Phosphatase and Tensin Homolog Deleted on Chromosome 10 (PTEN) Signaling Regulates Mitochondrial Biogenesis and Respiration via Estrogen-related Receptor α (ERRα)*

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Background: Aberrant PTEN/PI3K signaling and mitochondrial abnormalities are commonly associated with cancer. PTEN loss and activation of PI3K/protein kinase B up-regulate ERRα, increase mitochondrial mass, and induce a metabolic pattern similar to the “Warburg effect.”
Results: PTEN loss and activation of PI3K/protein kinase B up-regulate ERRα, increase mitochondrial mass, and induce a metabolic pattern similar to the “Warburg effect.”
Conclusion: PTEN/PI3K signaling controls mitochondrial mass and function by regulating ERRα through the AKT/CREB axis.
Significance: This study establishes a novel link between oncogenic signaling and dysregulated mitochondrial metabolism.

Mitochondrial abnormalities are associated with cancer development, yet how oncogenic signals affect mitochondrial functions has not been fully understood. In this study, we investigate the relationship between mitochondrial alterations and PI3K/protein kinase B (AKT) signaling activation using hepatocytes and liver tissues as our experimental models. We show here that liver-specific deletion of Pten, which leads to activation of PI3K/AKT, is associated with elevated oxidative stress, increased mitochondrial mass, and augmented respiration accompanied by enhanced glycolysis. Consistent with these observations, estrogen-related receptor α (ERRα), an orphan nuclear receptor known for its role in mitochondrial biogenesis, is up-regulated in the absence of phosphatase and tensin homolog deleted on chromosome 10 (PTEN). Our pharmacological and genetic studies show that PI3K/AKT activity regulates the expression of ERRα and mitochondrial biogenesis/respiration. Furthermore, cAMP-response element-binding protein, as a downstream target of AKT, plays a role in the regulation of ERRα, independent of PKA signaling. ERRα regulates reactive oxygen species production, and ERRα knockdown attenuates proliferation and colony-forming potential in Pten-null hepatocytes. Finally, analysis of clinical datasets from liver tissues showed no negative correlation between expressions of ERRα and PTEN in patients with liver cancer. Therefore, this study has established a previously unrecognized link between a growth signal and mitochondrial metabolism.

A hallmark of tumor cell metabolism is the elevation of glycolysis rate even in the presence of oxygen (1). Although this increase is not always coupled with a decrease in mitochondrial respiratory function, clinical studies have reported altered mitochondrial function and copy numbers in human cancers (2). Indeed, mitochondrial function is crucial for the transformation of tumor cells (3). Most tumor cells maintain functional mitochondria that are able to process oxidative phosphorylation (4). Enhanced mitochondrial function is also not uncommon in tumors (5–7). Particularly, recent evidence using immunohistochemistry and laser dissection suggests that tumor cells are highly dependent on mitochondrial respiration, whereas the surrounding stromas often rely on glycolytic respiration with low expression of mitochondrial genes (8, 9). One consequence of the enhanced mitochondrial respiration is the buildup of reactive oxygen species (ROS) (2), the byproduct of mitochondrial respiration and oxidative phosphorylation. By acting as both mutagens and mitogens, ROS is capable of inducing oncogenic transformation and promoting carcinogenesis and is proposed to play major roles in tumor cell transformation, progression, and metastasis (10). Growth factor and mitogenic signals that promote cancer cell growth and survival have been linked to altered mitochondrial functions and generation of ROS (11–16). However, the molecular mechanism of whether and how the aberrant growth factor/mitogenic signals in tumor cells may control mitochondrial mass and respiration still remains unclear.

In this study, we investigate the role of PI3K signals in the regulation of mitochondrial function. We have previously shown that mice lacking phosphatase and tensin homolog

2 The abbreviations used are: ROS, reactive oxygen species; ERRα, estrogen-related receptor α; PTEN, phosphatase and tensin homolog deleted on chromosome 10; PGC-1α, peroxisome proliferator-activated receptor γ coactivator 1α; CREB, cAMP-response element-binding protein; OCR, oxygen consumption rate; ECAR, extracellular acidification rate; IGF-1, insulin-like growth factor-1; FCCP, 7-(and-6)-chloromethyl-2,7’-dichlorodihydrofluorescein diacetate, acetyl ester; qPCR, quantitative PCR; cyt c, cytochrome c.
deleted on chromosome 10 (PTEN), the negative regulator of PI3K, develop liver steatosis and cancer (17, 18); both of these diseases have been reported to associate with mitochondrial alterations (19). High oxidative stress conditions also accompanied the steatosis and cancer phenotypes in this model. Here, we investigate whether mitochondrial functional regulation by PI3K/PTEN signals contributed to the oxidative stress condition and tumor transformation phenotypes. We also investigate the molecular mechanism for the PTEN-regulated mitochondrial functional alteration. Together, our study established a novel mechanism PTEN Signaling and Mitochondrial Metabolism.

**EXPERIMENTAL PROCEDURES**

*Animals—* PtenloxP/loxP; Alb−Cre+ (Pten-null, Pm) and PtenloxP/loxP; Alb−Cre− (control, Con) mice were previously described (18). All experimental procedures were conducted according to the Institutional Animal Care and Use Committee guidelines of the University of Southern California.

*Cell Culture—* Primary hepatocytes were isolated from livers of Con and Pm mice. Immortalized hepatocytes were previously described (20). Hepatocytes were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Mediatech) supplemented with 10% FBS (Atlas Biologicals), 5 μg/ml insulin (Sigma), and 10 ng/ml epidermal growth factor (Invitrogen).

*Reagents, Plasmids, siRNAs, and shPKA-expressing Lentivirus—* LY294002 and IGF-1 were purchased from Cell Signaling Technology. Antibodies against PTEN, p-AKT, CREB, and p-CREB were previously described (20). Two shPTEN targeting sequences, 5′-AGAGATCGTTAGCAGAAA-3′ (shPTEN1) and 5′-GATCTTGAACATGGCA-3′ (shPTEN2), were inserted into p-IRES CA-AKT, WT-AKT, DN-AKT, pSG5-WT PTEN and control vectors, p-IRES and pSG5, were previously described (20). Two shPTEN targeting sequences, 5′-AGAGATCGTTAGCAGAAA-3′ (shPTEN1) and 5′-GATCTTGAACATGGCA-3′ (shPTEN2), were inserted into vector pSilencer 3.1 Neo U6 (Invitrogen) and transformed with DH5α competent cells (Invitrogen). The sequences for siERRα are 5′-ATCGAGAGATAGTGGTCACCATCAG-3′ and 5′-GCCGACTAAATCAAG-3′. Positive plasmids were amplified by the plasmid midi kit (Qiagen). Luciferase reporter constructs, pATPSynB/−385Luc and pCytC/−686Luc, were generous gifts from Kralli and coworkers (21). The shPKA CA-expressing lentivirus was the generous gift from Dr. Wei Li (Keck School of Medicine, University of Southern California) (22).

*Mitochondrial Staining, ROS Staining, and Flow Cytometry—* Cells were grown on plates to 80% confluence and stained with 50 nM MitoTracker Red (Invitrogen, Molecular Probes) or 25 nM MitoTracker Green (Invitrogen, Molecular Probes) or 1 μM H2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (Invitrogen, Molecular Probes) for 20 min at 37 °C. For MitoTracker Red staining, cells were analyzed under a fluorescent microscope, and the relative mitochondrial area was measured using UN-SCAN-IT software. For MitoTracker Green and H2DCFDA staining, cells were then washed in PBS, trypsinized, and analyzed with a flow cytometer (BD Biosciences). Data were collected from 10,000 cells from each sample and analyzed with the software WinMDI.

*Mitochondrial Respiration Measurement—* Cells were stained with 250 nM MitoTracker Red (Invitrogen, Molecular Probes) or 25 nM MitoTracker Green (Invitrogen, Molecular Probes) or 1 μM H2DCFDA (5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (Invitrogen, Molecular Probes) for 20 min at 37 °C. Cells were analyzed using a fluorescent microscope, and the relative mitochondrial area was measured using UN-SCAN-IT software.

*Cell Lysate Preparation and Luciferase Assay—* Cell lysate preparation and luciferase activity measurement were done according to the manufacturer’s instructions (Promega, E1910).

*Western Blot and Immunoprecipitation—* Western blot and immunoblot analysis were described previously (20). Antibodies against PTEN, p-AKT, CREB, and p-CREB were purchased from Cell Signaling Technology. Antibodies against ERRα and PGC-1α were obtained from Abcam. Anti-actin was purchased from Sigma. p-AKT substrate antibody was from Cell Signaling Technology. For immunoprecipitation assays, p-AKT substrate antibody was incubated with cell extracts overnight. 30 μl of protein A beads (GE Healthcare) was then used to pull down p-AKT substrate antibody complexes (4 °C for 3 h). The lysates were spun down and washed five times with cell lysis buffer. Western immunoblotting was then performed.

*Seahorse XF-24 Metabolic Flux Analysis—* Seahorse XF-24 Metabolic Flux Analysis was performed using Seahorse-XF Analyzer (Agilent). Seahorse XF-24 Metabolic Flux Analysis was performed using Seahorse-XF Analyzer (Agilent). Seahorse XF-24 Metabolic Flux Analysis was performed using Seahorse-XF Analyzer (Agilent). Seahorse XF-24 Metabolic Flux Analysis was performed using Seahorse-XF Analyzer (Agilent).

*RNA Isolation, Reverse Transcription, and Quantitative Real Time PCR—* RNA isolation and reverse transcription were performed using Trizol (Invitrogen). Reverse transcription was performed using Moloney murine leukemia virus-reverse transcriptase system (Promega). Quantitative real-time PCR was performed using SYBR Green qPCR Master Mix (Fermentas) and 7900 HT fast real-time PCR system (Applied Biosystems). Gene-specific primers are as follows: ERRα forward 5′-CAAGAGCATCCCAGGCTT-3′ and reverse 5′-TCCCCAGATGATGCCT-3′; PGC-1α forward 5′-CAACACCCTGCCCAG-3′ and reverse 5′-GGTTGTTTCAC-3′; cyclophilin c forward 5′-CAGGCGAGAATTCA-3′ and reverse 5′-GGAAACTTGATGC-3′; GADPH forward 5′-GGTCAAGAGGAGAATTCA-3′ and reverse 5′-GGTTTCTGCACAGTGA-3′; and GAPDH forward 5′-GGTCAAGAGGAGAATTCA-3′ and reverse 5′-GGTTTCTGCACAGTGA-3′.

Plasmids and siRNA Transfections—Plasmids and siRNA transfections were conducted with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. 1 × 105 cells were plated in each well of 6-well plates 24 h before transfection. 4 μg of DNA and 10 μl of Lipofectamine were delivered into the cells. The combination of 100 pmol of siRNA and 5 μl of Lipofectamine was used for siRNA transfection.

Luciferase Reporter Assay—1 × 105 cells were plated in each well of 6-well plates 24 h before transfection. Luciferase constructs containing firefly luciferase driven by cytochrome c or ATP synthase β promoter and luciferase construct containing Renilla luciferase driven by thymidine kinase promoter (internal control) were cotransfected into the cells. The cell lysate preparation and luciferase activity measurement were done according to the manufacturer’s instructions (Promega, E1910).

Western Blot and Immunoprecipitation—Cell lysate preparation and immunoblot analysis were described previously (20). Antibodies against PTEN, p-AKT, CREB, and p-CREB were purchased from Cell Signaling Technology. Antibodies against ERRα and PGC-1α were obtained from Abcam. Anti-actin was purchased from Sigma. p-AKT substrate antibody was from Cell Signaling Technology. For immunoprecipitation assays, p-AKT substrate antibody was incubated with cell extracts overnight. 30 μl of protein A beads (GE Healthcare) was then used to pull down p-AKT substrate antibody complexes (4 °C for 3 h). The lysates were spun down and washed five times with cell lysis buffer. Western immunoblotting was then performed.

Seahorse XF-24 Metabolic Flux Analysis—Seahorse XF-24 Metabolic Flux Analysis was performed using Seahorse-XF Analyzer (Agilent). Seahorse XF-24 Metabolic Flux Analysis was performed using Seahorse-XF Analyzer (Agilent). Seahorse XF-24 Metabolic Flux Analysis was performed using Seahorse-XF Analyzer (Agilent). Seahorse XF-24 Metabolic Flux Analysis was performed using Seahorse-XF Analyzer (Agilent).
Statistical Analysis—Data in this study are presented as mean ± S.E. Differences between individual groups were analyzed by Student’s t test, with two-tailed p value, and <0.05 was considered statistically significant.

RESULTS

Levels of Reactive Oxygen Species in Hepatocytes Are Associated with PTEN Status—ROS are produced during various cellular processes and serve distinct biological functions (23). Depending on the levels, ROS has been proposed to promote cell growth and induce cell death (24). In mouse models where Pten is specifically deleted in the liver, we observed increased oxidative stress conditions, indicated as elevated H2O2 levels and trans-4-hydroxy-2-nonenal production (17). The expression patterns of glutathione peroxidase and GST differed likely due to the presence of different oxidative stress triggers at each stage of the phenotype development (25). Levels of the oxidized form of glutathione (GSSG) in the Pten null livers (0.52 ± 0.02 μM/g) are 2-fold higher than levels observed in the control livers (0.26 ± 0.008 μM/g) at 18 weeks, further confirming the presence of oxidative stress conditions. In addition, activity of thioredoxin reductase is also increased (123.4 ± 10.3 versus 91.8 ± 11.2 μM/min/mg, n = 4) in the Pten null livers, further indicating higher oxidative stress conditions. To determine whether the production of ROS is a direct consequence of PTEN loss or is secondary due to other cellular process changes occurring in the liver, we down-regulated PTEN in immortalized hepatocytes (20) established from the control mice. Inhibition of PTEN with two independent shRNAs led to increased ROS production in the hepatocytes (Fig. 1C), indicating that PTEN status is a determining factor for ROS levels.

PTEN and PI3K/AKT Signals Regulate Mitochondrial Respiration and Glycolysis in Hepatocytes—The major source of ROS in the cells is the mitochondria, which are the primary sites of aerobic respiration. To study whether the functions of mitochondria are affected by PTEN status, we determined the cellular respiration rate (OCR) in primary (Fig. 2, A and B) and immortalized hepatocytes (Fig. 2, C and D) from livers of Con and Pm mice. Primary hepatocytes from Pten-null mice exhibited markedly elevated OCR rates. Both basal and maximal OCRs were elevated when Pten was deleted. Maximal OCR was determined by the addition of FCCP, used to uncouple respiration from ATP production. An approximate 2-fold increase in the basal OCR and a 30% elevation in maximal OCR were
observed in Pten-null primary hepatocytes compared with the controls (Fig. 2, A and B). Similar inductions in immortalized hepatocytes were observed when Pten is deleted (Fig. 2, C and D), suggesting that the immortalized hepatocytes can be used to investigate the relationship between PTEN signal and mitochondrial respiration.

The rate of ECAR, an indicator of lactate levels in the culture produced from glycolysis, was simultaneously determined with OCR. Oligomycin treatment, which inhibits mitochondrial function, led to increased ECAR in Pten control and null cells, suggesting that glycolysis and mitochondrial activities remain coupled in response to metabolic alterations regardless of PTEN status. However, PTEN loss was able to induce both glycolysis (ECAR) and mitochondrial respiration (OCR) simultaneously. ECAR rate was 2–3-fold higher when PTEN was deleted in both primary (Fig. 2E) and immortalized (Fig. 2F) hepatocytes at base line and throughout the experiment. These observations suggest that PTEN and PTEN-regulated signals

![Figure 2](image-url)

**FIGURE 2. Increased mitochondrial respiration and glycolysis rate in Pten-deficient hepatocytes.** A, basal (first four time points) and maximal (with FCCP addition) oxygen consumptions (OCR) are higher in Pm primary hepatocytes versus Con. OCR was measured (Seahorse XF24 analyzer) in primary hepatocytes derived from Pten Con and null (Pm) mouse livers. B, quantification of base-line OCR in Pm and Con primary hepatocytes. C, basal and maximal OCR are increased in Pm immortalized hepatocytes. OCR was measured in Pm and Con immortalized hepatocytes. D, quantification of base-line OCR in Pm and Con immortalized hepatocytes. E and F, ECAR (indication of glycolysis rate) is constantly higher in both Pm primary and immortalized hepatocytes. ECAR was simultaneously measured with OCR. Oligomycin (1 μM), FCCP (1 μM), and rotenone (1 μM) were added sequentially after measuring basal OCR to inhibit ATP synthesis capacity, uncouple ATP synthesis and mitochondrial respiration, and completely block the respiration of mitochondrial, respectively. *, p ≤ 0.05, different from Con. n = 5.
may control mitochondrial respiration and glycolysis rate in hepatocytes independent of their metabolic coupling. This metabolic feature is highly similar to the “Warburg effect,” where accelerated glycolysis in the presence of high oxidative phosphorylation confers tumor cell growth advantage by supplying the necessary macromolecules for biosynthesis (26).

To confirm whether PTEN directly regulates mitochondrial function, we reduced PTEN expression using sequence-specific shRNA in immortalized Con hepatocytes. Knockdown of PTEN resulted in increased mitochondrial respiration compared with scrambled shRNA control (Fig. 3, left panels). Baseline OCR was ~2.5-fold higher when shRNA, against PTEN, was introduced, compared with the scrambled shRNA group (Fig. 3, right panels). Similarly, maximum OCR was also higher when PTEN was inhibited, suggesting that PTEN was responsible for regulating mitochondrial respiration in hepatocytes.

To explore the signals downstream of PTEN that may be involved in regulating mitochondrial function, we evaluated the role of PI3K, which is a major mitogenic kinase mediating essential cell events such as proliferation, growth, and survival (27). Immortalized hepatocytes were treated with IGF-1, a growth factor that induces PI3K signaling, and LY294002, a PI3K inhibitor capable of blocking signals downstream of PI3K, prior to OCR measurement. In Con hepatocytes, a 2-fold induction of baseline OCR was observed with a 2-h IGF-1 pretreatment (Fig. 4A); a 4-h pretreatment further increased OCR (Fig. 4A). Conversely, pretreatment of Pten-null cells with LY294002 for 4 h was sufficient to reduce the OCR (Fig. 4B, left panel). Base-line OCR rate was kept at a constant lower level (30%) in LY294002-treated groups compared with that of vehicle-treated groups (Fig. 4B, right panel). Inhibition of PI3K with LY294002 reduced the maximal OCR by 30% as well. LY294002 treatment also led to a persistent lower ECAR level in Pten-null hepatocytes compared with the vehicle-treated group (Fig. 4C). A 2.5-fold reduction in baseline ECAR is observed with LY294002 treatment compared with the vehicle-treated cells (Fig. 4C), confirming that both glycolysis and mitochondrial oxygen consumption are regulated by PTEN and its downstream PI3K signaling in the same fashion.

**FIGURE 3. PTEN regulates mitochondrial respiration.** A and B, PTEN knockdown leads to both increased basal and maximal OCR in immortalized Con hepatocytes. Con hepatocytes were transfected with shPTEN1/2 or shScramble. After 24 h, transfected and control cells were seeded for OCR measurement. Left panel, OCR results in shPTEN, shScramble, and naive Con hepatocytes. Right panel (bottom), quantification of baseline OCR value. Right panel (top), PTEN protein level is decreased in Con hepatocytes transfected with shPTEN1/2. *, p ≤ 0.05, different from shScramble. n = 3.
Quantification of the stain intensity showed that Pten-null hepatocytes exhibited increased mitochondrial intensity compared with control cells (Fig. 5A, left panel). The quantified ratio of fluorescent pixels versus whole cell area pixels was 10% higher in Pten-null hepatocytes. These results suggest that the mitochondrial mass might increase upon PTEN loss. To further confirm whether increased MitoTracker staining intensity was due to an increase in mitochondrial mass, we measured the expression of cytochrome c oxidase subunit II (COX II), a gene exclusively encoded by mitochondrial genome, using a non-transcribed intron region of globin as genomic DNA control. The ratio of COX II/globin (intron) is commonly used as an

**FIGURE 4. PI3K/AKT signal regulates mitochondrial respiration and glycolysis.**

A, IGF-1 treatment leads to increased OCR in Con immortalized hepatocytes. Con hepatocytes were serum-starved for 24 h followed by IGF-1 treatments for 1, 2, and 4 h. Cells were then subjected to OCR measurement. **Left panel,** representative OCR results of Con hepatocytes starved for 24 h and treated by IGF-1 for 4 h. **Right panel (top),** quantification of OCR base-line values of 24 h of starvation, 1, 2, and 4-h IGF-1 treatments. **Right panel (bottom),** P-AKT is induced by IGF-1 treatments in Con hepatocytes. *, $p < 0.05$, different from time 0. $n = 5$. B, LY294002 treatment leads to decreased OCR in Pm immortalized hepatocytes. Pm hepatocytes were treated with vehicle or LY294002 at 1, 2, and 4 h. Cells were then subjected to OCR measurement. **Left panel,** representative OCR results of Pm hepatocytes treated with vehicle and LY294002 for 4 h. **Right panel (top),** quantification of OCR base-line values of vehicle and 1-, 2-, and 4-h LY294002 treatments. **Right panel (bottom),** P-AKT level was suppressed by LY294002 treatments in Pm hepatocytes. *, $p < 0.05$, different from time 0. $n = 5$. C, left panel, LY294002 treatment leads to decreased ECAR in Pm hepatocytes. ECAR is simultaneously measured with OCR. **Right panel**, quantification of base-line ECAR. *, $p < 0.05$, different from time 0. $n = 5$. 

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indicator of mitochondrial content (21, 28, 29). As expected, the ratio of COX II/globin is 2–3-fold higher in both Pten-null hepatocytes and liver tissues than that of their control counterparts (Fig. 5B), confirming an increased mitochondrial mass under Pten-null condition. To further demonstrate that this observation occurs in vivo, we performed electron microscopic analysis of liver sections from 1-month-old mice. Mitochondria from control livers showed a relatively sparse number of cristae, whereas those from Pten-null livers showed densely packed cristae (data not shown). The mitochondria in the Pten-null

FIGURE 5, PTEN/PI3K signal controls mitochondrial mass. A, immortalized Pm hepatocytes showed larger relative mitochondrial area than Con hepatocytes. Con and Pm hepatocytes were stained by MitoTracker Red (50 nM, Invitrogen) at 37 °C for 20 min, and fluorescence signal was analyzed. Left panel, quantification of the ratio, fluorescence pixels/whole cell area pixels was performed by UN-SCAN-IT software. *, p ≤ 0.05, different from Con. n = 3. Right panel, representative of MitoTracker Red staining in Con and Pm hepatocytes. B, ratio of COX II/globin is higher in both Pm hepatocytes (left panel) and livers (right panel). Genomic DNA was extracted from Con and Pm hepatocytes and livers. qPCR was performed to analyze COXII and globin levels. *, p ≤ 0.05, different from Con, n = 5. C, IGF-1 treatment increases mitochondrial mass in Con immortalized hepatocytes. Con hepatocytes were treated with IGF-1 for 24 h and stained with MitoTracker Green (25 nM, Invitrogen). FACS was carried out to analyze the fluorescence intensity. Left panel, FACS results of mitochondrial density in unstained, vehicle-treated, and IGF-1-treated Con hepatocytes. Right panel, quantification of average fluorescence intensity. *, p ≤ 0.05, different from vehicle-treated group. n = 3. D, LY294002 decreases mitochondrial mass in Pm immortalized hepatocytes. Pm hepatocytes were treated with LY294002 for 4 h and stained with MitoTracker Green (25 nM, Invitrogen). FACS was used to analyze the fluorescence intensity. Left panel, FACS results of mitochondrial density in unstained, vehicle-treated, and LY294002-treated Pm hepatocytes. Right panel, quantification of average fluorescence intensity. *, p ≤ 0.05, different from vehicle-treated group. n = 3.
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liver also seemed to have occupied a relatively greater fraction of the cytoplasm, thereby supporting the DNA analysis results, indicating an increase in mitochondrial mass.

To investigate whether PTEN loss induces mitochondrial biogenesis via PI3K, immortalized hepatocytes were treated with either LY294002 or IGF-1 followed by staining of Mitotracker green, a mitochondria dye of which the accumulation is less dependent on mitochondrial membrane potential. Thus, the staining intensity reflects mitochondrial mass. Fluorescence-activated cell sorting (FACS) analysis was employed to evaluate whether mitochondrial density changes with these treatments. Substantially higher (>2.5-fold) fluorescent signal was observed in Con cells treated with IGF-1 (24 h) versus vehicle-treated cells, indicating an increase of mitochondrial mass upon PI3K activation (Fig. 5C). When the Pten-null cells were treated with LY294002 (4 h), the fluorescent peak shifted to the left, suggesting reduced Mitotracker staining and thus lower mitochondrial density when PI3K signal is inhibited (Fig. 5D). Quantification of fluorescent values showed that LY294002 treatment results in an ~2-fold decrease of fluorescence intensity in Pten-null versus Con hepatocytes. Together, these data suggest that the increased mitochondrial mass upon PTEN loss is dependent on PI3K activity.

PTEN Regulates Mitochondrial Respiration Partially by Reducing the Abundance of Transcriptional Factor ERRα—Mitochondrial biogenesis is largely dependent on the nuclear transcriptional machinery, where the nuclear receptor ERRα plays a predominant role in activating expressions of genes involved in the TCA cycle and oxidative phosphorylation (30). ERRα either directly activates genes of mitochondrial components or indirectly induces expressions of other transcription factors regulating mitochondrial biogenesis such as nuclear respiratory factor-1 (NRF-1) and -2 (NRF-2/GABP) (31, 32). ERRα is also a central transcription factor that is responsible for adaptive mitochondrial biogenesis in response to a variety of stimuli, including high fat diet, thermogenesis, etc. (30, 33, 34). The ERRα-null mice exhibited defective adaptive thermogenesis, a process that relies on induction of mitochondrial mass and energy production under cold exposure (30). To explore how PI3K/PTEN signal may control mitochondrial biogenesis, we compared expression levels of ERRα in Con and Pten-null hepatocytes and livers. The protein level of ERRα was dramatically up-regulated in Pten-null hepatocytes (Fig. 6A, left panel), and the level of ERRα transcript was 5-fold higher when compared with the Con (Fig. 6A, right panel). Similar induction of ERRα protein expression was observed in Pten-null liver, although ERRα was undetectable in Con liver lysates (Fig. 6B).

To confirm that the function of ERRα is induced with the elevated protein and mRNA levels, we evaluated the transcriptional function of ERRα using two luciferase constructs, pCycL/–686Luc and pATPsynβ/–385Luc. The pCycL/–686Luc consists of sequence from codon –686 to +55 of CycL gene that encodes cytochrome c. The pATPsynβ/–385Luc consists of sequence from codon –385 to +90 of ATP5b, which encodes ATP synthase β. Both CycL and ATP5b were found to be under the transcriptional regulation of ERRα (21). In comparison with Con hepatocytes, luciferase activity of pCycL/–686Luc was ~2-fold higher (~800 versus 400 units) in Pten-null cells (Fig. 6C, left panel), suggesting enhanced promoter activities when Pten was deleted. Similarly, more than a 2-fold induction of pATPsynβ/–385Luc luciferase activity was observed in the Pten-null cells (Fig. 6C, right panel), indicating that ERRs transcriptional activity was higher in Pten-null cells compared with controls. We further evaluated the mRNA levels of cytochrome c and ATP synthase β to verify the enhanced ERRα transcriptional activity on endogenous ERRα target genes. We show that mRNA expression of cyt c is 2-fold higher in the Pten-null cells versus controls (Fig. 6D), similar to what was observed with pCycL/–686Luc activity. The mRNA levels of ATP5b were also higher in the Pten-null cells versus controls (Fig. 6D). We also evaluated another endogenous gene, ACADM which encodes medium chain acyl-CoA dehydrogenase (MCAD), a fatty acid oxidation enzyme transcriptionally controlled by ERRα. We found that the MCAD mRNA level was 5-fold higher in Pten-null hepatocytes than the controls (Fig. 6D). Thus, ERRα levels and activities were both higher when Pten was deleted in liver hepatocytes.

These analyses suggested that PTEN and PTEN-regulated signals may control ERRα functions. To investigate whether the increased ERRα levels and activities may be responsible for the higher mitochondrial respiratory function observed in the Pten-null hepatocytes, we used two independent siRNAs to knock down ERRα in Pten-null hepatocytes and evaluated the respiration rate of these cells. Both siRNAs targeted at ERRα decreased its protein expression (Fig. 6, E and F, top right panels). Concomitantly, siERRα-transfected Pten-null hepatocytes displayed significantly decreased basal and maximal OCRs compared with scrambled siRNA-transfected ones, indicating that ERRα is crucial for mitochondrial function and may be responsible for the enhanced mitochondrial respiratory function observed in Pten-null hepatocytes (Fig. 6, E and F, left and bottom right panels).

PTEN Regulates ERRα Expression through PI3K/AKT Signaling Pathway—To elucidate the underlying mechanisms for ERRα up-regulation upon PTEN loss, we manipulated PTEN and its downstream signaling molecules and examined the corresponding change in the expression of ERRα. Compared with scrambled shRNA-transfected cells, shPTEN transfection in Con hepatocytes led to increased ERRα protein expression (Fig. 7A, left panel) whereas introduction of wild type PTEN into Pm hepatocytes down-regulated ERRα (Fig. 7A, right panel), reconstructing the expression pattern observed in the two genetically distinct cell lines.

Having shown that PTEN manipulations alter ERRα expression and that the PI3K/AKT signal, the primary PTEN target, controls mitochondrial biogenesis and respiration (Figs. 4 and 5), we next asked whether ERRα regulation by Pten is also PI3K/AKT-dependent. To test this, IGF-1 and LY294002 were used to treat Pten control and null cells to activate and inhibit the PI3K signal, respectively. After a 4-h treatment of LY294002 to inhibit PI3K in Pten null hepatocytes, we observed a dramatic reduction of ERRα expression together with a robust inhibition of p-AKT (Fig. 7B, left panel). Conversely, inductions of ERRα and p-AKT were observed in control hepatocytes treated with IGF-1 (Fig. 7B, right panel). These data suggested that activation of the PI3K signal leads to increased ERRα expression. To
confirm the involvement of AKT in this regulation, CA myristoylated AKT and dominant negative (DN) AKT constructs were introduced to the Con and *Pten*-null hepatocytes, respectively, to induce and inhibit total AKT activities (Fig. 7C). WT AKT construct and empty vectors were also used as controls. In control hepatocytes, overexpression of CA-AKT induced ERRα.
expression, whereas the ERRα protein expression was inhibited by DN AKT transfection in Pten-null cells. WT AKT also increased the expression of ERRα when introduced to the control hepatocytes. To further investigate the role of ERRα as a mediator for AKT-controlled mitochondrial function, we manipulated ERRα with siRNA in cells expressing CA-AKT. Introduction of CA-AKT alone led to robust induction of OCR in control hepatocytes as predicted. ERRα knockdown in these cells attenuated the induction and brought the OCR level back to a level comparable with that of the controls (Fig. 7D). Together, these data suggest that the expression of ERRα relies on AKT activity downstream of PI3K, and ERRα functions as a prominent PI3K/AKT target that regulates mitochondrial function.

**CREB Mediates ERRα Regulation Downstream of PI3K/AKT Independent of PKA**—To understand the molecular mechanisms leading to increased expression of ERRα, we examined the expression levels of PGC-1α, a nuclear coactivator of ERRα that also transcriptionally regulates ERRα expression (35). We show here that siRNA-mediated

**FIGURE 7. PTEN regulates ERRα expression through PI3K/AKT signaling pathway.** A, PTEN knockdown in Con hepatocytes results in elevated ERRα levels (left panel), and PTEN overexpression in Pm levels leads to decreased ERRα expression (right panel). shPTEN and WTPTEN constructs were introduced into Con and Pm hepatocytes, respectively. Total cell lysate was extracted, and Western blot was carried out using primary antibodies against PTEN, p-AKT, ERRα, and actin. N/A, naive cells. B, LY294002 treatment suppressed ERRα in Pm hepatocytes (left panel), and IGF-1 treatment leads to increased ERRα levels in Con hepatocytes (right panel). IGF-1 and LY294002 treatments were performed in Con and Pm hepatocytes, respectively. Western blot was employed to assess the protein levels of p-AKT, ERRα, and actin. C, AKT is involved in mediating ERRα abundance. Constitutively active AKT (CA-AKT) and dominant negative AKT (DN AKT) constructs were introduced into Con and Pm hepatocytes, respectively. Wild type AKT (WT AKT) and empty vector were also used as transfection controls. Western blot was employed to assess the protein levels of p-AKT, ERRα, and actin. The signal density ratio of ERRα/actin for each Western blot result is provided at the bottom. D, ERRα knockdown together with CA-AKT overexpression rescues the OCR induction by CA-AKT-only transfection. Con hepatocytes were transfected with CA-AKT or CA-AKT and siERRα or empty and siScramble as control. After 24 h, transfected and control cells were seeded for OCR measurement. Left panel, siERRα leads to decrease of OCR level from CA-AKT-transfected cells. Right panel (top), Western blotting results of ERRα, p-AKT, and actin to confirm the plasmid/siRNA transfections. Right panel (bottom), quantification of base-line OCR.
PGC-1α knockdown led to reduced ERRα expression in the Pten-null hepatocytes (Fig. 8B), suggesting that PGC-1α-mediated induction of ERRα transcription may play a role in the accumulation of ERRα when PTEN is lost.

CREB is proposed to directly induce PGC-1α expression upon phosphorylation at Ser-133 (37). To explore the potential PI3K/AKT downstream candidates mediating ERRα regulation in the nucleus, we evaluated CREB signals in hepatocytes. A remarkably higher p-CREB was observed in Pten-null hepatocytes versus controls (Fig. 8C), suggesting that Pten deletion might result in an elevated phosphorylation signal that activates CREB. A dominant negative CREB construct CREB133

![FIGURE 8. CREB mediates ERRα regulation downstream of PI3K/AKT signaling. A, qPCR (left panel) and Western blot (right panel) analyses of PGC-1α mRNA and protein levels in Con and Pm immortalized hepatocytes. *, p < 0.05, different from Con. n = 5. B, inhibition of PGC-1α expression with siRNA leads to reduced ERRα expression. Actin is used as loading control. C, Western blot analysis for p-CREB, CREB, and actin in Con and Pm immortalized hepatocytes. D, CREB induces PGC-1α expression. Wild type CREB construct (WT CREB), CREBS133A construct (CREB133), and empty pCMV vector were transfected into Con hepatocytes. Western blot shows levels of PGC-1α, p-CREB, and actin. E, CREB133 diminishes ERRα level in Pm hepatocytes. CREB133 and pCMV vector were transfected into Pm hepatocytes. Protein levels of p-CREB and ERRα were analyzed by Western blot. F, IGF-1 treatment stimulates p-CREB and ERRα levels. Con hepatocytes were serum-starved for 24 h and then treated with IGF-1 for 1, 6, and 24 h. Protein levels of ERRα, p-CREB, p-AKT, and actin were assessed by Western blot. G, LY294002 treatment suppresses p-CREB. Pm hepatocytes were treated with LY294002 for 4 and 8 h. Protein levels of p-AKT and p-CREB were analyzed by Western blot.]

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FIGURE 9. CREB serves as a putative AKT substrate and regulates ERRα levels independent of PKA. A, activated AKT signaling due to PTEN loss maintains p-CREB levels independent of PKA. Con and Pm immortalized hepatocytes were infected with lentivirus expressing shPKA (against catalytic α domain of PKA) or shLacZ (serves as control). Protein expression levels of PKA Cα, p-CREB, p-AKT, ERRα, and actin in Con and Pm cells were assessed by Western blot. IP; immunoprecipitation; IB, immunoblot. B, IGF-1 is able to induce p-CREB when the PKA Cα protein level is significantly reduced by lentivirus shRNA in Con hepatocytes. Con immortalized hepatocytes were infected with shPKA/LacZ lentivirus and then subjected to a 24-h serum starvation followed by IGF-1 treatment. P-AKT, p-CREB, and actin levels were assessed by Western blot. C, AKT and CREB physically interact. Potential AKT substrates were pulled down from Pm hepatocyte lysates by the antibody that specifically recognizes the AKT substrate motif. Total protein lysates (Input) and pulldown lysates were subjected to Western blot analysis using antibody against CREB.

(38), which has a S133A mutation, was used to abolish the phosphorylation event of CREB at Ser-133. We found that overexpression of wild type (WT) CREB, but not CREB133, was able to induce the expression of PGC-1α in Con hepatocytes (Fig. 8D). This observation corroborates with previous studies showing that CREB transcriptionally induces the expression level of PGC-1α (37). Concomitantly, expression of CREB133 dramatically repressed ERRα expression in Pten-null hepatocytes (Fig. 8E). These data suggested that CREB mediates the increased expression of ERRα that we observed in Pten-null hepatocytes.

To determine the dependence of CREB phosphorylation on PI3K/AKT signaling, Con hepatocytes were treated with IGF-1 to activate PI3K signaling (Fig. 8F). Treatment with IGF-1 for 1 h was sufficient to induce p-AKT, and this induction was sustained throughout the treatment. Meanwhile, a time-dependent induction of p-CREB by IGF-1 was also observed. ERRα protein starts to be detectable 6 h after IGF-1 treatment and sustained at 24 h concurrent with sustained AKT phosphorylation. In cells treated with IGF-1 for 24 h when AKT and CREB were maximally phosphorylated, significantly higher ERRα levels are also detected compared with other parallel treatment groups (Fig. 8F). Together, these data indicate that sustained p-AKT may be capable of transmitting an activation signal toward CREB in a phosphorylation-dependent manner. Conversely, applying LY294002 treatment in Pten-null hepatocytes showed a robust inhibition of p-CREB at 4 h accompanied by suppression of p-AKT (Fig. 8G). At 8 h post-treatment, p-AKT recovered back to a normal level. Interestingly, p-CREB followed the same trend (Fig. 8G). Thus, CREB phosphorylation in the Pten-null hepatocytes appears to depend on PI3K activity.

Phosphorylation of CREB has been shown to be a consequence of protein kinase A (PKA) activation upon the buildup of intracellular cyclic AMP (cAMP) (39). We therefore investigated whether PKA was involved in the PI3K/AKT-induced CREB phosphorylation. Con and Pten-null hepatocytes were infected with lentivirus expressing shRNA that targets the catalytic subunit of PKA (PKA Cα). Our data showed the lentivirus infection in control cells completely abolished PKA Cα expression, leading to a significant reduction of p-CREB without affecting the levels of p-AKT (Fig. 9A, left panel). However, PKA knockdown in Pten-null hepatocytes where p-AKT is constantly activated was unable to change p-CREB levels (Fig. 9A, right panel). In addition, consistent with our earlier data showing p-CREB mediates ERRα expression (Fig. 8), lentivirus-shPKA infection diminished ERRα expression in Pten-control hepatocytes but not in Pten-null ones (Fig. 9A). Together, these findings indicate that the hyper-activated PI3K/AKT signal is sufficient to induce phosphorylation of CREB independent of PKA.

To further interrogate this signaling relationship, we performed IGF-1 treatment in Con hepatocytes infected with lentivirus that express either shRNA targeting PKA Cα or LacZ as control. In shLacZ-infected cells, IGF-1 was able to activate p-AKT and p-CREB (Fig. 9B, left panel). As anticipated, a similar CREB phosphorylation response was also observed in IGF-1-treated hepatocytes when the PKA Cα level was dramatically
decreased by shRNA (Fig. 9B, right panel). Therefore, the activation of PI3K/AKT signal was capable of inducing CREB phosphorylation independent of PKA.

Screening of the CREB sequence revealed an imperfect AKT substrate consensus sequence, “RX(S/T).” It has been reported that chronic activation of AKT may lead to phosphorylation of “secondary substrates with imperfect consensus sequences” (40). We tested this potential kinase-substrate relationship by performing immunoprecipitation analysis using a p-AKT substrate antibody against a specific AKT phosphorylation motif. Immunoblot with CREB antibody revealed a positive binding signal within the pulldown sample (Fig. 9C), implicating that CREB might be a putative substrate for AKT kinase activity.

ROS Level Is Regulated by ERRα—To clarify the role of ERRα in the elevated ROS production upon PTEN loss, we either overexpressed or knocked down ERRα in control and Pten-null hepatocytes, respectively, and examined the corresponding ROS levels. H2DCFDA staining and FACS analysis revealed that knocking down ERRα in Pten-null hepatocytes significantly reduced ROS production by 2-fold compared with the scrambled cells (Fig. 10A and B). Conversely, overexpression of ERRα led to a 30% increase in ROS production in Con hepatocytes (Fig. 10C). Thus, ERRα plays a role in regulating ROS production downstream of PTEN signaling.

Knockdown of ERRα Attenuates Colony Forming Potential in Pten-null Hepatocytes and PTEN Level Is Negatively Correlated with ERRα Levels in Samples from Patients with Liver Cancer—ROS production has been shown to correlate with tumorigenicity and tumor cell transformation (41). We evaluated the role of ERRα in the tumorigenic potential of the Pten-null hepatocytes. We show here that siRNA inhibition of ERRα expression led to decreased cell growth compared with scrambled siRNA or naive cell controls (Fig. 11A). We have shown previously that the Pten-null hepatocytes are capable of forming colonies on soft agar (20). Here, we found that inhibition of ERRα with siRNA significantly inhibits the ability of Pten-null hepatocytes to form colonies (Fig. 11B). Thus, ERRα induction in Pten-null hepatocytes may support their ROS production and transformation potential.

To explore the physiological significance of the PTEN-ERRα signaling cascade, we analyzed gene expression data from five cohorts of samples from patients with liver cancer (42–45) (TCGA, tcga-data.nci.nih.gov) and assessed the coexpression pattern of PTEN and ERRα. Significantly negative correlation between expressions of PTEN and ERRα was observed, suggesting that the novel link between PTEN/AKT/CREB/ERRα may be present in human samples, and this signaling pathway may be relevant to liver cancer development (Fig. 11C and data not shown). Together these results support the crucial role of ERRα during the transformation of liver cancer cells. Moreover, the negative correlation between PTEN and ERRα revealed by the cohort analyses of datasets from patients with liver cancer confirmed the clinical relevance of the PTEN-controlled signaling pathway that regulates ERRα expression.

DISCUSSION

In this study, we investigated the roles of PTEN, a tumor suppressor and the mitogenic PI3K/AKT signaling negatively regulated by PTEN in the regulation of mitochondrial function. Broadly, the results suggest that activation of PI3K/AKT signal and loss of PTEN function lead to enhanced mitochondrial respiratory capacity and mitochondrial volume. PI3K/AKT/PTEN signaling was found to control the expression of ERRα, a critical nuclear transcription factor involved in adaptive mitochondrial biogenesis. Delination of the signaling events leading to induction of ERRα by PI3K showed that CREB, a putative AKT substrate, mediates the ERRα up-regulation in response to PI3K/AKT/PTEN signaling. ERRα induction in Pten-null hepatocytes promotes the transformation and growth potential of these cells. The clinical cohort analyses revealed that in tumor samples from patients with liver cancer, PTEN expression is negatively correlated with ERRα levels. These analyses further demonstrated the physiological relevance of the PTEN-ERRα signaling pathway established by this study.

Together with the PTEN mutation, PI3K amplification and AKT overactivation occur frequently in human malignancies (46). Studies in recent years have highlighted the metabolic regulatory function of the PI3K/AKT/PTEN signal in malignancy transformation in addition to its role in cell growth and survival (47). AKT, the prominent downstream function unit of PTEN/PI3K regulation, is a major metabolic enzyme besides being a cell survival kinase. AKT has been shown to regulate both glycolysis and oxidative phosphorylation (48, 49). Our data confirm this observation and demonstrate functionally that the rate of glycolysis (measured as ECAR) is induced when AKT activity is high and inhibited when AKT activation is blocked. Similarly, oxidative phosphorylation determined as oxygen consumption followed the same correlation with AKT. This observation suggests that AKT activation alters the cellular metabolic profile in a manner similar to that exhibited by tumor cells. In nontumor cells, oxidative phosphorylation is coupled with glycolysis. When oxidative phosphorylation is inhibited by the addition of oligomycin, glycolysis increases to maintain the supply of ATP production. In these cells, the two rates are only simultaneously increased when the two processes are uncoupled by the addition of an uncoupler to cause proton leakage (50). Tumor cells, however, were shown to metabolize large quantities of glucose via glycolysis and to generate excessive lactate while maintaining the high oxygen consumption through mitochondrial respiration (1). Our data show that the oncoprotein AKT is capable of producing such an effect.

Our data indicate that AKT is capable of regulating oxidative phosphorylation and glycolysis independent of each other. The effects of AKT do not depend on the negative coupling of the two processes as both processes increase simultaneously when AKT is activated. Activation of AKT has been previously shown to accelerate glycolysis by directly regulating molecules involved in glycolytic pathways (51, 52). AKT phosphorylates and promotes the translocation of hexokinase onto mitochondria where it binds to the voltage-dependent anion channel (51). This is proposed to allow hexokinase to gain direct access to high concentrations of ATP, thus facilitating accelerated gly-
These functions of AKT lead to increased aerobic glycolysis that supply both ATP and probably more importantly increase fluxes of substrates used in biosynthetic pathways. We show here that AKT activation also induces oxidative phosphorylation by inducing mitochondrial biogenesis and increasing the mass of the mitochondria. This process is mediated by a key transcription factor ERRα that orchestrates the mitochondrial bioenergetic response.

ERRα controls expressions of both nuclear and mitochondrial transcription factors involved in mitochondrial biogenesis (53). Expressions of other nuclear transcription factors necessary for mitochondrial biogenesis, including NRF-1 and -2 are also under the regulation of ERRα (54). In mouse models lacking ERRα, the ability to maintain the body temperature when exposed to cold is attenuated, and mice are unable to cope with cold environment. This is primarily due to the inability of the brown adipose tissue to induce mitochondrial biogenesis and energy production when ERRα is absent (30). Thus, ERRα plays crucial roles in the transcriptional paradigms for mitochondrial biogenesis.

**FIGURE 10. ERRα regulates ROS production.** A and B, ERRα knockdown decreases ROS production in Pm immortalized hepatocytes. siERRα1/2 and siScramble were delivered into Pm hepatocytes, which were then stained with H2DCFDA and subjected to FACS analysis. Left panels, FACS results of ROS production in siERRα-transfected, siScramble-transfected, and unstained Pm hepatocytes. Right panels (top), quantification of average fluorescence intensity. Right panels (bottom), ERRα level is diminished by siERRα in Pm hepatocytes. *, p = 0.05, different from siScramble (siScr) control. n = 3. C, ERRα overexpression in Con hepatocytes increases ROS production. WT ERRα was overexpressed in Con hepatocytes. H2DCFDA staining and FACS were performed to assess ROS levels (left panel). Right panel (top), quantification of average fluorescence intensity. Right panel (bottom), ERRα level after transfection in Con hepatocytes. *, p = 0.05, different from vector control. n = 3.
The expression of ERRα has been shown previously to correlate with that of ErB/Her2/EGFR, which signals through the PI3K/AKT signaling pathway (55). In tumor cells, particularly estrogen receptor negative breast cancer cells, ERRα level correlates with expression of EGF receptor including ErB2 and ErB3 and EGF receptors (56). In in vitro kinase assays, incubation of ERRα with MAPK or AKT led to gel mobility shifts, indicating a potential kinase-substrate relationship between the molecules (55). Phosphorylation status of ERRα is generally correlated with its transcriptional activity. However, phosphorylation of serine 19 is found to precondition ERRα for sumoylation, which suppresses its transcriptional activity (57). Major breakthroughs in ERRα regulation studies came from the realization that PGC-1α, the coactivator for a number of nuclear receptors, has structural

The expression of ERRα has been shown previously to correlate with that of ErB/Her2/EGFR, which signals through the PI3K/AKT signaling pathway (55). In tumor cells, particularly estrogen receptor negative breast cancer cells, ERRα level correlates with expression of EGF receptor including ErB2 and -3 and EGF receptors (56). In in vitro kinase assays, incubation of ERRα with MAPK or AKT led to gel mobility shifts, indicating
motifs specific for binding to ERKα. It was later discovered that the PGC-1α ERKα complex also acts as a transcriptional activator for ERKα itself, turning ERKα from a weak to a strong transcriptional activator (35). We found that Pten−/− deficient hepatocytes exhibited elevated levels of both ERKα and PGC-1α. The induction of these two factors when PI3K/AKT is activated confirms that ERKα transcriptional activity remains high in cells where their signal is induced. The high level of ERKα transcriptional activity is necessary to execute the mitochondrial biogenic program to increase mitochondrial mass in Pten−/− hepatocytes.

Our data indicate that ERKα expression is robustly up-regulated upon Pten deletion, and this process clearly depends on the activity of PI3K/AKT. We have identified CREB as a potential AKT substrate that mediates this induction of ERKα expression. Phosphorylation of CREB on serine 133 is a common consequence of PKA activation in response to cellular accumulation of cyclic AMP. In cells where AKT is overexpressed, phosphorylation of serine 133 has been observed (58). Whether this increased CREB phosphorylation in cultured cells depends on PKA activity was not clarified. Our data in hepatocytes indicates that PKA is dispensable in the phosphorylation of CREB regulated by the PI3K/AKT signal. Recombinant AKT is shown to induce phosphorylation of CREB polypeptides containing the Ser-133 residue in vitro kinase assay, suggesting a potential direct kinase-substrate relationship (58). CREB contains a motif of RX(S/T) similar to but different from the typical AKT recognition site RXRXX(S/T) (37). However, it was suggested that chronic activation of AKT is capable of phosphorylating the imperfect RX(S/T) sites (40). Our immunoprecipitation study using a p-AKT substrate antibody that recognizes potential AKT substrates shows that CREB can be effectively pulled down, suggesting that the two proteins physically interact with each other in vivo. Therefore, it is likely that AKT may directly phosphorylate CREB, particularly during a chronic adaptation to constitutively active AKT signals, like those in cancer cells.

In hepatocytes, a major role of CREB is to regulate hepatic glucose output during starvation. During fasting, CREB phosphorylation is induced in response to glucocorticoid induction and the buildup of cAMP. The presence of CREB is necessary for inducing expression of gluconeogenic programs (39). This process relies on the ability of CREB to induce the transcriptional coactivator PGC-1α. Chromatin immunoprecipitation analysis shows specific binding of CREB to the consensus CREB-binding element on the PGC-1α promoter (37). Thus, the phosphorylation of CREB induced by AKT activation may directly result in the induction of PGC-1α and the subsequent expression of ERKα observed in hepatocytes where PI3K/AKT signals are induced.

Mitochondrial function is crucial for the transformation of tumor cells. When transformed, human mesenchymal stem cells were found to exhibit an increased oxidative phosphorylation rate (6). In several breast cancer cell lines, reducing oxidative phosphorylation by knocking down the key mitochondrial protein p32 led to reduced transformation (5). In vivo studies using an oncogenic K-ras-driven mouse model of lung cancer showed that mitochondrial transcription factor A is essential for tumorigenesis (7). Thus, the AKT- pCREB-ERKα signal-driven mitochondrial mass increase is likely important for tumor cell transformation. Clinically, studies have reported increased mitochondrial DNA in liver cancer specimens. However, a decrease of mitochondrial DNA content has also been reported (59–61). As tumor samples in these clinical studies are primarily from patients with late stage liver cancer, it is still premature to determine the cause and consequence relationship between mitochondrial mass and tumor development based on the clinical findings. Nonetheless, these studies do support the notion that mitochondrial abnormalities are associated with tumor phenotypes clinically.

A primary link between mitochondrial function and cell transformation is proposed to involve the generation of ROS (62). By reacting with the cysteinyl thiols, ROS modify a variety of proteins involved in multiple cellular processes. In some cases, the thiol acts as a redox-sensitive switch to modulate protein activity. By modulating these proteins, ROS can control cell growth, survival, and tumorigenesis. At low levels, ROS promote cell proliferation and survival. Higher levels of ROS can lead to mutagenesis and senescence/cell death (63, 64). Mitochondria are the major source of ROS due to the generation of free electrons during the process of oxidative phosphorylation (65). The high metabolic rate (ECAR, glycolysis) and accelerated oxidative phosphorylation (OCR, oxygen consumption) induced by activation of AKT have the potential to lead to high levels of free electrons and thus ROS generation. In the Pten−/−null mouse model, high levels of ROS (H2O2) accompanied tumorigenesis (17). Similar results were observed when PTEN was manipulated in vitro in this study by using isolated hepatocytes. When we manipulated mitochondrial functions with ERKα, we observed reduction in ROS and also significant attenuation of cell proliferation and transformation ability. Thus, the ability of the PTEN/PI3K/AKT signal to control cell proliferation may be partially regulated by increased ROS production resulting from mitochondrial biogenesis.

In summary, our study demonstrates a novel molecular interaction between PI3K/AKT signaling and mitochondrial bioenergetics and establishes the PI3K/AKT-pCREB-PGC-1α/ERKα signaling relationship. Our study provides for the first time the molecular mechanisms by which PI3K/AKT/PTEN signaling may control the function of mitochondria concurrent with its role in glycolysis. We show that PTEN/PI3K/AKT signal contributes to enhanced mitochondrial biogenesis and respiration as well as ROS production.

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