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PDK2-enhanced glycolysis promotes fibroblast proliferation in thyroid-associated ophthalmopathy

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Abstract

The study aimed to investigate the role of pyruvate dehydrogenase kinase (PDK) in regulating glycolysis and proliferation of perimysial orbital fibroblasts (pOFs) during the pathogenesis of thyroid-associated ophthalmopathy (TAO). EdU and BrdU incorporation assays were performed to examine cell proliferation. Lactate production and oxygen consumption assays were conducted to evaluate glycolysis. Real-time PCR was adapted to quantify *PDK* mRNA levels. Capillary Western immunoassay was adapted to quantify PDK2, Akt, pAkt³⁰⁸ and GAPDH in protein samples. The TAO pOFs exhibited stronger proliferation activity, higher intracellular lactate concentration, and lower oxygen consumption rate than the control pOFs. The PDK inhibitor dichloroacetic acid (DCA) dose-dependently suppressed the proliferation of both TAO and control pOFs. DCA reduced lactate production and promoted oxygen consumption in the TAO pOFs but showed no significant effects on glycolysis in the control pOFs. Among four PDK isotypes, *PDK2* was overexpressed in the TAO pOFs. The potential PDK signaling mediator, cytoplasmic Akt, was more abundant in TAO pOFs than control pOFs. Knockdown of *PDK2* resulted in lower lactate production, stronger oxygen consumption, weaker proliferation activity, and less cytoplasmic Akt in the TAO pOFs but showed no significant effects in the control pOFs. The Akt inhibitor MK2206 suppressed proliferation in both TAO and control pOFs, and lactate production was inhibited by MK2206 in the TAO OFs but not the control pOFs. To conclude, PDK2 overexpression enhances glycolysis and promotes proliferation via Akt signaling in the TAO pOFs. These findings yield insights that PDK2 is a potential therapeutic target for TAO treatment.

Key Words

- ▶ PDK2
- ▶ glycolysis
- ▶ proliferation
- ▶ orbital fibroblasts
- ▶ thyroid-associated ophthalmopathy

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Introduction

Thyroid-associated ophthalmopathy (TAO), commonly co-occurrence with thyroid dysfunction (mostly Graves' disease), is an autoimmune disorder of orbital tissues. This condition is disfiguring and potentially sight-threatening,

because its pathological changes culminate in the expansion and remodeling of the soft tissues within the orbit. Only a few medical therapies have been proven safe and effective to alter the clinical outcome of TAO.

For instance, teprotumumab was recently approved by US FDA for the treatment of active, moderate to severe TAO (Smith 2019, Douglas *et al.* 2020). To develop more targeted treatments requires a more complete understanding of the mechanisms involved in the pathogenesis. Considerable effort has been expended in identifying the role of oxidative stress within the TAO orbit. Cigarette smoking, a putative cause of oxidative stress, is commonly blamed as a risk factor for developing TAO (Wiersinga *et al.* 2018). Reduction in tobacco use and supplement of selenium (an antioxidant agent) are frequently cited as effective strategies to reduce the incidence and morbidity of TAO (Taylor *et al.* 2020). Evidence from fundamental research suggests increased oxidative stress and upregulated antioxidant capacity in the serum and orbit of TAO patients (Korkmaz *et al.* 2015, Marique *et al.* 2015, Aass *et al.* 2016, Yuksel *et al.* 2020). Take together, these findings yield important insights into the role of oxygen metabolism during the pathogenesis of TAO.

The research findings thus far have established that orbital fibroblasts (OFs) represent the central target for immune reactivity in TAO development (Smith & Janssen 2019). Many studies have examined the behavior of OFs with regard to their responses to antioxidants. Significant differences between the TAO and control OFs have been identified in aspects of proliferation, cytokine production, reactive oxygen species (ROS) generation and so forth (Byun *et al.* 2016, Choi *et al.* 2018, Ko *et al.* 2018, Lee *et al.* 2019). Details concerning the oxygen metabolism and its regulatory factors have not been fully investigated. In mammalian cells, there are two basic pathways to generate energy, glycolysis in the cytoplasm (requires no oxygen) and oxidative phosphorylation in mitochondria (requires oxygen). The pyruvate dehydrogenase kinase (PDK), which has four isoforms (PDK1–4), is considered as the gatekeeper enzyme to trigger the switch from oxidative phosphorylation to cytoplasmic glycolysis (Stacpoole 2017). Growing evidence supports the concept that the glycolytic pathway dominates in proliferating cells (especially tumor cells) which present with reduced oxygen consumption and increased lactate production (Vander Heiden *et al.* 2009, Martinez-Outschoorn *et al.* 2017).

Perimysial orbital fibroblast (pOF) is a unique phenotype of OFs that resides within extraocular muscle fibers. Our previous study implied that the TAO pOFs possessed stronger proliferative capacity than the control pOFs obtained from healthy subjects (Ma *et al.* 2018). Based on these observations, we hypothesized that glycolytic pathway may be preferentially enhanced in the TAO pOFs. To test the hypothesis, this study compared the

differences between the TAO and control pOFs regarding proliferation, lactate production, oxygen consumption, PDK expression, cellular responses to PDK inhibition, and the mediator of PDK signaling transduction.

Materials and methods

Materials

Reagents included the PDK inhibitor dichloroacetic acid (DCA, Sigma-Aldrich, Cat# D54702) and the Akt inhibitor MK2206 (Selleck, Cat# S1078). Commercial kits included the Cell Counting Kit-8 (CCK-8, DOJINDO, Cat# CK04), EdU incorporation assay (Life Technologies, Cat# C10637), BrdU incorporation assay (Roche, Cat# 11647229001), Lactate ELISA kit (Abcam, Cat# ab65331), Deproteinizing Sample Preparation kit (Abcam, Cat# ab204708), Oxygen Consumption Assay (Abcam, Cat# ab197243), rtPCR kit (TaKaRa, Cat# RR047), SYBR qPCR kit (TaKaRa, Cat# RR420), Lipofectamine RNAiMAX Reagent (Invitrogen, Cat# 13778), Protein and RNA Isolation System (PARIS, Life Technologies, Cat# AM1921), and Capillary Western Immunoassay kit (ProteinSimple, 12–230 kDa). Primary antibodies targeted vimentin (clone GT7812; Abcam, Cat# ab184631), PDK2 (clone EPR1948Y; Abcam, Cat# ab68164), Akt (clone 11E7; CST, Cat# 4685), pAkt³⁰⁸ (clone L32A4; CST, Cat# 5106), and GAPDH (clone 14C10; CST, Cat# 2118). Validations of the primary antibodies are provided on the manufacturer's websites.

Subject recruitment

Extraocular muscle tissues were collected from six TAO patients during orbital decompression surgery and from six control volunteers during orbital or strabismus surgery (Supplementary Table 1, see section on [supplementary materials](#) given at the end of this article). The TAO subjects were patients at inactive stage with clinical activity score less than 3 points on a 7-point scale consisting of eyelid swelling, eyelid erythema, conjunctival redness, chemosis, caruncle or plical inflammation, spontaneous orbital pain, and gaze evoked orbital pain. The control subjects were volunteers without thyroid disease or other autoimmune disease. The exclusion criteria were diabetes or impaired glucose tolerance, thyroid dysfunction, orbital infection or orbital fracture within 6 months, and previous intraorbital malignant tumors. Written informed consent was obtained from each subject, and the study protocol was approved by the Institutional Review Board of Fudan Eye & ENT Hospital.

Cell culture

Primary pOFs were isolated from extraocular muscle tissues as previously described (Ma *et al.* 2018, 2019). After reaching 90% confluence, the primary pOFs were passaged with 0.25% trypsin and maintained in high-glucose DMEM (Gibco, 11965) supplemented with fetal bovine serum (FBS, 10%, v/v), penicillin (100 IU/mL) and streptomycin (100 µg/mL). Passages 3–8 were used in the study.

Cell viability

The CCK-8 colorimetric assay was adapted to measure cell viability. Passaged pOFs were seeded in 96-well plates at a density of 8000 cells/well and incubated overnight for recovery. After starvation for 6 h, the pOFs were incubated with different concentrations of DCA (0–150 mM) in 1% (v/v) FBS for 30 min, treated with CCK-8 reagent (10%, v/v) for 25–45 min, and processed for absorbance measurement at 450 nm.

EdU incorporation assay

The EdU incorporation assay is an immunofluorescence method to semi-quantify cell proliferation. Passaged pOFs were seeded on glass coverslips in 24-well plates and incubated for 24 h for recovery. The pOFs were starved for 12 h, treated with DCA (0–150 mM) in 1% (v/v) FBS for 48 h, and incubated with EdU labeling solution (10 µM) during the last 24 h. The incorporated EdU was detected according to the manufacturer's protocol, and the cellular nuclei were labeled with DAPI (1:1000) staining. A Leica TCS SP2 confocal microscope was used for imaging.

BrdU incorporation assay

The BrdU incorporation assay is an ELISA method to quantify cell proliferation. Passaged pOFs were seeded in 96-well plates and incubated overnight for recovery. The pOFs were starved for 12 h, treated with DCA (0–150 mM) in 1% (v/v) FBS for 48 h, and incubated with BrdU labeling solution (10 µM) during the last 12 h. The incorporated BrdU was conjugated with anti-BrdU antibody for 90 min, and the substrate solution was added for 5–10 min for color development. The absorbance values were measured at 370 nm with a reference wavelength at 492 nm.

Lactate production assay

Passaged pOFs were seeded in 15 cm dishes to reach 90% confluence, deprived of serum for 6 h, incubated with

DCA (0–15 mM) or MK2206 (0–3 µM) in 1% (v/v) FBS for 12 h, and harvested for intracellular lactate measurement according to the instruction of Lactate ELISA kit. In brief, the cell pellet was homogenized in 200 µL Lactate Assay Buffer. After centrifugation, the supernatant was collected and processed with the Deproteinizing Sample Preparation kit to remove endogenous lactate dehydrogenase. The lactate concentration in each sample was determined against the standard curve.

Oxygen consumption assay

Passaged pOFs were seeded in 96-well plates at a density of 4×10^4 cells/well and incubated overnight for recovery. The pOFs were starved for 6 h and treated with DCA (0–15 mM) or MK2206 (0–3 µM) in 1% (v/v) FBS for 12 h. All wells were then replaced with 150 µL fresh culture medium containing 10 µL Extracellular Oxygen Consumption Reagent and promptly sealed by pre-warmed High Sensitivity Mineral Oil. Measurement was repeated at 0.5 min intervals for 200 min at 380 nm excitation and 650 nm emission wavelengths. The oxygen consumption rate was calculated based on the slope of the ascending linear proportion of the signal profile.

Immunofluorescence staining

Passaged pOFs were grown on glass coverslips for immunofluorescence staining. The pOFs were fixed with paraformaldehyde, permeated with Triton (0.5%, v/v), blocked with BSA (3%, w/v), and incubated with the anti-Akt antibodies (1:100) and anti-vimentin antibody (1:500) at 4°C for 12–16 h. After extensive rinsing, the coverslips were incubated with secondary antibodies and stained with DAPI. The cell number on each coverslip was determined based on DAPI counting in nine random fields captured with a Leica TCS SP2 confocal microscope.

Quantitative real-time PCR

At least 10^6 cells in each experimental group were collected for quantitative PCR analysis. Total RNA was extracted with TRIzol (Invitrogen) and reverse-transcribed to cDNA with the rtPCR kit. Quantitative real-time PCR was performed on a ViiA™ 7 Real-Time PCR System (Applied Biosystems) to amplify *GAPDH* (housekeeping gene) and *PDK1–4* with the SYBR qPCR kit. The primer sequences are shown in Supplementary Table 2. The amplification efficiency was evaluated by the standard curve method and was identified as 99.7% for *GAPDH*, 99.6% for *PDK1*,

99.7% for *PDK2*, 99.9% for *PDK3*, and 97.0% for *PDK4*. The mRNA levels were measured in three replicates and normalized to *GAPDH* by the $-\Delta\Delta CT$ method.

siRNA transfection

Passaged pOFs were seeded in 6-well plates to reach 80% confluence and transfected with RNAiMAX reagent according to the manufacturer's instruction. Briefly, after 6-h starvation, the pOFs were incubated in Opti-MEM (Gibco, Cat# 31985088) containing RNAiMAX (vehicle group), RNAiMAX+scramble siRNA (Invitrogen, Cat# 12935200; scramble group), or RNAiMAX + *PDK2* siRNA (sense 5' → 3': GACCGAUGCUGUCAUCAUTT; antisense 5' → 3': AUAGAUGACAGCAUCGUUCTT) for 24 h. The transfected cells were harvested, replanted, or treated with other reagents for further experiments.

Cytoplasmic protein extraction

At least 10^6 cells in each experimental group were collected for protein extraction. The cytoplasmic fractions were prepared according to the instruction of PARIS kit. Notably, the phosphatase inhibitor PhosSTOP (1× concentration, Roche) and the protease inhibitor PMSF (1 mM, Solarbio) reagents were added to the cell fractionation buffer in the PARIS kit.

Capillary Western immunoassay (WES)

Protein samples were quantified by a BCA protein assay (Beyotime) and immunodetected with the ProteinSimple WES® System according to the manufacturer's instruction. The relative amount of immunoreactive band was normalized to *GAPDH* in the same sample. Different loading concentration of protein samples were tested by a titration experiment, and the suitable concentration was determined as 0.5 µg/µL for *PDK2* (1:50) and *GAPDH* (1:50) in total protein, 1.0 µg/µL for Akt (1:25) and *GAPDH* (1:25) in cytoplasmic protein, and 2.0 µg/µL for pAkt³⁰⁸ (1:25) and *GAPDH* (1:25) in cytoplasmic protein.

Statistics

Statistical software was Statistical Product and Service Solutions (version 19.0). All outlier data were included in data analysis. Student's t-test was applied to compare

two independent experimental groups. One-way ANOVA test was applied to compare three or more experimental groups from TAO pOFs or control pOFs. Two-way ANOVA test was applied to compare three or more experimental groups between the TAO and control OFs. If significant differences existed, Sidak test was applied for multiple comparisons. All data are expressed as the mean ± s.d. A *P* value less than 0.05 was considered statistically significant.

Results

Clinical characteristics of recruited subjects

Six TAO subjects and six control subjects were recruited in our study. The demographic characteristics including age (TAO: 54.0 ± 4.7 , control: 43.5 ± 5.5), sex (three male and three female in both groups) and smoking status (TAO: two smokers, control: one smoker) are comparable between the TAO and control groups (Supplementary Table 1). The TAO subjects were at inactive stage with an average CAS of 0.8 ± 0.3 (range 0–2). The control subjects included three cases of orbital cavernous hemangioma, two cases of uveal melanoma, and one case of intermittent exotropia. For TAO and orbital tumor subjects, the extraocular muscle tissues were sampled at the muscle belly of the lateral rectus; for uveal melanoma and strabismus subjects, the extraocular muscle tissues were collected adjacent to the scleral insertion session of the medial rectus. All muscle tissues were processed for primary culture of pOFs immediately after collection, and passages 3–8 were used in the following experiments.

DCA inhibits proliferation of TAO pOFs

According to the CCK-8 assay, a high concentration of DCA (15–150 mM) dose-dependently suppressed the viability of TAO and control pOFs, while low concentration of DCA (<15 mM) showed no significant effects on cell viability (Fig. 1A). The effects of 0–150 mM DCA on proliferation were investigated by EdU and BrdU assays. Since incubation time significantly affected the proliferation results, the incubation time of EdU and BrdU was adjusted as 24 and 12 h, respectively (Supplementary Fig. 1B, C and D). Both EdU and BrdU assays demonstrated that the TAO pOFs exhibited higher proliferation activity than the control pOFs (Fig. 1B, C, D and Supplementary Fig. 1B, C, D). DCA at 1–3 mM statistically suppressed TAO pOFs proliferation but showed no significant effects on control pOFs, while DCA at 5–15 mM inhibited proliferation of

Figure 1

DCA inhibits the proliferation of TAO pOFs. (A) The effects of different concentrations of DCA (0–150 mM) on cell viability in the TAO and control pOFs. Each cell strain was repeatedly measured in six replicates, and cell viability was expressed as a percentage to the data in the 0 mM group. (B and C) Comparison of cell proliferation based on EdU incorporation assay of the TAO and control pOFs treated with or without DCA (0–15 mM). The initial seeding density was 10^4 cells/well in 24-well plates. The EdU-positive (green) nuclei (DAPI, blue) were counted in nine random fields of each cell strain. Scale bars, 100 μ m. (D) Comparison of cell proliferation based on BrdU incorporation assay of the TAO and control pOFs treated with or without DCA (0–15 mM). The initial seeding density was 3500 cells/well in 96-well plates. The absorbance value of each cell strain was calculated across six replicates and normalized to the data of control pOFs in the 0 mM group. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.000$; n indicates the number of cell strains in each experimental group.

both TAO and control pOFs (Fig. 1B and C). Notably, DCA at 50–150 mM resulted in large amounts of nonspecific binding in the EdU assay and significant increase of absorbance values in the BrdU assay, suggesting that 50–150 mM DCA is not suitable for cellular biological analysis (Supplementary Fig. 1E and F). The CCK-8 absorbance values were statistically lower in the TAO pOFs (Supplementary Fig. 1A), indicating that the TAO pOFs exhibited lower activity of dehydrogenases than the control pOFs. DCA is a specific inhibitor of glycolysis, and glycolytic cells are characterized with strong activity of proliferation and weak activity of pyruvate dehydrogenase (Stacpoole 2017). We, therefore, hypothesized that the glycolytic pathway may be enhanced in the TAO pOFs and the enhanced glycolysis, in turn, promotes proliferation.

DCA suppresses the enhanced glycolysis in TAO pOFs

Enhanced glycolytic pathway features increased lactate production and decreased oxygen consumption (Vander Heiden *et al.* 2009). The lactate production assay demonstrated a higher level of intracellular lactate in the TAO pOFs than the control pOFs, and DCA

dose-dependently suppressed lactate production in the TAO pOFs (Fig. 2A). The oxygen consumption assay confirmed a lower oxygen consumption rate in the TAO pOFs than the control pOFs, and 1 mM DCA statistically enhanced oxygen consumption in the TAO pOFs (Fig. 2B). Longer incubation time resulted in increased intracellular lactate concentration (Supplementary Fig. 2A), and therefore DCA treatment was fixed at 12 h for both lactate and oxygen measurement. After 12-h incubation, the cell numbers of TAO pOFs in the 5 mM and 15 mM DCA groups were statistically lower than the 0 mM and 1 mM DCA groups (Fig. 2C). Since oxygen consumption rate decreased as cell number reduced (Supplementary Fig. 2B), the uneven cell numbers may contribute to the paradoxical results that the oxygen consumption rate of TAO pOFs in the 5 and 15 mM DCA groups were lower than the 1 mM DCA group (Fig. 2B). The targets of DCA include four isoforms of PDK (Ki PDK2 < PDK1 $\approx$ PDK4 < PDK3) (Wu *et al.* 2019), and reverse-transcription PCR confirmed the existence of all four PDK mRNAs in both TAO and control pOFs (Supplementary Fig. 2C). Quantitative PCR revealed a higher level of PDK2 in the TAO pOFs than the control pOFs (Fig. 2D), suggesting that PDK2 overexpression may contribute to the enhanced glycolysis in the TAO pOFs.

Figure 2

DCA suppresses the enhanced glycolysis in TAO pOFs. (A) Comparison of glycolysis based on intracellular lactate production of the TAO and control pOFs treated with or without DCA (0–15 mM). The lactate concentration of each sample was calculated across three replicates and expressed as a percentage to the data of control pOFs in the 0 mM group. (B) Comparison of glycolysis based on the oxygen consumption rate of the TAO and control pOFs treated with or without DCA (0–15 mM). The line charts show representative signal profiles of one TAO (red) and one control (green) cell strain. The oxygen consumption rate was calculated based on linear slopes in three replicates and expressed as a percentage to the data of control pOFs in the 0 mM group. (C) Corresponding cell numbers of each experimental group in (B). The cells were quantified by DAPI staining after measurement of oxygen consumption rate. (D) Comparison of *PDK* mRNA levels in the TAO and control pOFs by quantitative PCR. Before RNA extraction, the cells were processed with 6-h starvation and 12-h incubation in 1% (v/v) FBS. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n indicates the number of cell strains in each experimental group.

PDK2-enhanced glycolysis promotes proliferation of TAO pOFs

The effects of PDK2 on glycolysis and proliferation were explored by siRNA transfection. Quantitative PCR confirmed that 24-h transfection with *PDK2* siRNA (the siPDK2 group) significantly suppressed *PDK2* transcription in both TAO and control pOFs (Fig. 3A) and showed no significant effects on other *PDK* mRNA isotypes (Supplementary Fig. 3A). WES assay verified that the TAO pOFs expressed a higher level of PDK2 than the control pOFs, and *PDK2* siRNA transfection for 24 h statistically decreased PDK2 protein levels in both TAO and control pOFs (Fig. 3B, C and Supplementary Fig. 3B, C, D). CCK-8 assay revealed that cell viability was not affected by siRNA transfection in both TAO and control groups (Fig. 1D). Congruent with the results in Supplementary Fig. 1A, the CCK-8 absorbance values of TAO pOFs were statistically lower than the control pOFs in the vehicle and scramble groups (Fig. 1D). Knockdown of *PDK2* resulted in decreased lactate production (Fig. 4A) and increased oxygen consumption (Fig. 4B) in the TAO pOFs but showed no significant effects in the control pOFs, providing evidence that the enhanced glycolysis in the TAO pOFs can be partially attributed to PDK2 overexpression. Knockdown of *PDK2* also exerted inhibitive effects on the proliferation of TAO pOFs, resulting in a significant decrease of EdU and BrdU incorporation in the siPDK2 group compared with

the scramble group (Fig. 4C, D and E). The proliferation of control pOFs, however, was not affected by *PDK2* siRNA transfection. Collectively, the above results establish that PDK2 overexpression enhances glycolysis and in turn promotes proliferation in the TAO pOFs.

Cytoplasmic Akt participates in PDK2 signaling transduction in TAO pOFs

Akt is a canonical signaling molecule to regulate proliferation, and cytoplasmic Akt has been recently identified as a candidate to regulate glycolysis (Chae *et al.* 2016). Immunofluorescence revealed Akt expression in both nucleus and cytoplasm, and the cytoplasmic Akt of the TAO pOFs was relatively lower in the siPDK2 group compared with the vehicle and scramble groups (Fig. 5A). The WES assays quantified Akt and its active form pAkt³⁰⁸ in the cytoplasmic fractions of TAO and control pOFs (Fig. 5B, C and Supplementary Figs 4, 5). Based on data analysis, TAO pOFs expressed higher levels of Akt and pAkt³⁰⁸ than the control pOFs, and *PDK2* knockdown significantly suppressed Akt and pAkt³⁰⁸ expression in the TAO pOFs (Fig. 5D and E). These results collectively imply that cytoplasmic Akt may mediate the signaling transduction of PDK2.

A highly selective Akt inhibitor MK2206 was adapted to explore the role of Akt in PDK2 pathway. Quantitative PCR showed that *PDK1–4* transcription was not affected

Figure 3

Verification of PDK2 knockdown in the TAO and control pOFs. (A) Verification of *PDK2* knockdown by quantitative PCR in the TAO and control pOFs. The *PDK2* mRNA levels were quantified in the vehicle (RNAiMAX reagent), scramble (scramble siRNA) and siPDK2 (*PDK2* siRNA) groups and normalized to the data of control pOFs in the vehicle group. (B and C) Verification of *PDK2* knockdown by WES chemiluminescence assay in the TAO and control pOFs. The *PDK2* protein levels were quantified by the signal intensity in the virtual panels and normalized to GAPDH in each protein sample. Lanes 1, 4, 7, 10, 13, 16 represent one cell strain; lanes 2, 5, 8, 11, 14, 17 represent a second cell strain; lanes 3, 6, 9, 12, 15, 18 represent a third cell strain. (D) The effects of *PDK2* knockdown on cell viability in the TAO and control pOFs. Each cell strain was repeatedly measured in six replicates, and cell viability was expressed as a percentage to the CCK-8 absorbance values of control pOFs in the vehicle group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.000$; n indicates the number of cell strains in each experimental group.

by MK2206 treatment (Fig. 6C and Supplementary Fig. 6A). The cell counting results confirmed that MK2206 significantly suppressed pOFs proliferation in both TAO and control groups (Fig. 6A and B), providing evidence that cytoplasmic Akt participates in PDK2 regulation of TAO pOFs proliferation. Regarding glycolysis, 3 μ M MK2206 statistically suppressed lactate production in the TAO pOFs but exerted no significant effects on control pOFs (Fig. 6D), indicating that Akt may play a role in promoting glycolysis in the TAO pOFs. The oxygen consumption rate of TAO pOFs slightly increased to 1.26-fold after incubation with 1 μ M MK2206, but data analysis detected no statistical significance (Fig. 6E). The incongruity between lactate and

oxygen assays may result from the uneven cell numbers among different experimental groups after MK2206 treatment (Supplementary Fig. 6B). Taken together, cytoplasmic Akt in part mediates the promotive effects of PDK2 on glycolysis and proliferation in the TAO pOFs.

Discussion

Recently, the rapid advancement toward the understanding of oxygen homeostasis has yielded important insights into the pathogenesis of autoimmune disease. An inspiring concept has emerged that glycolysis plays a role in immune processes by promoting cell proliferation and strengthening antioxidant defense (Mills *et al.* 2017). With regard to TAO, there is increasing awareness of elevated oxidative stress within patient's orbit and peripheral blood (Korkmaz *et al.* 2015, Marique *et al.* 2015, Aass *et al.* 2016, Yuksel *et al.* 2019). It is reasonable to speculate that glycolysis is preferentially enhanced in TAO OFs so as to sustain prosperous proliferation and resist oxidative stress. Our observation, along with several previous studies, supported the speculation that enhanced glycolysis may be involved in TAO pathogenesis. For instance, orbital adipose-derived stem cells (OASCs) exhibited higher viability in the TAO group than the control group, and the increased viability is probably associated with a relatively higher expression of UCP1 which uncouples the oxidative phosphorylation and in turn, promotes glycolysis (Choi *et al.* 2018). High-throughput studies also disclosed potential candidates that may facilitate the glycolytic shift in TAO patients, such as downregulation of ALDH, downregulation of mitochondrial-encoded NADH and upregulation of AOC3 (Matheis *et al.* 2015, Tao *et al.* 2017, Kishazi *et al.* 2018). Furthermore, recent findings, albeit mostly circumstantial, supported the assumption that strong resistance to oxidative stress contributes to TAO development. According to in vitro studies, regardless of the H_2O_2 treatment to induce ROS production, the TAO OFs generated a lower amount of GSSG (product of ROS) and higher amount of GPX (enzyme to eliminate ROS) than the control OFs (Rotondo Dottore *et al.* 2017, 2018a,b). Both histopathological study and proteomic analysis detected upregulation of antioxidant proteins in the tear and orbital tissues of TAO patients, suggesting stronger protection from oxidative damage (Marique *et al.* 2015, Aass *et al.* 2016). Given the lack of direct evidence, more investigation is needed before definitive conclusions can be drawn. Another ambiguous area concerns the proliferative capacity of TAO and control OFs. Some experts advocated that TAO OFs possessed a stronger

Figure 4

PDK2 knockdown inhibits glycolysis and proliferation in the TAO pOFs. (A) The effects of *PDK2* knockdown on intracellular lactate production in the TAO and control pOFs. After transfection, approximately 2×10^6 cells in the vehicle, scramble and siPDK2 groups were harvested for lactate measurement. The lactate levels were calculated across three replicates and expressed as a percentage to the data of control pOFs in the vehicle group. (B) The effects of *PDK2* knockdown on oxygen consumption rate in the TAO and control pOFs. The transfected cells were replanted to 96-well plates at a density of 4×10^4 cells/well and processed with oxygen consumption assay. The line charts show representative signal profiles of one TAO (red) and one control (green) cell strain. The oxygen consumption rate was calculated across three replicates and expressed as a percentage to the data of control OFs in the vehicle group. (C and D) The effects of *PDK2* knockdown on cell proliferation based on EdU incorporation assay in the TAO and control pOFs. Passaged cells were seeded at a density of 2×10^4 cells/well in 24-well plates, processed with siRNA transfection, and incubated in 1% (v/v) FBS for 48 h. The vimentin (red) staining demonstrates cell morphology, and the DAPI (blue) staining demonstrates nucleus. The EdU-positive (green) nuclei were counted in nine random fields of each cell strain to semi-quantify proliferation. Scale bars, 100 μ m. (E) The effects of *PDK2* knockdown on cell proliferation based on BrdU incorporation assay in the TAO and control pOFs. The transfected cells were replanted to 96-well plates at a density of 4000 cells/well and incubated in 1% (v/v) FBS for 48 h. Each cell strain was measure in six replicates, and the absorbance values were normalized to the data of control pOFs in the vehicle group. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$; n indicates the number of cell strains in each experimental group.

capacity of proliferation than the control OFs, whereas others reported no significant differences between the TAO and control groups (Kau *et al.* 2016, Choi *et al.* 2018, Hikage *et al.* 2019, Woeller *et al.* 2019). Our study demonstrated a positive correlation between enhanced glycolysis and active proliferation in the TAO pOFs. We, therefore, assumed that the paradoxical conclusions may be attributed to a kinetic equilibrium between glycolysis and oxidative phosphorylation under different experimental conditions. Further study is necessary to investigate the oxygen homeostasis and its impact on cellular activities in the pathogenesis of TAO.

The progress in elucidating the function of PDK has invigorated novel therapeutic targets in various diseases. Inhibition of PDK with DCA blocks the glycolytic pathway and shows a beneficial effect in the treatment of diabetes, myocardial ischemia, lactic acidosis, and cancer (Stacpoole 2017). Cytoplasmic Akt was recently identified as a potent activator of PDK enzymes, and inhibition of Akt by MK2206 also enables a metabolic switch from cytoplasmic glycolysis to oxidative

phosphorylation (Chae *et al.* 2016). In our study, the data of intracellular lactate concentration aligned with the above theories, while the data of oxygen consumption rate remains unexplained. The paradoxical results of oxygen consumption assay may be partially attributed to uneven cell density among different experiment groups. In addition, these results lead to the supposition that mitochondrial function may be impaired in the TAO pOFs. The influence of PDK activity on oxygen consumption is largely dependent on mitochondrial function. In cells with unimpaired mitochondria, inhibition of PDK activity restores mitochondrial glucose oxidation and subsequently promotes oxygen consumption (Denko 2008). In cells with defective mitochondrial activity, DCA treatment suppresses the energy flux by reducing oxygen consumption and consequently induces mitochondrial apoptosis (Martinez-Outschoorn *et al.* 2017). Growing evidence embraces the concept that mitochondrial defect may play a role in TAO pathogenesis. Electron microscopy studies directly observed mitochondrial abnormalities in the extraocular muscles obtained from TAO patients (Wall

Figure 5

PDK2 knockdown suppresses cytoplasmic Akt activity in the TAO pOFs. (A) Akt immunofluorescence of the TAO and control pOFs in the vehicle (RNAiMAX reagent), scramble (scramble siRNA) and siPDK2 (*PDK2* siRNA) groups. Scale bars, 100 μ m. (B and C) The virtual panels of WES chemiluminescence assay to quantify Akt, pAkt³⁰⁸ and GAPDH in the cytoplasmic fractions of the TAO and control pOFs. The corresponding lanes of each immunostaining target (such as lanes 1 and 10) are results from a same protein sample. Lanes 1, 4, 7, 10, 13, 16 represent one cell strain; lanes 2, 5, 8, 11, 14, 17 represent a second cell strain; lanes 3, 6, 9, 12, 15, 18 represent a third cell strain. (D and E) Corresponding data analysis of (B) and (C). The relative amount of Akt and pAkt³⁰⁸ are quantified by the signal intensity and normalized to GAPDH in the same sample. **P* < 0.05; ***P* < 0.01; *n* indicates the number of cell strains in each experimental group.

et al. 2006, Tani *et al.* 2007). Autophagic vacuoles, which probably originated from defective mitochondria, were more abundant in the TAO OFs than the control OFs (Yoon *et al.* 2015). Several mitochondrial-specific genes, proteins and autoantibodies were dysregulated in the orbit and serum of TAO subjects compared with healthy control (Cheng *et al.* 2015, Matheis *et al.* 2015, Tao *et al.* 2017, Yuksel *et al.* 2019). Further investigation is required to determine whether mitochondrial impairment participates in TAO development.

Our data demonstrate a higher level of *PDK2* mRNA in the TAO pOFs than the control pOFs. Further study is necessary to explore the mechanism of *PDK2* overexpression in the TAO pOFs. According to previous findings, the transcription factor HIF1 α is a potential candidate. Stable expression of HIF1 α enables constitutive transactivation of target genes including PDKs and stimulates metabolic switch from oxidative phosphorylation to cytoplasmic glycolysis (Denko 2008). Previous research discovered an increase of HIF1 α in the

TAO OFs, and the HIF1 α level positively correlated with the clinical activity score of TAO patients (Görtz *et al.* 2016, Hai *et al.* 2020). In a recent study conducted with a three-dimensional (3D) culture system, however, the authors failed to detect the increased expression of HIF1 α but observed HIF2 α activation in the TAO group (Hikage *et al.* 2019). The incongruent findings may be attributed to the disparity of experimental conditions between 2D (cell layer) and 3D (cell organoid) culture settings. Thus, more effort should be directed in developing standardized approach to modeling TAO pathogenesis and exploring the regulatory network of oxygen homeostasis. Moreover, several pieces of evidence indicate that the cross talk between IGF-IR (insulin-like growth factor-I) and TSHR (thyrotropin receptor) may also participate in regulating oxidative stress in TAO orbit. In recent decades, a great progress in understanding TAO pathogenesis is the discovery of a physical and functional complex formed between the two receptor proteins. According to previous findings, TSHR downstream signaling is

Figure 6

MK2206 inhibits proliferation and glycolysis in the TAO pOFs. (A) Akt (red) and DAPI (blue) immunofluorescence of the TAO and control pOFs treated with or without MK2206 (0–3 μM). Cells were incubated with different concentrations of MK2206 in 1% (v/v) FBS for 48 h. Scale bars, 50 μm. (B) Corresponding cell numbers of each experimental group in (A). The cells were quantified by DAPI staining. The initial seeding density was 2×10^4 cells/well in 24-well plates. (C) The effects of MK2206 (0–3 μM) on *PDK2* mRNA levels in the TAO and control pOFs. Cells were treated with or without MK2206 in 1% (v/v) FBS for 12 h. The *PDK2* mRNA levels were quantified by quantitative PCR and normalized to the data of control pOFs in the 0 μM group. (D) The effects of MK2206 (0–3 μM) on intracellular lactate production in the TAO and control pOFs. Cells were treated with or without MK2206 in 1% (v/v) FBS for 12 h. The lactate levels were expressed as a percentage to the data of control pOFs in the 0 μM group. (E) The effects of MK2206 (0–3 μM) on the oxygen consumption rate of the TAO and control pOFs. The line charts show representative signal profiles of one TAO (red) and one control (green) cell strain. The oxygen consumption rate was calculated based on linear slopes in three replicates and expressed as a percentage to the data of control pOFs in the 0 μM group. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$; n indicates the number of cell strains in each experimental group.

strongly enhanced by IGF-IR activation and vice versa (Smith 2019). These findings collectively suggest that IGF-IR may play a critical role in mediating at least a subset of actions initiated through TSHR. IGF-IR is a membrane-spanning tyrosine kinase receptor that provokes Akt signaling and in turn, activates multiple biological processes including aerobic glycolysis (DiToro *et al.* 2020). Our study discovered that cytoplasmic Akt participated in promoting glycolysis and proliferation in the TAO group. These observations collectively provide the speculation that oxygen homeostasis may be part of the IGF-IR and TSHR signaling picture during TAO pathogenesis.

Pitfalls of our study stem from the control group chosen. Many factors, such as malignant tumor and

tissue crowding, may interfere with oxygen homeostasis in extraocular muscles (Denko 2008, Vander Heiden *et al.* 2009). To minimize the effects of malignancy, the control group only recruited subjects with intrabulbar tumors (uveal melanoma), benign orbital tumors (cavernous hemangioma) and concomitant strabismus. To compromise the space-occupying effects, the muscle samples were collected from lateral recti to avoid orbital mass at supranasal quadrant. However, we anticipate more suitable control samples in future studies to investigate the regulation of aerobic glycolysis in extraocular muscle tissues. To conclude, PDK2 overexpression enhances glycolysis and promotes proliferation via Akt signaling transduction in the TAO pOFs, suggesting that PDK2 is a potential therapeutic target for TAO treatment.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JME-20-0143>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Data availability

The datasets generated and analyzed during the present study are available from the corresponding author on reasonable request.

Author contribution statement

R M and L G conceived and designed the experiments. R M, L G, and H R performed the experiments. R M and L G analyzed the data. A H and J Q supervised the study. R M and L G wrote the paper. All authors revised the final version of the manuscript. J Q is the principal investigator of this work and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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