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NOK mediates glycolysis and nuclear PDC associated histone acetylation

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1. ABSTRACT

NOK is a potent oncogene that can transform normal cells to cancer cells. We hypothesized that NOK might impact cancer cell metabolism and histone acetylation. We show that NOK localizes in the mitochondria, and while it minimally impacts tricarboxylic acid (TCA) cycle, it markedly inhibits the process of electron transport and oxidative phosphorylation processes and dramatically enhances aerobic glycolysis in cancer cells. NOK promotes the mitochondrial-nuclear translocation of pyruvate dehydrogenase complex (PDC), and enhances histone acetylation in the nucleus. Together, these findings

show that NOK mediates glycolysis and nuclear PDC associated histone acetylation.

2. INTRODUCTION

Cancer cells consume glucose more heavily as compared to the non-cancer cells. More than 90 years ago, the German scientist Dr. Otto Heinrich Warburg reported that cancer cells principally metabolize glucose into the end product, lactic acid even in presence of sufficient oxygen (1–3). The lactate is subsequently transported to the extracellular compartment, which will generate an acidic microenvironment around the cancer cells. In 1972, this phenomenon has been termed as Warburg effect by American scientist Efraim Racter (4). Later studies indicated that the aerobic glycolysis is a more potent energy production pathway and can generate ATP 100~200 times faster than mitochondrial oxidative phosphorylation which is sufficient to meet the huge energy demands for cancer cell proliferation.

Cancer cells usually undergo an enhanced aerobic glycolysis. Upon being taken up into the cytosol, the glucose can be catabolized to pyruvate and later lactate through eleven enzyme-catalyzed reactions (5, 6). For one molecule of glucose catabolized by aerobic glycolysis, a total of two molecules of ATP can be generated. Among the eleven glycolytic enzymes, three of them, hexokinase (HK), phosphofructokinase (PFK) and pyruvate kinase (PK), are rate-limiting enzymes. Most of the catalytic enzymes in aerobic glycolysis are up-regulated in cancer cells (7, 8). The diverse glycolytic intermediates can be further converted into various biological building blocks such as nucleic acids, amino acids, fatty acids and lipids needed for the rapid growth of cancer cells (6, 9).

Warburg proposed that the cause for glycolytic switch to lactate is mainly due to the mitochondrial dysfunction in cancer cells, suggesting that the aerobic glycolysis has to occur in cancer cells since the entry of pyruvate into TCA cycle might be blocked. This hypothesis was first questioned by Dean Burk in 1939 who found the Pasteur effect in some tumor cells, indicating that glycolysis can be inhibited by O_2 (10). In addition, the mitochondrial TCA cycle can be driven by c-Myc-mediated glutaminolysis, indicating that mitochondrion is potentially functioning even under the Warburg effect (11, 12). A recently study further demonstrated that both the Warburg effect and the mitochondrial function can be highly intertwined (13). For example, the glucose uptake in cancer cells could be stimulated by AMP-activated protein kinase (AMPK), and the latter is directly activated by the reduced mitochondrial ATP due to the impaired mitochondrial functions (14). Moreover, mutations of oncometabolites in the TCA cycle such as isocitrate dehydrogenses (IDH1 and IDH2) can lead to carcinogenesis (15).

The novel oncogene with kinase-domain (NOK) is a potent oncogene that induces both tumorigenesis and metastasis in animal model systems. Accumulated data demonstrate that both highly proliferative normal cells and cancer cells exhibit a similar profile of metabolic re-programming. However, whether NOK could induce the Warburg effect and impact the metabolic gene expression profile is completely elusive. In this study, we systematically examined the effects of NOK on the expression profiles of the key enzymes involved in the glycolysis, TCA, and electron transport/oxidative phosphorylation process. We demonstrated that NOK could induce the Warburg effect. Specifically, NOK promotes glucolytic flux by up-regulating key enzymes involved in aerobic glycolysis. Despite a minimal effect on TCA, NOK significantly inhibits electron transport and mitochondrial oxidative phosphorylation. More significantly, our data provide a functional link between the mitochondrion-nucleus translocation of PDHA and transcriptional activation mediated by epigenetic modification.

3. MATERIAL AND METHODS

3.1. Cell lines, antibodies and chemicals

Human embryonic kidney cell line HEK293T, murine fibroblast cell line NIH-3T3, murine myelomonocytic leukaemia cell line WEHI-3B, and murine pre-B cells BaF3 were obtained from the Cell Culture Center of Basic Institute of Medical Sciences, Chinese Academy of Medical Sciences (Beijing, China). HEK293T and NIH-3T3 cells were grown in DMEM (Thermo-Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum at 37°C plus 5% CO_2. BaF3 and WEHI-3B cells were cultured in RPMI1640 (Thermo-Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum at 37°C with 5% CO2. Antibodies used include: anti-HA, anti-STYK1, anti-HK1 and 2, anti-PDHA1, anti-PKM1 and 2, anti-HIF1α, anti-VDAC1, anti-c-myc, anti-ACL and anti-ACSS2 antibodies from Proteintech Group Inc. (Rosemont, IL, USA); anti-mTOR, anti-p-mTOR, anti-Histone H3, anti acetyl Histone H3 (K18), anti-Lamin B, anti-β-actin and anti-PFKP antibodies from Bioworld Technology Inc (St. Louis Park, MN, USA); anti-OGDH, anti-SDHA and anti-IDH1 antibodies from ABclonal Biotechnology Co., Ltd (Wuhan, China); anti-β-tubulin and anti-COX4 from Cell Signaling Technology (Danvers, MS, USA, HRP-conjugated secondary antibody and FITC-conjugated secondary antibody from Zhongshan Biotechnology (Beijing, China). Thiazolyl Blue Tetrazolium Bromide (MTT) was purchased from Sigma-Aldrich Corp (St. Louis, MO, USA).

3.2. Plasmid construction and transient transfection

The plasmids pcDNA3.0.-NOK-HA, pEGFP-N3-NOK, pcDNA3.1.-NOK-ICD-HA and pcDNA3.1.-

Table 1. Primers used in qRT-PCR analysis

Gene Name	GenBank ID	Forward primer (5'→3')	Reverse primer (5'→3')	Product size (bp)
β-actin	BC009275	TCCATCATGAAGTGTGACGT	CTCAGGAGGAGCAATGATCT	161
ATP5A	NM_007505	TCTCCATGCCTCTAACACTCG	CCAGGTCAACAGACGTGTCAG	101
COX5B	NM_009942	GCTGCATCTGTGAAGAGGACAAC	CAGCTTGTAATGGGTTCCACAGT	98

NOK-KD-FLAG were laboratory-preserved. The NOK-KD-EGFP plasmid was constructed by inserting the kinase domain sequence of NOK into the Hind III and Kpn I sites of pEGFP-N1. All reagents used for plasmid construction were purchased from Takara Biotechnology (Dalian, Liaoning, China). For transfection, HEK293T or NIH-3T3 cells at about 80% confluence were transiently transfected with plasmid DNAs using Neofect™ DNA transfection reagent (Neofect biotech Co. Ltd, Beijing, China).

3.3. Generation of a NOK-expressing stable cell line

HEK293T/N packaging cells were co-transfected with the lentiviral vectors (pCMV-VSV-G, pMD2.G and pRSV-Rev) plus the pCDH-CMV-MCS-EF1-CD511B containing the NOK gene. After 48 h transfection, the lentivirus-containing medium was collected and supplemented with 8mg/ml Polybrene Sigma-Aldrich Corp (St. Louis, MO, USA). Murine BaF3 cells were incubated with the lentivirus-containing medium for 12 hrs. NOK-expressing stable cell clones were identified based on the expression of lentiviral EF1-driven GFP by flow cytometric analysis, followed by RT-PCR and western blot confirmation.

3.4. Generation of NOK transgenic mice

NOK transgenic mice were created by standard microinjection approach (16) under the guideline of animal care and use policy of the Institute of Basic Medical Sciences, Chinese Academy of Medical Sciences. Briefly, the NOK expression cassette was microinjected into fertilized oocytes. About 25 injected oocytes were implanted into the oviduct of each pseudopregnant Kunming mouse. The founder mice were identified by PCR using genomic DNA as a template. The pair of primers 5'-tggcccgcctg gcattatgcccagtacatg-3' and 5'-agccacaggatgaccccaag aaggatgagg-3' was used to amplify a 478bp fragment corresponding to a region between 3' end of CMV promoter and 5' end of the NOK gene.

3.5. Isolation of mitochondria and nuclei

Mitochondria were isolated using a mitochondria isolation kit for culture cells from Thermo Scientific Inc. (Waltham, MA, USA). Nuclei were isolated using a nucleoprotein extraction kit from Sangon Biotech (Shanghai, China) following the manufacturer's instruction.

3.6. Real time quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNAs were extracted from the cultured cells with TRIzol (Invitrogen, Carlsbad, CA, USA) and subjected to qRT-PCR analysis using One Step SYBR PrimeScript RT-PCR Kit II (Perfect Real Time, Takara Biotechnology, Dalian, China). The primers used in the qRT-PCR reactions were listed in Table 1. The level of β-actin expression was served as internal control. All primers were designed using the PrimerBank online program (https://pga.mgh.harvard.edu/primerbank/). Primer sequences were listed in Table 1.

3.7. Glucose consumption and lactate production assay

To examine glucose consumption and lactate production *in vivo*, serum samples were collected from wild type mice and NOK transgenic mice. About 10 ul of serum from each mouse was examined for both lactate and glucose using Biosen C-line analyzer (EKF Diagnostics, Cardiff, UK). For the *in vitro* assay, cells with and without ectopic NOK expression were cultured in a 12-well culture plate for 48 h, prior to the measurement of total levels of lactate and glucose in the medium.

3.8. (^{3}H)-2-deoxyglucose (DG) uptake assay

For the (^{3}H)-2-DG uptake assay, cells were seeded onto a 12-well plate. After transfection for 48 h, the cells were harvested and gently washed twice with KRP buffer (140 mM NaCl, 2 mM KCl, 1 mM KH_2PO_4, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 5 mM L-alanine, 5 mM indomethacin, and 10 mM HEPES (pH 7.4.)). Following an incubation with 1 μCi/mL (^{3}H)-2-DG in KRP buffer at 37°C for 30 min, the cells were washed three times with ice-cold KRP buffer and then lysed with 1 mL of 0.1.% sodium dodecyl sulfate (SDS). The (^{3}H)-2-DG uptake was determined with 1450 microBeta TriLux liquid scintillation counter (Beckman Instruments, Fullerton, CA, USA).

3.9. Cell proliferation assay by MTT

For cell proliferation assay, cells with either transient (HEK293T) or stable (BaF3) expression of ectopic NOK were plated onto 96-well plates at a

density of 5,000 cells per well and allowed to grow for 48 h. Cell proliferation was assessed by addition of MTT at a final concentration of 0.5. mg/mL for 4 h, followed by complete dissolving the MTT crystal with 150 uL of dimethyl sulfoxide and reading OD570 nm with a spectrophotometer.

3.1.0. Western blot analysis

Cells were harvested for protein extraction using a lysis buffer containing 20 mM Tris-HCl (pH7.4.), 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 1 mM Na3VO4, 2.5. mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, and 5μg/ml leupeptin (pH7.5). Equal amounts of protein, determined by BCA (bicinchoninic acid) protein assay kit (Pierce Biotechnology, Rockford, IL, USA), were loaded onto and resolved by 10–15% SDS-PAGE. Following protein transfer onto a nitrocellulose membrane (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA), the blot was probed first with primary antibodies at 4°C overnight and then with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature, prior to final development using an enhanced chemiluminescence kit (Santa Cruz, CA, USA).

3.1.1. Immunoprecipitation assay

NIH-3T3 cells were transiently transfected with the indicated plasmids. At 48 h post-transfection, cells were collected, washed with cold 1×PBS and re-suspended in RIPA lysis buffer (50 mM Tris, at pH7.4., 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with protease and phosphatase inhibitors (Thermo Scientific Inc., Waltham, MA, USA). After sonication, the lysates were incubated with a primary antibody at 4°C overnight. After incubation with protein G beads (Thermo Scientific Inc., Waltham, MA, USA) at 4°C for 5 h, the immunoprecipitates were washed four times with RIPA washing buffer (without protease and phosphatase inhibitors) and subjected to SDS-PAGE and Western analysis as described above.

3.1.2. Immunofluorescence

NIH-3T3 cells transiently transfected with indicated plasmids were fixed with 4% formaldehyde and then penetrated with 0.2.% Triton X-100, followed by immunofluorescent staining. After blocking for 1 h, cells were first incubated with the primary antibody at 4°C overnight and then with fluorescein isothiocyanate (FITC)- or tetramethyl rhodamine isocyanate (TRITC)-labeled secondary antibodies for 1.5. h. Finally, the cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) for observation. under an Olympus FV1000 confocal microscope.

3.1.3. Mitochondrial stress assay

Oxygen consumption rate (OCR) was measured in intact cells using a Seahorse Bioscience XF24 extracellular flux analyzer by following the manufacturer's instructions. Briefly, about 3×10^4 cells were seeded into XF24 cell culture plates and cultured at 37°C for 24 h. The cell culture medium was replaced with warmed assay medium and continuously incubated in a 37°C non-CO_2 incubator for 1 h. Subsequently, OCR was measured using the mitochondrial stress assay according to the user's manual. The combination of injections with multiple inhibitors of mitochondrial respiration enables the determination of non-mitochondrial respiration produced from the outside of mitochondria. The assay parameters (Basal, ATP, Maximal and Spare) were calculated by the XF Stress Test Report Generators and the values are represented as absolute OCR in pmol O2/min.

3.1.4. Statistical analysis

Data were presented as mean ± standard deviation (SD) from at least three independent experiments and subject to two-tailed, unpaired student's t test. A p value less than 0.05 was considered statistical significant. .

4. RESULTS

4.1. NOK induces aerobic glycolysis

To investigate whether NOK induces the Warburg effect, HEK293T cells with and without NOK over-expression were assayed for glucose and lactate. Over-expression of NOK significantly reduced the glucose level but increased lactate excretion (Figure 1A), indicating enhanced glycolysis. In addition, the intracellular level of (^{3}H)-2-DG-6-phosphate was significantly increased by NOK over-expression (Figure 1B), indicating that NOK promoted the glucose uptake. To further consolidate these results, we also measured serum levels of both glucose and lactate in either wild type or NOK transgenic mice. We found that glucose consumption in NOK transgenic mice has been enhanced significantly (Figure 1C, the left), which is accompanied by an increase of lactate production (Figure 1C, the right). Overall, these results demonstrate that NOK induces the Warburg effect in both transfected cells and transgenic animals.

4.2. Relative importance of glucose and glutamine for NOK-mediated cell proliferation

Cell culture medium contains two main carbon sources: glucose and glutamine. To evaluate the relative contribution of these carbon sources to NOK-mediated cell proliferation, MTT assay was

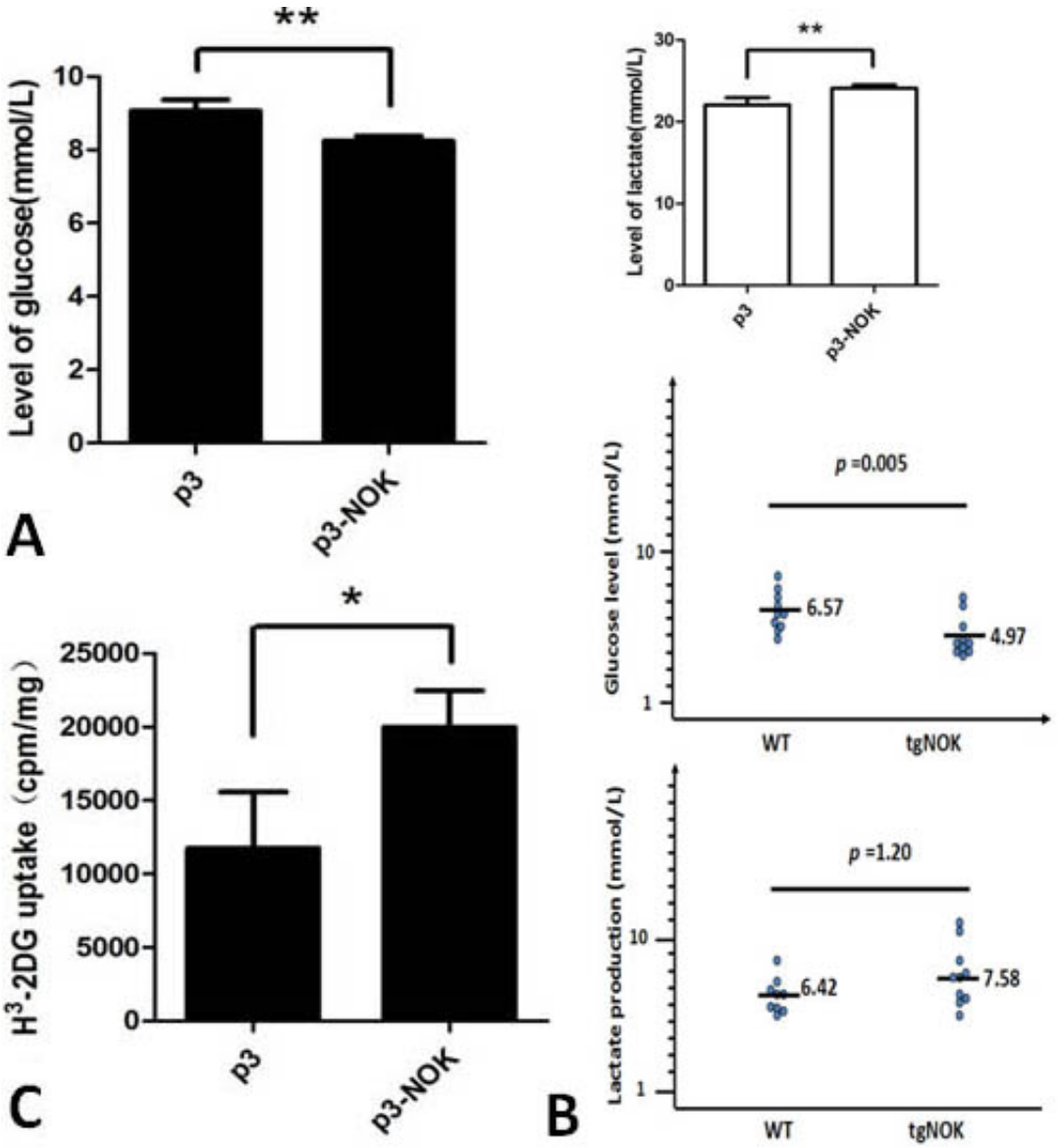

Figure 1. NOK over-expression induces the Warburg effect. (A) NOK promotes aerobic glycolysis in transfected HEK293T cells. HEK293T cells cultured in a 12-well culture plate were transiently transfected with 2 μg/well of either pcDNA3 or pcDNA3-NOK-HA. After 48 h transfection, supernatants were harvested and subjected to the detection of glucose or lactate using an assay kit. (B) NOK promotes the glucose uptake in HEK293T cells. HEK293T cells transfected with either pcDNA3 or pcDNA3-NOK-HA as described in (A) were labeled with 1 μCi/mL of (^{3}H)-2-DG and subjected to measurement of radioactivity. (C) NOK induces aerobic glycolysis in transgenic animals. The levels of glucose (left) and lactate (right) in the serum of wild type or NOK transgenic mice were detected by using an assay kit. Data are presented as mean ± SD from at least three independent experiments. *, $p < 0.0.5$; **, $p < 0.0.1$; ***, $p < 0.0.05$.

performed in NOK-overexpressing cells cultured in either complete medium (G+/Gln+), glucose depleted medium (G-/Gln+) or glutamine depleted medium (G+/Gln-). Our results demonstrate that both transient (in HEK293T cells) and stable (in BaF3 cells) expression of NOK significantly increased cell proliferation (Figure 2). More interestingly, glucose served as a major carbon source in HEK293T cells, which is manifested by a more significant impact of glucose depletion on cell viability as compared to glutamine depletion (Figure 2A). In contrast, BaF3 cells with a stable over-expression of NOK relied on both glucose and glutamine for proliferation (Figure 2B). These data indicate that persistent expression of NOK in BaF3 cells enhances the requirement of glutamine, in addition to glucose, as a major carbon source for cell proliferation.

4.3. Effects of NOK on the expression of key molecules in glycolysis

It is known that most of the enzymes involved in glycolysis are up-regulated in cancer or proliferative cells. To investigate the expression profile of the

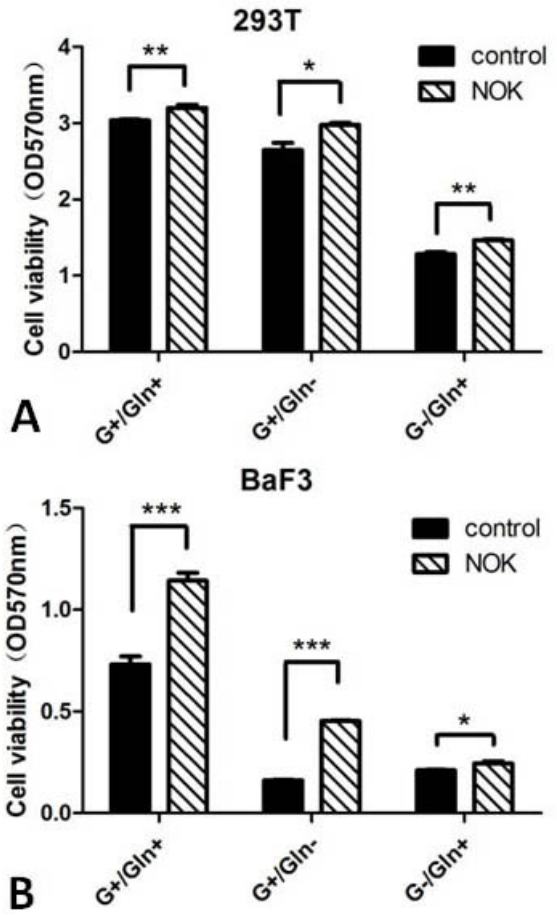

Figure 2. The influence of glucose and glutamine on NOK-mediated cell proliferation. HEK293T cells with transient NOK expression (A) and BaF3 cells with stable NOK expression (B) were cultured in three different media: the complete medium with both glucose (Glc+) and glutamine (Gln+); the culture medium with glucose (Glc+) but without glutamine (Gln-); the culture medium without glucose (Glc-) but with glutamine (Gln+). After 24 h incubation, cell proliferation in each culture condition was evaluated by MTT assay. Data are presented as mean ± SD from at least three independent experiments. *, $p < 0.0.5$; **, $p < 0.0.1$; ***, $p < 0.0.05$.

enzymes in NOK-induced glycolysis, we analyzed the expression of three rate limiting enzymes (HK, PFK and PK) as well as lactate dehydrogenase (LDH), the enzyme involved in the interconversion of pyruvate and lactate. In transiently transfected HEK293T cells, NOK promoted the expression of HK2, PFKP, PKM1 and LDHA, but not that of HK1, PKM2 & LDHB (Figure 3A). A different expression profile was found in BaF3 cells stably expressing NOK, in which NOK up-regulated the expressions of HK1, PFKP, PKM1 and LDHA, but not that of HK2, PKM2 and LDHB (Figure 3B). These results indicate that NOK may program glycolysis in a tissue specific manner. To further examine the signaling pathways that potentially control NOK-mediated glycolysis, the expressions of mTOR, HIF1α and Myc were evaluated. Over-expression of NOK markedly activates mTOR and HIF1α signaling pathways in both HEK293T and BaF3 cells, but only augments c-Myc expression in BaF3 not in HEK293T cells (Figure 3C & 3D). Overall, our data indicate that NOK-mediated Warburg effect can be manifested in different patterns of signaling events.

4.4. Effects of NOK on mitochondrial functions

The up-regulation of LDHA by NOK (Figure 3A & 3B) promotes the conversion of pyruvate, the end product of glycolysis, to lactate (Figure 1A & 1C). Alternatively, pyruvate can also serve as a substrate for the TCA cycle. To determine whether NOK may impact TCA, the expression of pyruvate dehydrogenase alpha 1 (PDHA1) was detected. Our data showed that NOK up-regulated PDHA1 expression in BaF3-NOK stable cells but not in HEK293T cells with transient NOK expression (Figure 3A & 3B), suggesting that intracellular acetyl-CoA might be potentially stimulated in a cell type dependent manner. WoLF PSORT analysis predicts that 21.7% of NOK is localized in mitochondria (data not shown). Indeed, NOK was found in mitochondria, as shown by immunoblotting and immunofluorescence analysis (Figure 4A & 4B). To address whether NOK can influence the mitochondrial function, we examined the expressions of three main enzymes involved in TCA cycle in NOK transfected NIH3T3 cells. NOK

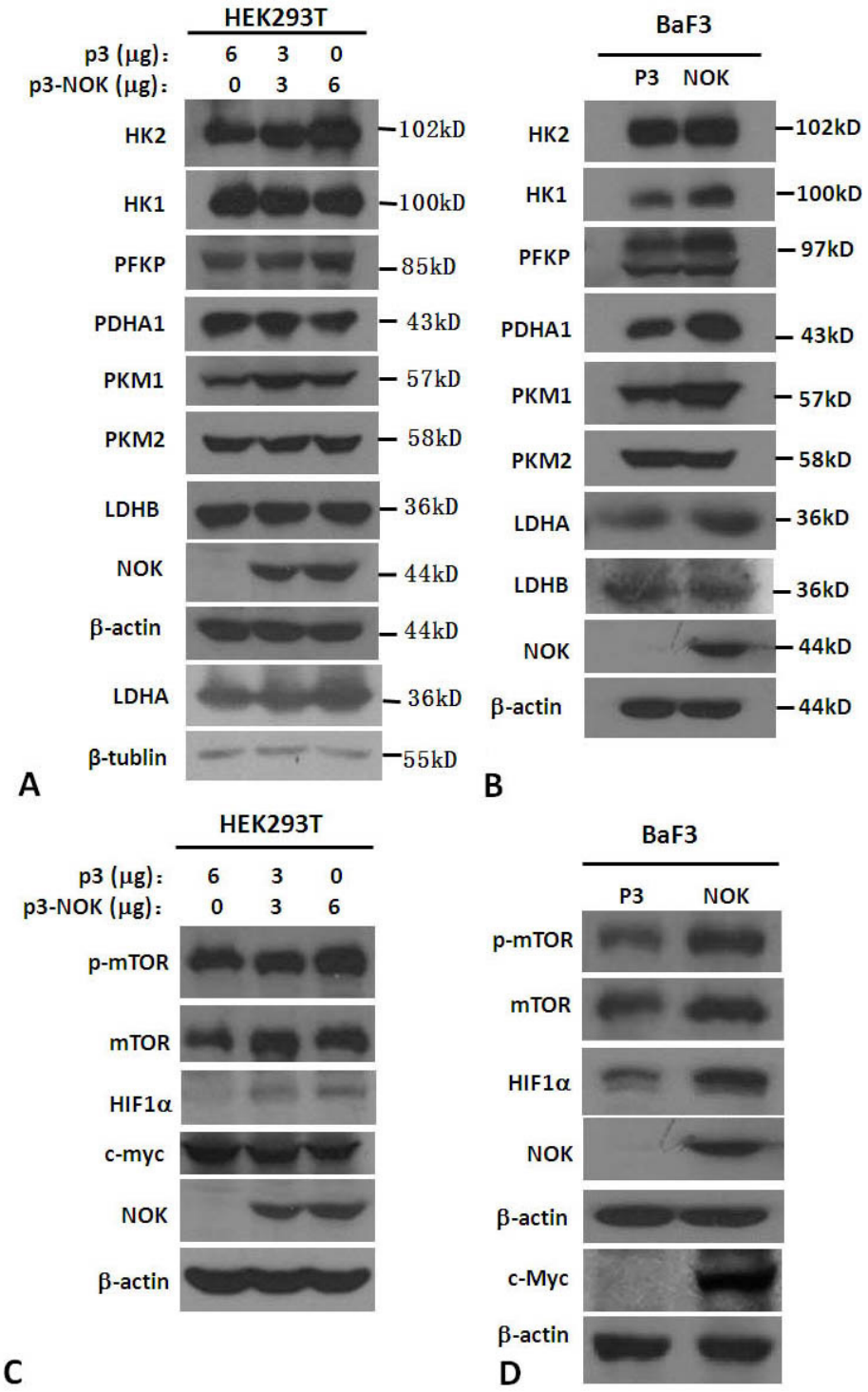

Figure 3. The expression profiles of key enzymes and regulators of glycolysis in response to NOK over-expression. (A) The effect of NOK on key enzymes of glycolysis in HEK293T cells. HEK293T cells were transiently transfected with increasing doses (0, 3, and 6 μg) of pcDNA3-NOK-HA. After 48 h following transfection, cell lysates were prepared and subjected to western blot analysis using anti-HK1, anti-HK2, anti-PFKP, anti-PKM1, anti-PKM2, anti-LDHA, anti-LDHB, anti-PDHA1, and anti-NOK antibodies. β-actin or β-tubulin expression was used as a loading control. (B) The effect of NOK on key enzymes of glycolysis in BaF3 stable cells. Cell lysates prepared from BaF3-CD511B and BaF3-CD511B-NOK-HA were subjected to western blot analysis using anti-HK1, anti-HK2, anti-PFKP, anti-PKM1, anti-PKM2, anti-LDHA, anti-LDHB, anti-PDHA1, and anti-NOK antibodies. β-actin was used as a loading control. (C) The effect of NOK on regulators of glycolysis in HEK293T cells. Cell lysates from HEK293T cells transfected with the increasing doses (0, 3, and 6 μg) of pcDNA3.-NOK-HA. After 48 h transfection were prepared and subjected to western blot analysis using anti-mTOR, anti-p-mTOR, anti-HIF1α, anti-c-myc and anti-NOK antibodies. β-actin was used as a loading control. (D) The effect of NOK on regulators of glycolysis in BaF3 stable cells. Cell lysates prepared from BaF3-CD511B and BaF3-CD511B-NOK-HA were subjected to western blot analysis using anti-mTOR, anti-p-mTOR, anti-HIF1α, anti-c-myc and anti-NOK antibodies. β-actin expression was served as an internal control.

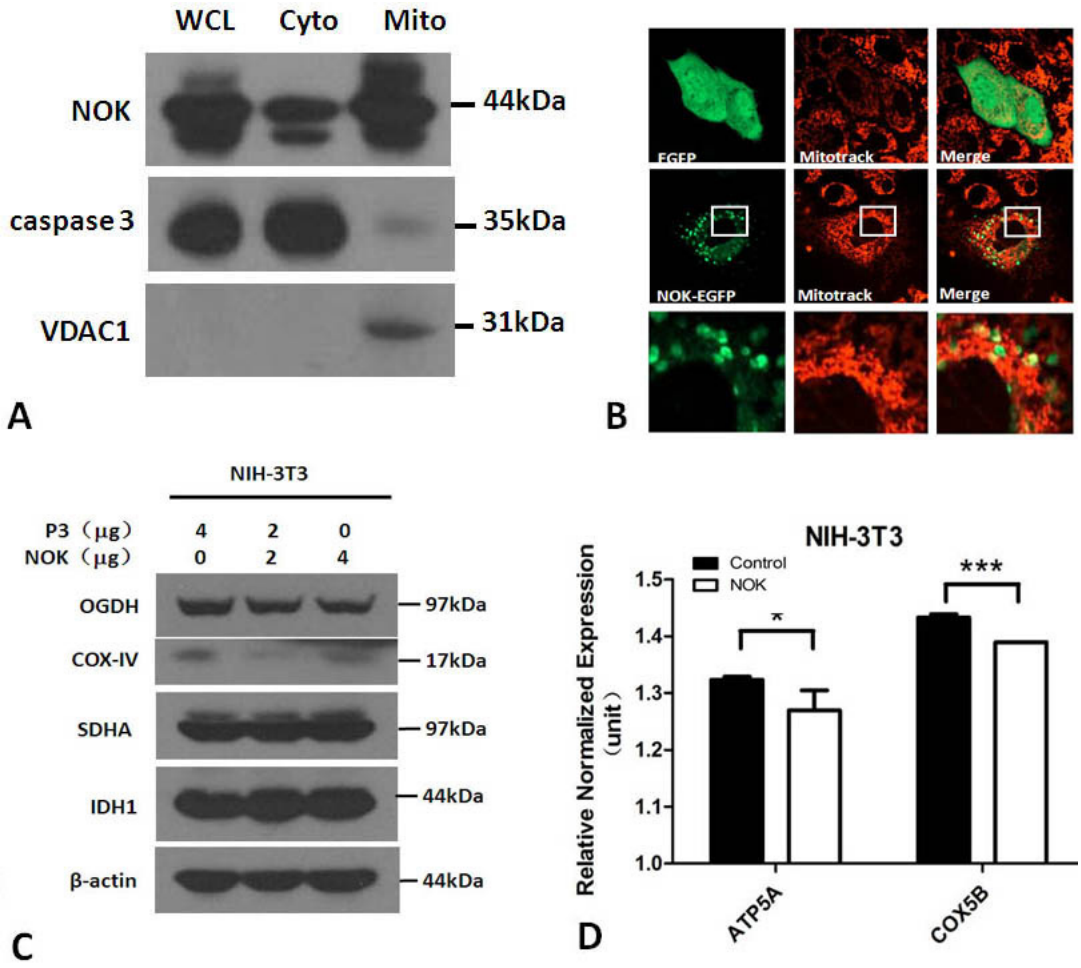

Figure 4. The effect of NOK on mitochondrial functions. (A) NOK can be detected in mitochondria. HEK293T were transiently transfected with 12 μg pcDNA3 or pcDNA3-NOK-HA. After 48 h, the whole cell lysates (WCL), the cytoplasmic fraction (Cyto) and the mitochondrial fraction (Mito) were prepared and subjected to western blot analysis using anti-NOK antibody. Caspase 3 and VDAC1were used as a cytoplasmic marker and a mitochondrial marker respectively. (B) Immunofluorescent detection of mitochondrial NOK. NIH-3T3 cells were transiently transfected with pEGFP-N3 or pEGFP-N3-NOK. After 48 h, MitoTracker-red dyes were added to each well for 30 min, followed by microscopic analysis of NOK expression using a confocal microscope. (C) The effect of NOK on the expression of enzymes involved in TCA and oxidative phosphorylation (OxPhos). NIH-3T3 cells were transiently transfected with increasing doses (0, 2, and 4 μg) of pcDNA3-NOK-HA. After 48 h, whole cell lysates were subjected to western blot analysis using anti-ODGH, anti-COX 4, anti-SDHA and anti-IDH1 antibodies. β-actin expression was used as a loading control. (D) RT-PCR analysis of the expressions of enzymes involved in OxPhos. Total mRNA was extracted from cells as described in (C) and subjected to real time RT-PCR analysis using primers specific for ATP5A and COX5B. Data from at least three independent experiments are presented as mean ± SD. *, $p < 0.0.5$; ***, $p < 0.0.05$.

had no effect on the expressions of both succinate dehydrogenase complex A (SDHA) and isocitrate dehydrogenase 1 (IDH1) except for a minor reduction of 2-oxoglutarate dehydrogenase (OGDH), indicating that NOK has a minimal impact on the function of TCA (Figure 4C). We next asked whether NOK might influence the mitochondrial oxidative phosphorylation. Our data showed that the over-expression of NOK suppressed the protein levels of cytochrome c oxidase 4 (COX4), COX5B, as well as mitochondrial ATPase α chain (ATP5A) (Figure 4C and 4D), indicating that NOK over-expression impaired the electron transfer and ATP production.

4.5. Mitochondrial NOK mediates impaired mitochondrial oxidative phosphorylation

To further evaluate the importance of NOK kinase activity in regulating the mitochondrial function, we compared the localization of a truncated NOK mutant containing only its kinase domain (NOK-KD) with that of wild type NOK. While EGFP-tagged wild type NOK exhibited a substantial overlap with mitotracker, NOK-KD-EGFP lost its co-localization with mitotracker (Figure 5A). Consistently, NOK-KD failed to induce the Warburg effect when over-expressed in HEK293T cells (Figure 5B). We also examined the

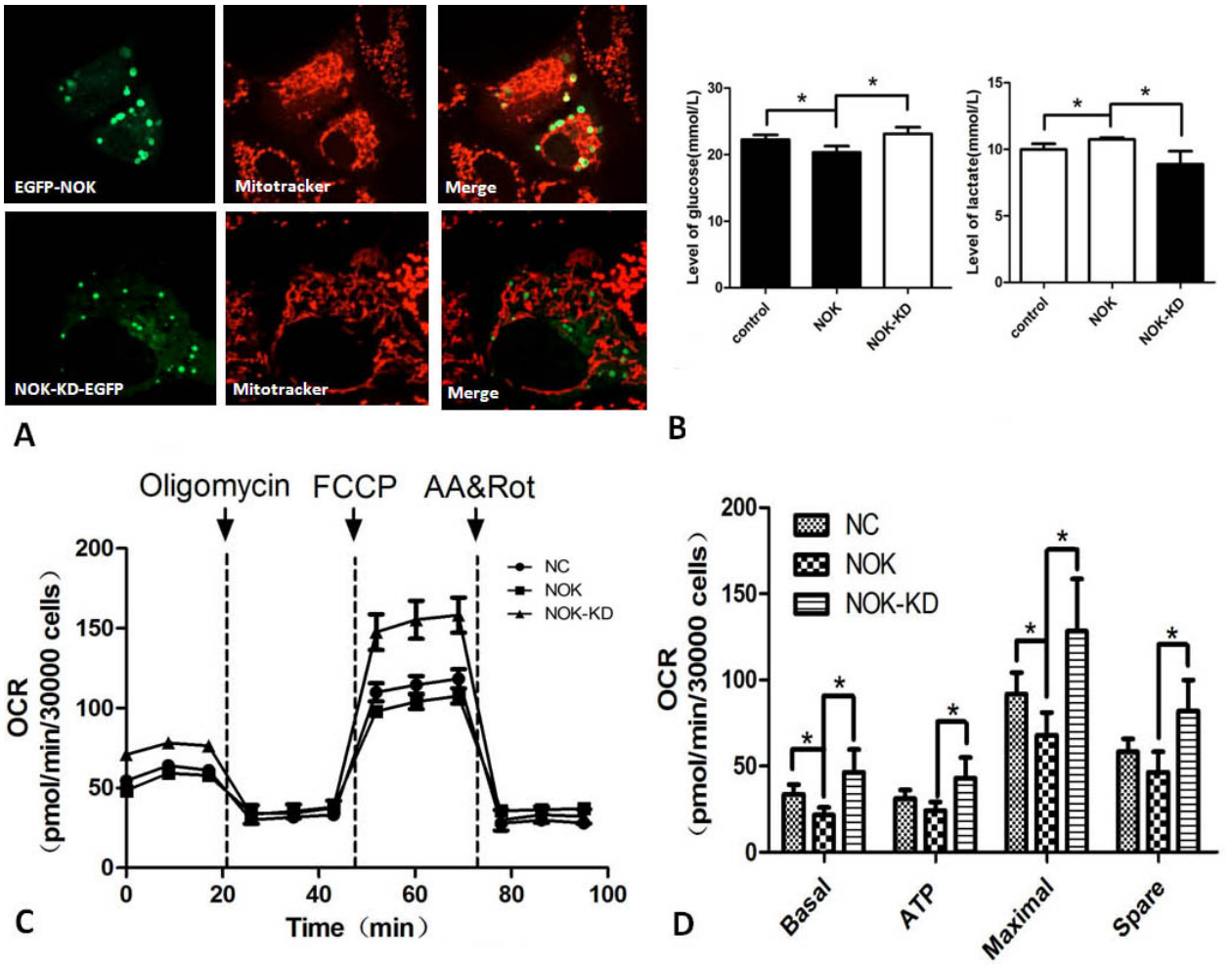

Figure 5. NOK inhibits mitochondrial oxidative respiration. (A) Immunofluorescent analysis of the mitochondrial localization of NOK and its mutant NOK-KD. NIH-3T3 cells were transiently transfected with 4 μg/well of pEGFP-NOK or pEGFP-NOK-KD. After 48 h, cells were incubated with MitoTracker-red dyes for 30 min and subsequently subjected to immunofluorescent analysis using a confocal microscopy. (B) The effect of NOK and NOK-KD on the production of glucose and lactate. NIH-3T3 cells were transiently transfected with 2 μg/well of pcDNA3, pcDNA3-NOK or pcDNA3-NOK-KD. After 48 h, cultural media were harvested and subjected to detection by a Glucose/Lactate assay kit. (C) The effects of NOK and NOK-KD on mitochondrial oxygen consumption rate (OCR). NIH-3T3 cells transfected with either empty vector, NOK or NOK-KD were seeded into XF24-well plates. Then OCR was analyzed using the Seahorse analyzer. (D) The effects of NOK and NOK-KD on mitochondrial OxPhos respiration. Cells as described in (C) were employed for analysis of key parameters of mitochondrial OxPhos respiration, including basal, ATP, maximal, and spare calculated from the OCR curves. Data from at least three independent experiments are presented as mean ± SD. *, $p < 0.0.5$.

effect of NOK and NOK-KD on ATP synthesis and mitochondrial respiratory by using an XF Cell Mito Stress Test Kit. Seahorse analysis showed that both basal and maximal respirations were significantly inhibited by NOK but not by NOK-KD. These results suggest that NOK mediates the impairment of oxidative phosphorylation in mitochondria, likely in a manner independent of its kinase activity.

4.6. NOK promotes histone acetylation by inducing nuclear transportation of pyruvate dehydrogenase complex (PDC)

Pyruvate can be reduced to lactate in cytoplasm or oxidatively decarboxylated to acetyl-CoA in mitochondria. Alternatively, pyruvate can be converted to acetyl-CoA in the nucleus (17). Based on the observations of NOK-mediated PDHA1 up-regulation (Figure 3B) and OGDH reduction (Figure 4C), we hypothesized that NOK might regulate the subcellular localization of pyruvate as well as the pyruvate dehydrogenase complex **(**PDC). As expected, NOK stimulated the mitochondria-nucleus translocation of PDHA1 (Figure 6A), indicating an increased nuclear function of PDC. It is well known that nuclear acetyl-CoA is derived from three main sources (17). In addition to being converted from pyruvate after the oxidative decarboxylation by PDC, nuclear acetyl-CoA could also be generated from citrate by ATP-citrate lyase (ACL) or from acetate by acetyl-CoA synthetase (ACS) (17–19). To test the involvement of NOK in these processes, we examined the subcellular localization

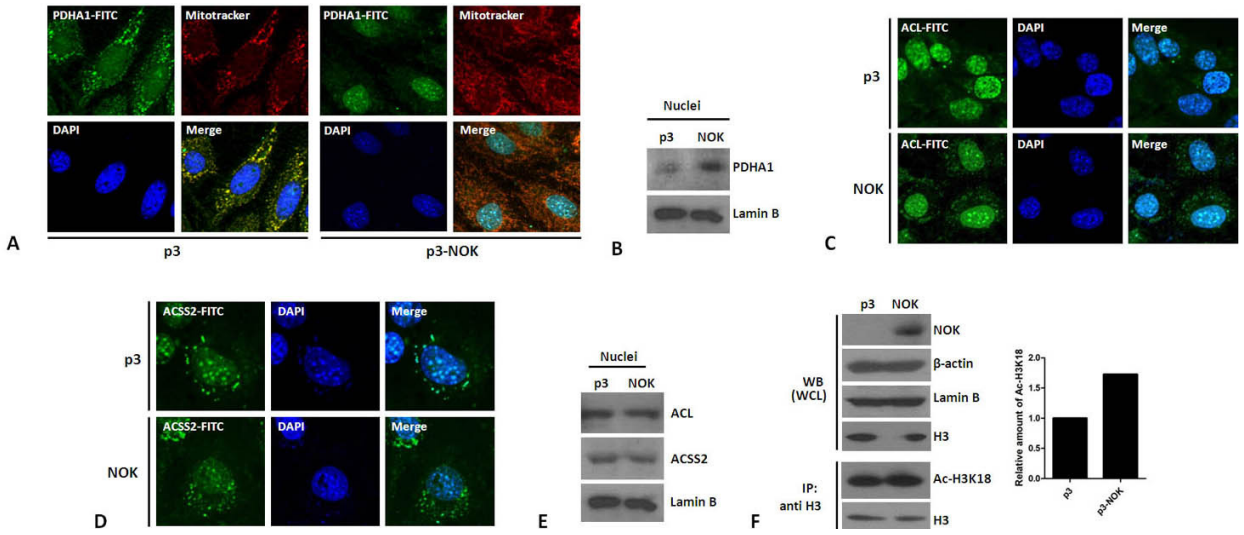

Figure 6. NOK promotes nuclear translocation of the pyruvate dehydrogenase complex (PDC). (A) NOK promotes the translocation of mitochondrial PDHA1 to the nucleus. NIH-3T3 cells were transiently transfected with pcDNA3 or pcDNA3-NOK, followed by staining with MitoTracker-red dyes and immunostaining of PDHA1 for microscopic analysis. The cell nuclei were counterstained with DAPI. (B) Western blotting analysis of NOK-mediated PDHA1 expression in the nucleus. Nuclei were isolated using a Nucleoprotein Extraction Kit and then subjected to western blot analysis using anti-PDHA1 antibody. Lamin B was used as a nuclear marker and a loading control. (C) Immunofluorescent analysis of the NOK impact on nuclear ACL expression. NIH-3T3 cells were transiently transfected with 12 μg of pcDNA3 or pcDNA3-NOK-HA. After 48 h, the cells were fixed and probed with a primary antibody against ACL, followed by incubation with a FITC-labeled secondary antibody. The nuclei were stained with DAPI. The immunostained cells were analyzed with a confocal microscopy. (D) Immunostaining analysis of the effect of NOK on nuclear ACSS2 expression. Cells as described in (C) were fixed and probed with a primary antibody of ACSS2, followed by incubation with a FITC-labeled secondary antibody for microscopic analysis. (E) Western blotting analysis of NOK-mediated ACL and ACSS2 expressions in the nucleus. Nuclear extracts prepared from transfected cells from (C) or (D) were subjected to western blot analysis using anti-ACL or anti-ACSS2 antibody. Lamin B was used as a nuclear marker and a loading control. (F) The effect of NOK on acetylation of histone 3. NIH-3T3 cells were transiently transfected with 12 μg of pcDNA3 or pcDNA3-NOK-HA. After 48 h, cells were disrupted by sonication. Cell lysates were subjected to immunoprecipitaion with an anti-H3 antibody, followed by immunoblotting analysis of H3 acetylation using a site-specific anti-Ac-H3(K18) antibody. Alternatively, cell lysates were directly subjected to Western analysis using indicated antibodies. β-actin and lamin B were used as loading controls for whole cell lysates and nuclear extracts respectively.

of multiple regulators of acetyl-CoA dynamics. Over-expression of NOK dramatically promoted the nuclear translocation of PDHA1 (Figure 6A and 6B) without affecting that of ACL and ACSS2 (Figure 6C, 6D and 6E), suggesting that NOK primarily regulates nuclear PDC-mediated pyruvate conversion to acetyl-CoA. To further explore the mechanism underlying NOK-mediated nuclear translocation of PDC, we examined the acetylation status of nuclear histone H3 by using a site-specific H3 acetylation antibody. The level of H3 acetylation at lysine 18 was significantly increased by NOK (Figure 6F), indicating that NOK promotes epigenetic modification of histone and nuclear localization of PDC, leading to cell proliferation.

5. DISCUSSION

The Warburg effect is a highly conserved phenomenon observed not only in cancer cells but also in proliferating cells. According to Warburg's hypothesis, the continuous excretion of lactate from cancer cells is largely due to the impaired mitochondrial function and subsequent failure of pyruvate to facilitate the entry of acetyl-CoA into the TCA cycle. In cancer cells, this phenotype is mainly driven by the activation of oncogenes or loss of tumor suppressor genes (5, 20). The Warburg effect can also be induced by oncogenic receptor protein tyrosine kinases (RPTKs), in which a constitutively active form of RPTK is usually required. For example, the expression of an epidermal growth factor receptor (EGFR) mutant (EGFRvIII) or a fibroblast growth factor receptor type 1 fusion gene mutant (ZNF198-FGFR1) could result in the Warburg effect through the activation of H-RAS or inactivation of pyruvate kinase M2 (PKM2), respectively (21, 22). In this report, we demonstrate that NOK represents another example of RPTK that directly promotes aerobic glycolysis and metabolic reprogramming. In agreement with previous observations, our study shows that the enhanced glycolytic flux in cancer cells is directly associated with the up-regulation of catalytic enzymes of glycolysis. The rate limiting enzymes of glycolysis usually play a critical role in tumorigenesis. For example, PKM2, a spliced isoform of pyruvate kinase in muscle important for glycolysis, promotes cell proliferation and tumor formation (23). Nevertheless, contradictory data suggest that PKM2 is dispensable for tumorigenesis (24). It is well known that PKM1 is more catalytically active than PKM2. The fact that PKM2 is favored by cancer cells has been attributed to the bottleneck issue generated at the end intersection of glycolytic flux (13). However, our data demonstrate

that NOK up-regulates PKM1 but not PKM2, which suggests that tumor cells can use the highly active PKM1 to catalyze the final reaction of glycolysis and subsequently facilitate tumor cell growth.

Both HIF1α and c-Myc can promote the conversion of pyruvate to lactate by activating LDHA (25–27). We observed the differential regulation of HIF1α and c-Myc by NOK in HEK293T versus in BaF3 cells: NOK up-regulates both HIF1α and c-Myc in BaF3 cells, but only enhances HIF1α in HEK293T cells. These observations may have functional implications in understanding NOK-mediated aerobic glycolysis and mitochondrial functions. Glucose and glutamine are two major carbon sources for energy generation and cell proliferation (5, 13). As compared to BaF3-NOK cells, NOK transfected HEK293T cells demand less glutamine, representing a reduced mitochondrial function. This notion is further supported by the fact that NOK up-regulates c-Myc in BaF3 cells but not in HEK293T cells. It is known that c-Myc, a master regulator of glutaminolysis, can induce glutaminase expression (11). We therefore propose that c-Myc-mediated glutaminolysis might compensate the lack of pyruvate-mediated TCA functional status. Specifically, NOK induces c-Myc in BaF3-NOK cells to maintain a functional TCA through glutamine addiction, whereas the TCA cycle may enter an inert status in NOK transfected HEK293T cells in the absence of c-Myc-mediated glutaminolysis. There are three c-Myc isoforms, including two major ones (c-Myc1, c-Myc2) and one short form (MycS). Interestingly, the two major isoforms, c-Myc1 and c-Myc2, may carry out opposite cellular functions in terms of cell growth versus cell arrest (28). However, it remains unclear how different c-Myc isoforms contribute to NOK-mediated glutaminolysis in both cell types, which needs further characterization.

In cancer cells, the impairment of mitochondrial function may result from multiple metabolic mechanisms. Mutations of the metabolic genes involved in the TCA cycle have been shown to increase cell proliferation or prevent cell death (29). A recent study indicates that RPTK such as ErbB2 can translocate into mitochondria to disrupt the mitochondrial oxygen consumption and electron transport (30). Our results are consistent with the above observation and indicate that the mitochondrial localization of NOK can result in the impairment of mitochondrial functions. However, NOK might function differently from ErbB2 in at least two aspects: 1) NOK may be an intrinsic component of mitochondria; 2) the kinase activity of NOK is insufficient for its mitochondrial localization. Therefore, our data suggest that NOK-mediated aerobic glycolysis in cancer cells has a limited effect on TCA but instead, markedly inhibits mitochondrial oxidative phosphorylation.

Our study demonstrates that NOK promotes nuclear translocation of PDHA1, indicating that PDC may function in the nucleus. We also show the concurrent histone H3 acetylation during the nuclear translocation of PDC. It is therefore plausible that abnormal chromatin relaxation and histone modification may alter the expression profile and subcellular localization of the important metabolic enzymes in cancer cells. Among various sources to produce nuclear acetyl-CoA, the nuclear translocation of PDC may be the major driving force of NOK-mediated production of nuclear acetyl-CoA. An earlier study reported that the elevated level of nuclear acetyl-CoA serves as an important metabolite that mediates histone modification and subsequent cellular transformation (31). It is also known that acetylation of histone H3 at K18 promotes cell cycle progression and proliferation (19, 32). Our data further confirm these observations by showing that NOK promotes this type of histone acetylation, which might be critical for NOK-mediated cell proliferation and/or transformation. In summary, our data presented in this study suggest that over-expression of NOK can induce the Warburg effect in a manner independent of cellular transformation and cancer, and that NOK-mediated carcinogenesis might be directly linked to metabolic reprogramming associated with epigenetic modification via nuclear acetyl-CoA.

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Abbreviation: NOK, novel oncogene with kinase-domain; TCA, tricarboxylic acid cycle; PDC, pyruvate dehydrogenase complex; PGI, phosphoglucose isomerase; HK, hexokinase; LDH, lactate dehydrogenase; AMPK, AMP-activated protein kinase; IDH, isocitrate dehydrogenses; OCR, oxygen consumption rate; TRITC, tetramethyl rhodamine isocyanate; DAPI, 4',6-diamidino-2-phenylindole; PDHA1, pyruvate dehydrogenase alpha 1; SDHA, dehydrogenase complex A; OGDH, 2-oxoglutarate dehydrogenase; ATP5A, ATPase α chain; COX4, cytochrome c oxidase 4; COX5B, cytochrome c oxidase 5B; NOK-KD, NOK kinase domain; ACL, ATP-citrate lyase; ACS, acetyl-CoA synthetase; RPTKs, receptor protein tyrosine kinases; FGFR1, fibroblast growth factor receptor type 1; EGFR, epidermal growth factor receptor.

Key Words: RPTK, NOK, Warburg Effect, Aerobic Glycolysis, Mitochondria-Nucleus Translocation, Mitochondria, acetyl-CoA, Acetylation, Histone, Pyruvate Dehydrogenase Complex, Tricarboxylic Acid Cycle

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