and a highly reactive agent. Under certain conditions hydrogen peroxide can react with a variety of cellular components including phospholipid cell membrane, DNA and proteins. It can freely diffuse out of the mitochondria into the cytosol, causing lipid and protein oxidation [27]. Lipid peroxidation disrupts the normal structure and permeability of membrane and dramatically alters cell integrity [28]. Malondialdehyde (MDA) the end-product of lipid peroxidation, appears to be the most mutagenic and an effective marker for lipid peroxidation [29]. Hence, MDA production is a marker for lipid peroxidation in pCMV and UCP2 cells using the thiobarbituric acid (TBARS) assay. As shown in the Figure 2A, higher levels of MDA were observed after TPA treatment, and UCP2 over-expression enhanced this increase, which is in agreement with elevated levels of hydrogen peroxides. Similar trends were also observed in the levels of oxidatively modified proteins (protein carbonyls), which show increased oxidation levels of multiple proteins (Figure 2B).

UCP2 overexpressed enhanced PLC γ -1 signaling

To identify the potential signaling molecules regulated by UCP2 during skin cell transformation, a protein microarray has been performed previously [21]. Interestingly, as shown in Figure 3A, PLC γ -1 (Phospholipase C gamma-1) was activated (as the measurement of Phospho-Tyr771) in the skin tumor samples of the wild-type mice, but not in the UCP2 knockout mice. PLC γ -1 is ubiquitously expressed, and plays an important role in regulating lipid signaling, cell proliferation, and differentiation [30–31]. PLC γ -1 can be activated in response to oxidant injury, and cellular stress, and once activated, PLC γ -1 cleaves membrane phospholipid generating two second messengers, DAG and IP3, further stimulating intracellular calcium [32]. In the same protein array study, the calcium binding protein Calmodulin also was found to be increased in the tumor tissues of the wild-type, but not in the UCP2 knockout mice (Figure 3B). Although the physiological role of PLC γ -1 activation in tumor cell is unclear, studies have demonstrated that PLC γ -1 is often overexpressed in cancers [33–37]. To detect whether UCP2 overexpression enhanced PLC γ -1 expression, we examined the protein levels of PLC γ -1 and its phosphorylation status in UCP2 over-expressed and control pCMV vector cells. Since PLC γ -1 is a tyrosine kinase substrate, its activation requires the phosphorylation of tyrosine (Y) residue. Hence, we measured PLC γ -1 phosphorylation (Y783) using a phosphotyrosine-specific antibody. As shown in Figure 3C, PLC γ -1 and pPLC γ -1 expression were indeed enhanced in UCP2 overexpressed cells, and in response to TPA treatment. To verify the activation of PLC γ -1, we measured its downstream targets. Both IP3 and DAG were elevated after TPA treatment, and UCP2 overexpressed enhanced these increases (Figure 4A&B). Therefore, intracellular calcium levels were increased in UCP2 overexpressed cells with or without TPA treatment as measured by either ELISA (Figure 4C) or fluorescent imaging (Figure 4D&E).

UCP2-induced hydrogen peroxide is essential for PLC γ -1 activation and lipid signaling

Since high levels of H₂O₂ play an important role in the onset of lipid peroxidation and downstream PLC γ -1 signaling, we wanted to detect whether removal of H₂O₂ suppressed lipid peroxidation and PLC γ -1 activation. Accumulating evidence suggests that an increase in cellular levels of H₂O₂ is implicated in tumor development, progression and metastasis [38–40]. It is not surprising therefore, that an increase in the levels of H₂O₂ -detoxifying enzymes could inhibit tumorigenesis. Hence, to detect whether removal of H₂O₂ suppressed lipid peroxidation and PLC γ -1 activation, catalase one the common and longest known enzyme for the removal of H₂O₂ was added to the cultured UCP2 overexpressed cells similarly to what has been described previously [41–43]. Addition of exogenous catalase to the growth culture media significantly inhibited the levels of hydrogen peroxide (Figure 5A), lipid peroxidation (Figure 5B), the increased expression of PLC γ -1 and pPLC γ -1 (Figure 5C), and its downstream targets IP3 and DAG (Figure 5D&E). 3D spheroid growth was also inhibited by adding catalase (Figure 5F). These data suggest that UCP2-induced H₂O₂ stimulates phosphorylation and activation of PLC- γ 1 and is essential for downstream PLC γ -1 mediated signaling.

Inhibition of PLC γ -1 suppressed cell proliferation, colony formation and 3D culture of UCP2 cells *in vitro*

To study the physiological relevance of PLC γ -1 activation in UCP2 overexpressed cells, and to determine the importance of PLC γ -1 in skin cell transformation, we inhibited PLC γ -1 expression by either the siRNA approach or by the PLC γ -1 inhibitor U73122. Using anchorage independent growth assay on control and siRNA transfected cells, we found that inhibition of PLC γ -1 (Figure 6A) significantly decreased the number of colonies formed in soft agar (Figure 6B). Inhibition of PLC γ -1 significantly decreased cell proliferation (Figure 6G). Similarly, inhibition of PLC γ -1 with U72133 (Figure 6C) significantly decreased the 3D spheroid formation (Figure 6F), and suppressed the downstream targets IP3 and DAG levels (Figure 6D&E), suggesting that PLC γ -1 could be a dominant pathway in UCP2-induced tumor promotion.

Knockdown of PLC γ -1 inhibited fatty acid oxidation in UCP2 overexpressed cells

It is well known that cancer cells rewire their metabolism to support their rapid growth [44]. While the dependence of tumor cells on aerobic glycolysis leading to lactate production (the Warburg effect) has been well characterized in cancer research [45–50], little is known about the effect of fatty acid oxidation (FAO) in cancer cells. Fatty acids are a rich source of energy that can produce nearly two times more ATP than sugar. Energy is released from fatty acids mainly through β -oxidation (FAO) which occurs in the mitochondrial matrix. The rate of β -oxidation is in turn controlled by intracellular concentration of free fatty acids. New findings reveal that fatty acid oxidation promotes cancer cell proliferation and survival [51–52]. Since UCP2 is a critical regulator of energy metabolism, we investigated the impact of UCP2 overexpression on FAO. The rate of palmitate oxidation was measured using control pCMV and UCP2 overexpressed cells by the Seahorse XF analyzer. As shown in Figure 7A, UCP2 overexpressed cells oxidized more palmitate, suggesting that UCP2 upregulation enhanced fatty acid oxidation. Additionally, to determine whether this enhanced FAO was a result of PLC γ -1 upregulation, we used siRNA to knockdown PLC γ -1 in UCP2 overexpressed cells. As shown in Figure 7B, small interfering RNA (siRNA)-mediated knockdown of PLC γ -1 suppressed the FAO in UCP2 overexpressed cells. Therefore, this suggests that PLC γ -1 promotes lipolysis in UCP2 overexpressed cells.

Discussion

Cancer cells often adapt their metabolism to suit their growth requirements, and metabolic reprogramming is one of the hallmarks of cancer [53]. More emphasis is being made on the avariciousness of cancer cells towards enhanced glycolysis (the Warburg Effect), enhanced glutamine oxidation, and fatty acid metabolism, thus, integrating multiple aspects of cancer metabolism, and bioenergetics [54–57]. Since mitochondria play important roles in maintenance of cell growth, cell proliferation, and development, much attention has been dedicated towards mitochondrial dysfunction in cancer [58]. Deregulated cell proliferation, survival and death are often associated with cancer. Since tumor cells employ metabolic pathways to promote their growth and survival, metabolic reprogramming is firmly established as a hallmark of cancer cells [49,59].

Here we focus on the effects of UCP2 overexpression on mitochondrial redox regulation and metabolic regulatory networks. ROS are produced in mitochondria as byproducts of oxidative phosphorylation. Superoxide, the primary and the most abundant ROS is generated at complex I and complex III of the ETC. Superoxide is readily dismutated to hydrogen peroxide in the mitochondrial matrix by the enzyme MnSOD. H_2O_2 , a bona fide second messenger, can diffuse into cytosol causing damaging to biological tissues [60–63]. Elevated levels of ROS have been detected in almost all tumor cells [64–65]. ROS are involved in both the initiation, and progression of cancers [66]. However, some tumor cells also express high levels of antioxidant enzymes [67–70]. Therefore, a delicate balance between pro-oxidants and anti-oxidants is optimal.

UCP2, a mitochondrial uncoupling protein, functions as an adaptive antioxidant defense to protect against ROS. In fact, attenuation of ROS is the physiological role of UCP2 and interestingly, UCP2 is overexpressed in human cancers. We have previously demonstrated that knockout of UCP2 significantly suppressed tumor growth *in vivo* [21], suggesting that UCP2 up-regulation may promote tumorigenesis. Nevertheless, the mechanistic role of UCP2 overexpression in cancer still remains unclear. Hence, to effectively target such cancers, understanding the fine tuning of intracellular ROS signaling by UCP2 is of utmost importance. The significance of elevated ROS levels, oxidative stress and oxidative damage to macromolecules is well recognized in carcinogenesis. In addition to the classical view of free radicals causing mutations and, hence, evolution of cancer, many signaling pathways are directly activated by free radicals leading to enhanced cell proliferation, differentiation and tumorigenesis [71]. Using JB6 cell lines that overexpress UCP2, we showed that UCP2 differentially regulates superoxide, and hydrogen peroxide during skin cell transformation. An interesting result of our study is that UCP2 overexpression decreases superoxide production but increases hydrogen peroxide with a concomitant increase in MnSOD expression, and activity. Dichotomy of MnSOD in cancer is very interesting, particularly because it may be viewed both as a tumor suppressor and a tumor promoter [72–75]. Based on consistent reports, MnSOD seems to have a dual role in cancer. Abundant evidence suggests MnSOD is essential for life. Various studies have shown that complete knockout of MnSOD is embryonically lethal in mice [76–77] while various other studies have demonstrated that overexpression of MnSOD have far-reaching implications in cancer [78]. Hydrogen peroxide, a product of MnSOD, has also been shown to play important roles in controlling cancer cell proliferation, differentiation, and cell cycle [70]. While the role of elevated hydrogen peroxide in cancer have yielded conflicting results, and there is a possibility that hydrogen peroxide is protective against cancer [79]; our results provide direct evidence in support of the concept that high levels of MnSOD, and increased hydrogen peroxide serves as the tumor promoting mechanism of UCP2. It would be further interesting to study further if by specifically targeting MnSOD, treatments may develop to inhibit UCP2 overexpression in cancers and, thereby, diminish tumorigenesis. Moreover, despite the increase in MnSOD, and hydrogen peroxide, it appears that catalase and GPx remain unresponsive to the increase in hydrogen peroxide. This lack of antioxidant protection elicited from catalase and GPx, therefore, gives evidence for the shift of antioxidant response in UCP2 overexpressed cells. Furthermore, high levels of H_2O_2 can stimulate lipid peroxidation and lipid signaling and is detrimental to biological molecules.

Similarly, the present study supports hydrogen peroxide as a contributor to lipid peroxidation, subsequent PLC γ -1 activation, and downstream lipid signaling. We further studied the role of PLC γ -1 activation in UCP2 overexpressed cells, and we demonstrated for the first time that UCP2 upregulation induced PLC γ -1 signaling during skin tumorigenesis, and knockdown of PLC γ -1 suppressed colony formation, and 3D growth *in vitro*. Taken together, it is possible that UCP2-induced H₂O₂ acts as a second messenger for PLC γ -1 activation and downstream lipid signaling. Through these studies, we will be one step closer to understanding how UCP2 fine-tunes mitochondrial ROS levels in favor of cell transformation. In summary, our results demonstrate that hydrogen peroxide and PLC γ -1 activation could play a critical role in mediating UCP2's action. Therefore, manipulation of hydrogen peroxide may be a therapeutic utility in UCP2 overexpressed cancers.

Acknowledgments

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Abbreviations

AP-1	activator protein 1
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
FBS	fetal bovine serum
FCCP	carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazine
genipin	methyl (1S,2R,6S)-2-hydroxy-9-(hydroxymethyl)-3-oxabicyclo[4.3.0]nona-4,8-diene-5-carboxylate
PGx	glutathione peroxidase
IP3	Inositol triphosphate
MDA	malondialdehyde
MnSOD	manganese superoxide dismutase
PBS	phosphate buffered saline
PLCγ-1	phospholipase C gamma 1
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBARS	thiobarbituric acid
TPA	12- <i>O</i> -tetradecanoylphorbol 13-acetate

ROS	reactive oxygen species
UCP2	uncoupling protein 2

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Figure 1. UCP2 differentially regulates ROS production

Control pCMV and UCP2 overexpressed JB6 P+ cells were treated with TPA (5 nM) or vehicle control (DMSO) for 24 h. Superoxide levels (**A**) were measured by quantifying the fluorescence of the dye MitoSox and hydrogen peroxide levels (**B**) were measured with the Amplex Red Hydrogen Peroxide Kit. (**C**) Measurement of manganese superoxide dismutase expression levels. The MnSOD/GAPDH bands were detected on the same gel but the lanes were not adjacent. The ratios of MnSOD/GAPDH were presented. MnSOD (**D**), GPx (**E**) and Catalase (**F**) activities were measured in pCMV and UCP2 cells.

N=4 per group, data were presented as the mean $\pm$ SD. *, $p < 0.05$ when compared with its own control (except in Figure 1A); #, $p < 0.05$ when compared with the control/TPA group.

Figure 2. UCP2 enhanced lipid and protein oxidation

Detection of lipid peroxidation by the MDA assay (**A**, N=4 per group) and protein oxidation by Oxiblot (**B**) in UCP2 overexpressed and control JB6 cells. These cells were treated with TPA (5 nM) or vehicle control (DMSO) for 24 h. For Oxiblot, the bands were detected on the same gel but the lanes were not adjacent. *, $p < 0.05$ when compared with the its own control group. #, $p < 0.05$ when compared with the control/TPA group.

Figure 3. UCP2 upregulation enhanced PLCγ-1 activation

UCP2 knockout mice and wild-type (WT) mice were subjected to DMBA/TPA-induced skin carcinogenesis, and skin epidermal tissues were collected for this protein array analysis (21). Detection of p-PLCγ-1 (**A**) and Calmodulin (**B**) levels in skin tissue lysates. *, $p < 0.05$ when compared to the WT-DMSO group. (**C**) Detection of PLCγ-1 and phospho PLCγ-1 expression levels in UCP2 overexpressed and control JB6 cells. These cells were treated with TPA (5 nM) or vehicle control (DMSO) for 24 h. SDHB served as the loading control.

Figure 4. UCP2 enhanced PLC- γ -1 signaling

The levels of IP3 (A), DAG (B), and intracellular calcium either by spectrophotometer (C) or fluorescent imaging (D) (Fluorescent imaging quantification results shown in E) levels in UCP2 overexpressed cells and control JB6 cells. These cells were treated with TPA (5 nM) or vehicle control (DMSO) for 24 h. N=3 per group, data were presented as the mean $\pm$ SD. *, $p < 0.05$ when compared with its own control group. #, $p < 0.05$ when compared with the control/TPA group.

Figure 5. Hydrogen peroxide was critical for PLCγ-1 activation in UCP2 overexpressed cells
Removal of hydrogen peroxide by catalase (A) suppressed lipid peroxidation (B, N=4 per group), PLCγ-1 induction (C, Fra-I is a subunit of the activator protein 1, which is activated during skin carcinogenesis) and the levels of its downstream targets: IP3 (D, N=3 per group) and DAG (E, N=3 per group), as well as 3D spheroid formation (F, N=6 per group) of UCP2 overexpressed cells. *, p<0.05 when compared with its own control group. #, p<0.05 when compared with the control/TPA group.

Figure 6. Inhibition of PLC γ -1 suppresses cell proliferation and colony formation of UCP2 overexpressed cells

Knockdown of PLC γ -1 by the siRNA approach (A) suppressed anchorage-independent growth (B) of UCP2 overexpressed cells (N=3 per group). The PLC γ -1 inhibitor U73122 suppressed PLC γ -1 expression (C), the levels of its downstream targets: IP3 (D, N=3 per group) and DAG (E, N=3 per group), as well as 3D spheroid formation (F, N=6 per group) of UCP2 overexpressed cells. (G) Knockdown of PLC γ -1 by a second set of siRNA significantly decreased cell proliferation of UCP2 overexpressed cells. A Two-way ANOVA revealed significance effects. *, indicates significant differences ($p < 0.001$) between control TPA and control DMSO group. #, indicates significant differences ($p < 0.001$) between siRNA PLC γ -1 DMSO and control DMSO group. +, indicates significant differences ($p < 0.001$) between siRNA PLC γ -1 TPA and control DMSO group. For Figure 6C–E, UCP2 cells were treated with TPA (5 nM) or vehicle control (DMSO) for 24 h. *, $p < 0.05$ when compared with its own control group. #, $p < 0.05$ when compared with the control/TPA group.

Figure 7. Knockdown of PLC γ -1 inhibited fatty acid oxidation in UCP2 overexpressed cells
(A) FAO was significantly higher in UCP2 overexpressed cells with palmitate compared to control pCMV cells. **(B)** siRNA knockdown of PLC γ -1 inhibited fatty acid oxidation in UCP2 overexpressed cells. *, $p < 0.0001$ by Two-way ANOVA. Results were the representatives of three independent experiments.